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Mutagenesis and self-ligation of the self-cleavage domain of the satellite RNA minus strand of tobacco ringspot virus and its binding to polyamines

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ABSTRACT

Several mutants for the minus strands of the self-cleaving domain of the satellite RNA of tobacco ringspot virus have been synthesized by joining chemically synthesized oligoribonucleotides with RNA ligase. Kinetic properties of the enzyme strands (50 nucleotides) against substrates (15-mer and 18-mer) were investigated. Structural properties of the unpaired part in the cleavage region were estimated from mutagenesis. The catalytic domain alone was proved to be responsible for the rejoining reaction of cleaved substrates. It was also found that the ribozyme could be divided into two strands without loss of activity. Effects of concentration of magnesium ion and polyamines on the cleavage reaction for the two-stranded ribozyme are also reported.

INTRODUCTION

Several types of catalytic RNA's are known to cleave and join RNA strands. Catalytic domains in group I or II introns involve large numbers of nucleotides, at least about 400 (1, 2). The RNA component of RNase P (M1 RNA) also contains over 300 nucleotides (3). On the other hand, self cleaving domains found in RNA's infectious to plants (4–7) and in transcripts of a satellite DNA of newt (8) consist of a rather small number of nucleotides.

The satellite RNA of tobacco ringspot virus (sTobRV) contains 359 nucleotides and replicates by a rolling cycle involving self-cleavage reactions. In contrast to the cleaving domain of the plus strand of sTobRV, which has the hammerhead secondary structure, the catalytic site of the minus strand ((−)sTobRV) does not have the hammerhead structure (5). The minimum catalytic domain of (−)sTobRV has been reported to contain a 50-base catalytic RNA sequence and a 14-base substrate RNA (9). The secondary structure of this domain is called the hairpin model.

We have previously reported site-directed mutagenesis of a hammerhead ribozyme using chemically synthesized oligoribonucleotides (10) and also designed ribozymes that distinguished a single base mutation in RNA (11, 12). In this paper, we report mutations and re-joining reactions of the catalytic domain of (−)sTobRV using chemically synthesized oligoribonucleotides (Figure 1, 2). Although the (−)sTobRV itself has been shown to undergo both catalytic cleavage and ligation (5), we show that the joining reaction occurs in the minimum catalytic domain. We also describe how the minimum domain can be divided into two parts, and how a two-stranded ribozyme (E21 and E29 in Figure 1) shows binding to Mg^{2+} and polyamines.

MATERIALS AND METHODS

Enzymes

T4 polynucleotide kinase (*E. coli* A19) and T4 RNA ligase were purchased from Takara Shuzo Co. Ltd. RNase U_2 was purchased from Sankyo Co. Ltd.

Oligoribonucleotides

Oligoribonucleotides were synthesized by the phosphoramidite method using 2′-*O*-tetrahydropyranyl and 5′-*O*-dimethoxytrityl protecting groups as described previously (10, 13). Oligoribonucleotides containing 2′-*O*-methyladenosine were prepared as described (12). These oligonucleotides were synthesized with a DNA synthesizer, Applied Biosystems 380A. The oligonucleotides were purified with reverse-phase and anion-exchange high performance liquid chromatography (HPLC). Reverse-phase column: YMC A-324, 10 mm I.D.×300 mmL. (Yamamura Chemical Laboratories) or TSK gel ODS-80 TM, 4.6 mm I.D.×250 mmL. (Tosoh). Anion-exchange column: TSK gel DEAE-2SW, 4.6 mm I.D.×250 mmL. (Tosoh)

Construction of catalytic RNA

F3 (4.8 nmol) was incubated in 30 μl of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.33 U/μl of T4 polynucleotide kinase, and 0.73 mM ATP at 37°C for 1 hr. After phenol-chloroform extraction, the phosphorylated product was desalted by gel filtration on a column (20 mm I.D.×475 mmL.) of Sephadex G25, dissolved in 20 μl of water and 2.5 μl of 0.1 M $NaIO_4$, then placed in an ice bath in the dark. After 70 min, 25 μl of 0.1 M ethylene glycol was added and the mixture was kept in the same ice bath for 30 min. Two M Lysine-HCl (pH 8.2, 50 μl) was added, the mixture was incubated at 45°C

