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Modification and Application of Self-Trimming Hairpin Ribozymes to Targeting a Transcribed RNA *In Vitro*[1]

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The three-domain hairpin ribozyme was improved by increasing the number of linker bases connecting domain I′ with domain II from six to seven, and a new ribozyme was designed to release a *trans*-acting ribozyme that is able to bind with target RNAs by three hybridizing arms. The *trans*-cleavage activities of the trimmed ribozyme were used to cleave a long target RNA, with 85 bases transcribed from a synthetic gene encoding induced nitric oxide synthase (iNOS). Although the ribozyme with three-hybridizing arms did not efficiently cleave the long target RNA, the overall activity, from self-trimming to the *trans*-cleavage reaction of the target RNA, was higher than that of the ribozyme with two hybridizing arms.

Key words: catalytic RNA, hairpin ribozyme, iNOS, self-trimming, three-way structure.

The hairpin ribozyme consists of 50 bases, which form two stem-loop domains (domains I and II) (*1-3*), and is able to cleave a target RNA by binding at two stems (helix 1 and helix 2). Domain I has a cleavage site, and consists of two stems (helix 1 and helix 2) and a symmetrical internal loop. Although domain II also has two stems (helix 3 and helix 4), the internal loop is asymmetrical. The base requirements for catalysis have been extensively probed by *in vitro* selection (*4, 5*) and point mutation experiments (*6-8*). Most of the bases that are essential for the cleavage activity, are in these internal loops. It is of particular interest that the 2-amino group of the guanosine at the 3′-side of the cleavage site plays an essential role in ribozyme catalysis (*9*). Another guanine base in the internal loop of domain I has also been revealed to be essential from an experiment using a *cis*-cleavage system (*10*). Since the sequences of both helices 1 and 2 can be changed, hairpin ribozymes have been applied to the cleavage of HIV RNA for gene therapy (*11, 12*).

As the cleavage products, 5′-hydroxyl and 2′,3′-cyclic phosphate groups are produced, similar to the hammerhead ribozyme, and divalent metal ions greatly enhance the catalysis of the ribozyme (*13*). The substitution of a sulfur for the non-bridging oxygens of the cleavage phosphate had little effect on either the cleavage or the ligation (*14, 15*), and recently, cobalt hexamine, which is an exchange-inert metal complex, was also shown to induce catalytic cleavage (*16, 17*). This suggests that the hairpin ribozyme catalyzes transesterification by a unique mechanism.

Folded structures of the ribozyme are thought to be involved in the catalytic mechanism; however, the active conformation is still unknown. The structure of domain I alone has been investigated by an NMR study (*18*), while that of domain II has been suggested from the results of cross-linking experiments (*19*) as well as chemical modification (*20*). Grasby *et al.* proposed another secondary structure for the internal loop of domain II, based on chemical substitutions of the purine functional groups (*8, 21*). Although the active conformation of the ribozyme is still unknown, linker insertion experiments suggest that these domains are aligned in parallel to show RNA cleavage activity (*22, 23*). Hairpin ribozyme analogs with replaced (*24*) and completely separated domains remained active (*25, 26*), confirming an active bent conformation. Gait and co-workers probed the active conformation by inter-domain cross-linking (*27*). They introduced modified bases with attached thiol groups into specific sites and formed disulfide bonds under oxidative conditions. The cleavage activities of the cross-linked ribozymes were investigated, and the tertiary structure of the hairpin ribozyme was modeled from investigation of the correlation between the cross-linked bond lengths and the cleavage activities of cross-linked ribozymes. These studies revealed the active bent conformation, and suggested that a ribose-zipper, such as that in the group I ribozyme (*28*), might be a key to inter-domain docking.

Based on the nature of the interaction between domains I and II, we constructed a three-way hairpin ribozyme, as shown in Fig. 1a (*29*). This ribozyme was able to bind with a target RNA by three hybridizing arms instead of two arms as in the wild type, and provided higher cleavage activities than the wild type with two arms. In addition, we have constructed the three-domain hairpin ribozyme (TriRz) shown in Fig. 1b (*30*), which can process the 3′-side extra sequence from domain I′ by interacting with domain II during transcription. In this report, we describe improved

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Abbreviations: NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; iNOS, inducible nitric oxide synthase; TriRz, a hairpin ribozyme with trimming activity; 5′FTriRz and 3′FTriRz, 5′- and 3′-side fragments processed from TriRz.