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for 2 hr and it was desalted as described above. The quantity was estimated by UV absorbance. 5', 3'-Bisphosphorylated F3 (3.3 nmol) and synthesized F2 (5 nmol) were dissolved in 50 μl of a solution containing 50 mM HEPES-NaOH (pH 7.5), 20 mM $MgCl_2$, 3.3 mM DTT, 0.005% bovine serum albumin, 10% dimethyl sulfoxide, 0.16 mM ATP, and 1.38 U/μl of T4 RNA ligase and then incubated for 12 hr at 6°C. After phenol extraction, the ligation products were purified by HPLC. The second ligation between F2−3 and F1 was done by a similar procedure. E50 was purified by anion-exchange HPLC and the sequence of E50 analyzed by partial digestion with base specific RNases (14, 15).

5' or 3'-labeled oligoribonucleotides

For 5'-end labeling, purified substrate RNA was incubated in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol with [γ-^{32}P]ATP, and T4 polynucleotide kinase for 1 hr at 37°C. The labeled oligonucleotides were isolated and desalted with NENSORB 20 (Du Pont). To prepare 3'-end-labeled RNA, 500 pmol of the oligoribonucleotide was incubated in 10 μl of 50 mM HEPES-NaOH (pH 7.5), 20 mM $MgCl_2$, 3.3 mM DTT, 0.001% bovine serum albumin, 10% dimethyl sulfoxide, 0.1 mM ATP, and [5'-^{32}P]pCp with 4.6 U/μl of T4 RNA ligase at 37°C for 1 hr. The 3'-end-labeled RNA was purified and desalted by the same procedure used for 5'-end-labeling.

Cleavage reaction

5'-End-labeled substrate RNA and RNA enzyme (E50) were dissolved separately in 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, and 2 mM spermidine-3HCl and heated for 2 min at 65°C, then immediately transferred to an ice bath. To start the reaction, the two solutions were mixed. The reaction for complex 7 was done in the same way after the complex was heated at 65°C for 2 min and annealed to 32°C. The reaction was stopped by addition of 50 mM EDTA-2Na (pH 8.0) and the mixture electrophoresed by 20% PAGE containing 8 M urea. Cleaving rates were estimated from the radioactivity of the sliced gel after autoradiography.

Partial digestion by RNase U_2

5'-End-labeled substrates were incubated with 0.002 or 0.001 U/μl of RNase U_2 in 20 mM citric acid (pH 3.5), 0.1 mg/ml $tRNA^{phe}$, 5.6 M urea, 0.8 mM EDTA, 0.8 mM xylene cyanol, and 0.8 mM bromophenol blue at 55°C. After 18 min, the mixture was transferred into an ice bath immediately and used as a marker to identify the cleavage site. (Figure 3)

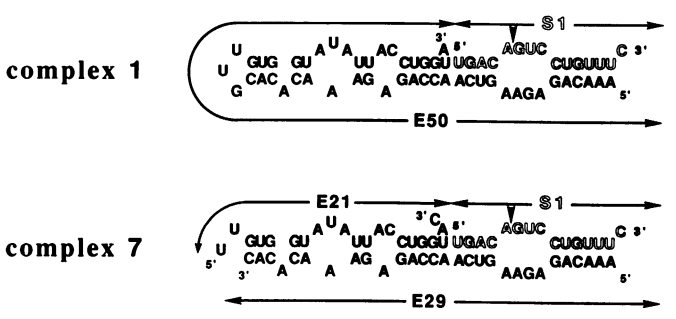

Figure 1. A hairpin RNA domain (complex 1) and a mutant with two-stranded ribozyme parts (complex 7).

Self-ligation reaction

5'-End-labeled 18-mer was cleaved by the ribozyme. The 5' fragment bearing a 2', 3'-cyclic phosphate (P1) was separated by 20% PAGE containing 8 M urea and purified with NENSORB 20. The 8-mer (P1) and an equal amount of the synthesized 10-mer (P2) were dissolved in 40 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 2 mM spermidine-3HCl, then an equal amount of E50 was also dissolved in the same buffer. Each solution was heated at 65°C for 2 min, then chilled in ice immediately. The 2 solutions were mixed for 1 hr, and the stop solution that was used for the cleavage reaction described above was added. The mixture was electrophoresed by 20% PAGE and the ligation rate was estimated from radioactivity of the sliced gel after autoradiography.

RESULTS AND DISCUSSION

Synthesis of substrates and ribozymes

A substrate 15-mer, UGACAGUCCUGUUUC (S1) and its sequence isomers as well as the 5' extended substrate 18-mer, GCGUGACAGUCCUGUUC (S2) were synthesized chemically as described (10). The approach involved 2'-*O*-tetrahydropyranyl and 5'-*O*-dimethoxytrityl groups as protecting groups (10, 16). The product prepared by a DNA synthesizer was released from the support and treated successively with thiophenol and concentrated aqueous ammonia. The dimethoxytritylated 15-mer

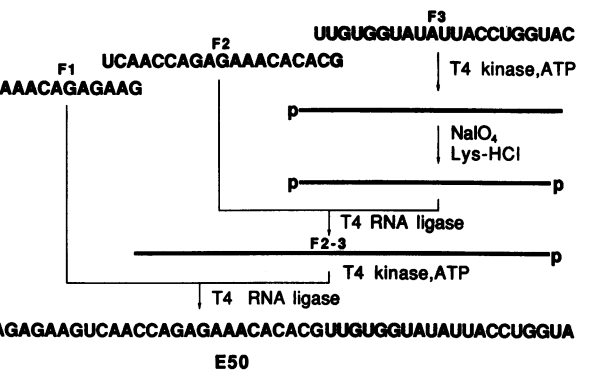

Figure 2. Construction of catalytic RNA (E50).

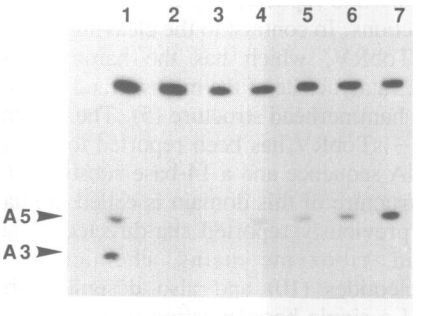

Figure 3. PAGE analysis of self-cleavage of complex 1 and the identification of the cleavage site. Lane 1, the partial digestion product of S1 by RNase U_2; lane 2, 0 min; lane 3, 5 min; lane 4, 10 min; lane 5, 15 min; lane 6, 30 min; lane 7, 60 min. The reaction was done in a solution (20 μl) containing 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-3HCl, 81 nM S1 and 19 nM E50 at 37°C.

was separated from the other short oligonucleotides by reverse-phase HPLC and the completely deblocked oligomers were also purified by anion-exchange HPLC.

Three fragments corresponding to the catalytic part were prepared by similar procedures and joined by RNA ligase. The strategy for construction of the catalytic 50-mer is shown in Figure 2. A donor 21-mer (F3) was 5'-phosphorylated by polynucleotide kinase and ATP. The 3'-end of this donor oligonucleotide was protected with 3'-phosphate, which was introduced by elimination of the extra nucleoside by periodate oxidation, and the products joined to an acceptor 19-mer (F2) using RNA ligase. The joined molecule (F2−3) was 5'-phosphorylated and ligated to the 5'fragment (F1). The product (E50) was purified by anion-exchange HPLC and analyzed by digestion with nucleases (14, 15).

Catalytic activities of (−)sTobRV domain and its mutants

The 5'-end labeled substrate 15-mer (S1) was cleaved by the catalytic 50-mer (E50) at 37°C in the presence of 12 mM magnesium chloride. The 2', 3'-cyclic phosphate at the cleavage site was characterized by electrophoresis on polyacrylamide gel (PAGE) using markers obtained by partial digestion of the 5'-labeled substrate with RNase U_2, which gives a 2', 3'-cyclic adenylate preferentially (Figure 3) (11, 14). The cleavage site was found to be at the 3'-side of A5 as found in the natural complex. The amount of the product cleaved in complex 1 was found to be 26% and 56% after 30 min and 60 min. (Figure 3).

The catalytic activity of E50 was tested by altering the concentration of E50 as shown in Figure 4a. Kinetic parameters of the reaction with the substrate 15-mer were deduced from a Lineweaver-Burk plot (Figure 4b). The K_m for the 15-mer (S1) was 0.084 μM, which was larger than the value of 0.03 μM reported for the transcript (17-mer) (9). We tested the reaction of E50 with a 18-mer (S2) which had GCG at the 5'-end of the 15-mer. The K_m for S2 was 0.022 μM. However, k_{cat} values for the (S1) and (S2) in this reaction were 0.21 min^{-1} and 0.30 min^{-1}, respectively. These values were one order of magnitude smaller than the value reported for the 17-mer (9, 17). The difference between K_m for S1 and S2 may be derived from the participation of the 3 bases of S2 in a favorable complex that has a kinked structure (unpublished results by Y. Komatsu *et al.*).

To investigate structural requirements in the cleavage reaction, the base sequence of the substrate was altered. Adenosine at the 5'-site of the cleavage site in S1 was replaced by guanosine, cytidine, uridine, or 2'-*O*-methyladenosine. The cleavage percent after 30 min of these substrates (81 nM) with E50 (16 nM) were: A (wild type), 45%; G, 88%; C, 44%; U, 26%; Am, not detected. The cleavage site could be altered to the other three bases as had already been shown qualitatively (18). In contrast to the case of the hammerhead ribozyme, which had an extremely slow reaction with guanosine in the cleavage site (10), guanosine 2', 3'-cyclic phosphate was generated more efficiently in the hairpin ribozyme. This indicates that complex 1 may have a different coordination structure from that of the hammerhead ribozyme. The 2'-*O*-methyl group prevented the reaction, which was expected from the transesterification with the 2'-hydroxyl group by the in-line mechanism (19).

Effects of the loop size in the cleavage domain on the activity were tested by constructing cleavage domains containing various combinations of substrates and mutated ribozymes. The structures for complex 1−6 and cleavage percentage are shown in Table 1. To examine whether or not two bases (U7 and C8) pair with A24 and G23 (U7:A24 and C8:G23) and bulged A22 exist in an active form, complexes 3, 4, and 6 were tested. These complexes showed no cleavage, indicating that A22 did not form a bulge in the cleavage domain. This result is consistent with that with an inactive double mutant (18). On the other hand,

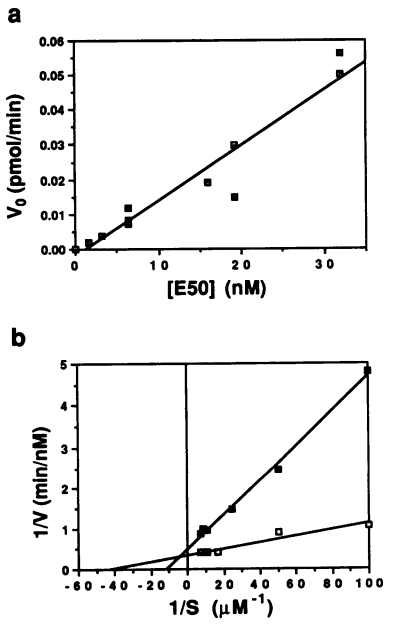

Figure 4. Kinetic data with S1 and S2. a) Initial rate of cleavage with S1 (81 nM). b) Lineweaver-Burk plot of kinetic data for S1 (solid square) and S2 (open square) with 10 nM of E50 at 37°C.

Table 1. Mutants of the catalytic domain

	sTobRV(-)RNA mutants	cleavage rate (%) [a]	
		30 min.	15 hr
1	wt 5' UGAC AGUC CUGUUU C 3' 3' ACUG AAGA GACAAA 5'	45	89
2	5' UGAC AGUA CUGUUU C 3' 3' ACUG AAGA GACAAA 5'	trace	9
3	5' UGAC AGUA CUGUUU C 3' 3' ACUG AAG_ GACAAA 5'	N.D. [b]	trace
4	5' UGAC AGUC CUGUUU C 3' 3' ACUG AAG_ GACAAA 5'	N.D.	trace
5	5' UGAC AGUU CUGUUU C 3' 3' ACUG AAGA GACAAA 5'	3	52
6	5' UGAC AGUU CUGUUU C 3' 3' ACUG AAG_ GACAAA 5'	N.D.	trace

a) Using conditions shown in Fig.3

b) N.D.=Not Detected

complex 5 containing U8 instead of C8 maintained a reduced activity although A8 mutant (complex 2) showed only a trace of activity and a G8 mutant was reported to be inactive (18). These may indicate that an unusual base pair, C8:A22, is essential and U7:(G23 is also involved for cleavage since any deletion mutants (complexes 3, 4, and 6) were inactive. A similar sequence in this loop except that positions for C and A are opposite has also been found in the loop of the minus strand of the satellite RNA of arabis mosaic virus (20). These unusual pairs might be favorable to the accommodation of magnesium ions.

Self-ligation of the substrate in the (−)sTobRV domain

Although (−)sTobRV RNA has been shown to have a joining activity (5), the minimum domain for the joining activity has yet to be resolved. The 5′-side fragment of the cleavage product (P1) from S2 by E50 was isolated by 20% PAGE and mixed with the chemically synthesized 10-mer (P2), then reacted with E50 in the presence of $MgCl_2$ at 37°C. As shown in Figure 5b, the products were obtained in yields of 0.4% (lane 3) and 1.5% (lane 4) in the presence of 6 mM $MgCl_2$ and 12 mM $MgCl_2$, respectively. When the concentration of RNA complex was increased from 0.5 μM to 1 μM, the yield was 2% (lane 5). And the ligation rate was 3.0% by lowering the reaction temperature from 37°C to 4°C. This slight increase might be due to suppression of the cleavage reaction at lower temperature. Furthermore, when the concentration of Mg^{2+} was increased from 12 to 40 and 80 mM, the yields of the ligation products increased from 3.0% to 4.6% and to 8.0%, respectively. The genomic RNA of hepatitis delta virus (HDV) has also shown self-ligation reaction when Mg^{2+} ions were removed (21). However, the RNA of HDV has been shown not to catalyze the *trans*-ligation reaction (21). The mechanism of the catalytic function of (−)sTobRV seems to be different from that of the RNA of HDV. Comparison of the three-dimensional structures of these ribozymes is of interest.

The joining reaction was not catalyzed by the complementary 14-mer in E50 from the 5′-end, 3′ACUGAAGAGACAAA5′ (22). This indicated that the self-ligation is not a template-dependent reaction. To characterize the structure of the phosphodiester linkage at the joining site, the product was reacted with a reverse transcriptase. The diester bond was identified as the 3′-5′ linkage by its ability to be a template of the reverse transcriptase reaction (23). These results showed that the domain that catalyzed the cleavage reaction was also involved in the self-ligation, and these catalytic functions should be important in the rolling-circle mechanism for the replication of this satellite RNA (7).

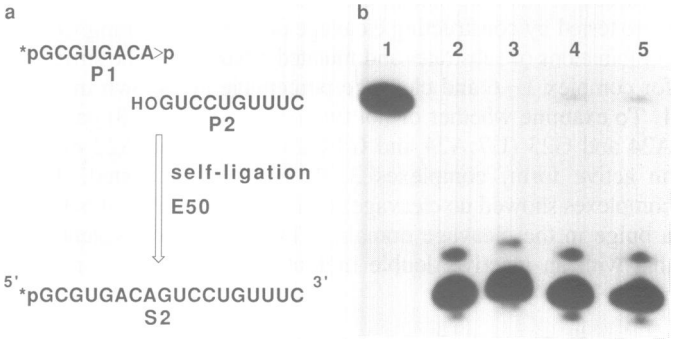

Figure 5. Self-ligation of P1 and P2. a) Scheme for joining reactions. b) Autoradiogram for self-ligation of P1 (8-mer) and P2 (10-mer) by E50. Lane 1, S2; lane 2, P1; lane 3, reaction mixture, [RNA complex] = 0.5 μM, [Mg^{2+}] = 6 mM; lane 4, [RNA complex] = 0.5 μM, [Mg^{2+}] = 12 mM; lane 5, [RNA complex] = 1.0 μM, [Mg^{2+}] = 12 mM. The reaction was done at 37°C for 1 hr.

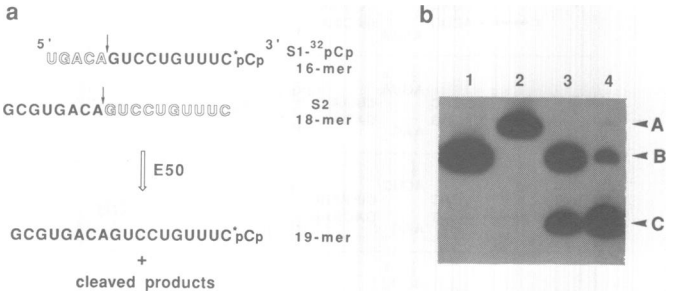

Figure 6. Cross-ligation of S1-^{32}pCp (16 mer) and S2 (18 mer). a) Scheme for cross-ligation reaction. b) Autoradiogram of the cross-ligation reaction. Lane 1, S1-^{32}pCp (16-mer); lane 2, S2 (18-mer); lane 3 and 4, reaction mixture. Lane 3, 4 min; lane 4, 15 min. The reaction mixture (10 μl) contained 40 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 2 mM spermidine-3HCl, 1 μM S1-^{32}pCp, 1 μM S2 and 2 μM E50. The reaction was done a 37°C. The cross-ligation product, S1-^{32}pCp and P2-^{32}pCp are indicated as A, B and C, respectively.

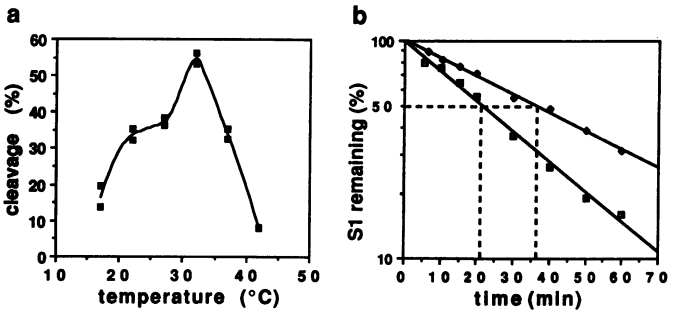

Figure 7. Optimum temperature and half-life of S1. a) Effects of temperature on cleavage activity of the two-stranded ribozyme complex 7. The reaction was done in a solution containing 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-3HCl, 81 nM S1 and 32 nM E29-E21 for 20 min. b) Course of the cleavage reaction for complex 1 (open square) and complex 7 (solid square) at 32°C. The reaction conditions are described above.

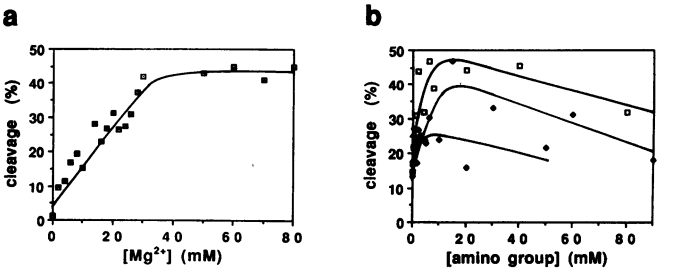

Figure 8. Effects of magnesium ions and polyamines on the cleavage of complex 7. a) Plots of the cleavage percentage with complex 7 vs the concentration of Mg^{2+}. The reaction conditions are described in Figure 7b except for concentrations of Mg^{2+}. b) Plots of the cleavage percentage with complex 7 vs concentration of amino groups for spermine (open squares), spermidine (open diamonds) and ammonium chloride (solid diamonds). The reaction conditions are described in Figure 7b except for concentrations of amines.

Self-ligation and self-cleavage reactions seem to be reversible. To verify this hypothesis, it was examined whether these reactions occurred in the same reaction system. In the presence of 100 mM $MgCl_2$ at 37°C, two oligoribonucleotides of different lengths, UGACAGUCCUGUUUC*pCp (S1) and GCGUGAC-AGUCCUGUUUC (S2) were mixed with E50 at 37°C for 2 hr (Figure 6a). The product 19-mer which appeared to be the joined product of the 3'-fragment of S1 and the 5'-fragment of S2, was detected on PAGE. Figure 6b shows the re-joined 19-mer besides a large amount of the cleaved product of S1 and a small amount of the unchanged S1 (lane 4). These results indicated that the two reactions of (−)sTobRV are reversible and catalyzed by the same catalytic domain in the hairpin ribozyme.

Binding properties of a two-stranded ribozyme to Mg^{2+} and polyamines

E50 was divided into two strands by deletion of G45 (E29 and E20, Figure 1) to find whether or not the loop containing GUU (45−47) was essential for the hydrolysis of S1 (15-mer). First, the two-stranded ribozyme in complex 7 was examined for its ability to cleave S1 under the standard conditions (40 mM Tris-HCl, 12 mM $MgCl_2$, and 2 mM spermidine-3HCl, at 37°C) without annealing. The substrate was cleaved very slowly (data not shown). When E29 and E20 were annealed before the reaction, the rate of cleavage was increased, but the cleavage rate for complex 7 was smaller than that for complex 1. The optimal temperature for self-cleavage in complex 7 was measured by increasing the reaction temperature from 17°C to 42°C and found to be 32°C (Figure 7a). This temperature was lower than 37°C for the reported hairpin domain (9) and it may be due to a destabilized structure of complex 7 by loss of the hairpin loop. Figure 7b shows a comparison of the cleavage rates for complex 1 and complex 7 at 32°C. The t1/2 was found to be 37 min and 22 min for complex 1 and 7, respectively.

Although complex 7 showed a slightly less efficient cleavage activity, the active domain in complex 7 seems to restore essentially the same tertiary structure as complex 1. The effects of Mg^{2+} or polyamines on the cleavage activity were examined using complex 7. Binding properties of complex 7 with Mg^{2+} were tested by changing the Mg^{2+} concentration from 0 to 80 mM. The cleavage rate also increased until the concentration of Mg^{2+} reached to 30 mM (Figure 8a). With a Hill plot, the association constant (K_a) and the number of binding Mg^{2+} (n) to complex 7 were found to be 87 M^{-1} and 1, respectively. Since K_a and n were obtained from the cleavage rate, they were persistently related to the activity of the ribozyme. This association constant is much smaller than that of the hammerhead ribozyme (24). It has been reported that when the association constant is larger than 10^4 M^{-1}, the mode of Mg^{2+} binding to RNA appears to be mediated by solvent molecules (25, 26). Thus, this small association constant may show that this divalent cation binds to the hairpin ribozyme directly.

It is known that biological polyamines such as spermidine and spermine bind to various nucleic acids and stabilize their conformations (27). It is important to study whether or not these polyamines interact with the hairpin ribozyme and increase the catalytic activity. The effects of these polyamines on the ribozyme activity have not been investigated.

The effects of spermine and spermidine on the cleavage activity of complex 7 were examined by changing the concentration of the polyamines. To find whether these polyamines act only as counter cations for phosphate anions, ammonium chloride was tested as a control. The pH was kept around pH 7.5. All amino groups in these polyamine molecules was protonated under the conditions investigated and the concentration of the polyamines was counted as that of the cations (the protonated amino groups) as shown in Figure 8b. Each amine had the optimal concentration. The optimal concentrations of spermine, spermidine, and ammonium chloride were 6−8 mM, 12−15 mM, and 1.5−2 mM, respectively. The cleavage rate increased most efficiently when the reaction buffer contained spermine which has four amino groups. In the presence of spermidine which has three amino groups, in the reaction buffer, the cleavage rate also increased but the efficiency was not as large as that of spermine. The activity for ammonium chloride was about half of that for spermidine. From these results, it seems that the number of amino groups in the molecule increased, the cleavage activity of the ribozyme was enhanced. The effects of spermine and spermidine on the cleavage activity were much larger than that of ammonium chloride. This may mean that these polyamines might not only act as counter cations for phosphate anions but also bind to certain regions of the ribozyme and stabilize the complex. Since the cleavage activity of complex 7 was reduced above each optimal concentration of polyamines, extra polyamines may bind to the complex and decrease the turnover of the ribozyme.

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