TriRz molecules, which release three-way ribozymes more efficiently. In our previous report in which we described a hairpin ribozyme with a three-way junction, we evaluated the *trans*-cleavage activity against a 19-base target RNA, which is part of the mRNA of the inducible nitric oxide synthase (iNOS) from a human glioblastoma cell line (*29*). We now describe the application of the new TriRz to the cleavage of a long substrate RNA (85 mer) transcribed from a synthetic gene for human inducible nitric oxide synthase (iNOS).

MATERIALS AND METHODS

Construction of Self-Trimming Ribozyme Cassette—DNAs were synthesized using phosphoroamidite blocks purchased from Perkin-Elmer Applied Biosystems and were purified by reversed phase and anion exchange HPLC. Eight single-stranded DNA fragments encoding the ribozymes and the T7 RNA promoter were assembled and ligated using T4 DNA ligase as previously reported (*31*). In the same manner, the DNA encoding iNOS85 was constructed. All ribozymes were prepared by transcription from the DNA, using T7 RNA polymerase. The sense sequence of the T7 promoter and the TriRz-1 fragments is 5′G(−19)TTAATACGACTCACTATAG(+1)GTCACTGAGAAGGAGACCAGAGAAACACACGCGGTTCGCGTGGTATATTACCTGGTAAAAAAGTGGCGTCCTGTTTGTTAAACAGAG(+88)AACCAC(+94)3′. To investigate the effect of base pair G67·C91 and linker length on the self-trimming reaction, the underlined sequence of TriRz-1 was changed to GTGGCA in TriRz-2. The sense sequence of the T7 promoter and iNOS85 is 5′G(−19)TTAATACGACTCACTATAG(+1)GAACAACAATGTGGAGAAAGCCCCCTGTGCCACCTCCAGTCCAGTGACACAGGATGACCTTCAGTATCACAACCTCAGC3′. *Eco*RI and *Bam*HI sites were introduced in the DNA template of iNOS85 by polymerase chain reaction, using two primers (5′CGGCGAATTCTTAATACGACTCACTATA3′ for the *Eco*RI restriction site; 5′GCACGGATCCGCTGAGGTTGTGATACTG3′ for the *Bam*HI restriction site). After the PCR products were digested by *Eco*RI and *Bam*HI, the DNA cassettes were ligated into pUC118, which had been cleaved by *Eco*RI (Takara Shuzo) and *Bam*HI (Takara Shuzo). The DNA template was transformed into *Escherichia coli* strain DH5α. After cloning, the plasmid was recovered using a QIAGEN column (QIAGEN) and was used as an *in vitro* transcription template.

Preparation of iNOS85 from Run-Off Transcription—The plasmid DNA encoding iNOS85, which had been digested by *Bam*HI, was used for the run-off transcription. The linearized plasmid DNA (1 μg), 7.5 mM of each NTP, 10 mM DTT, and T7 RNA polymerase from the AmpliScribe™ T7 kit (A.R. Brown Ltd; 2 μl) were dissolved in the transcription buffer (AmpliScribe™ T7 kit) and incubated in a total volume of 20 μl at 37°C. After an incubation for 2 h, DNase I (1 unit) was added to the reaction, which was incubated at 37°C for 15 min. After phenol-chloroform and chloroform extractions, ethanol precipitation was carried out. The transcripts were dissolved in distilled water and loading solution (10 M urea, 50 mM Na_2EDTA, 0.1% bromophenol blue), and were then fractionated and purified from 8% polyacrylamide gels (acrylamide:bisacrylamide, 19:1) containing 8 M urea.

To remove the triphosphate at the 5′-end, the purified transcripts (300-500 pmol) were dissolved in 0.1 M Tris-HCl (pH 8.0; 9 μl) and were treated with alkaline phosphatase from *E. coli* A19 (0.4 unit, 1 μl; Takara Shuzo) at 37°C for 1 h. After phenol-chloroform and chloroform extractions and ethanol precipitations, the transcripts were dissolved in distilled water. The 5′-end of iNOS85 was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara Shuzo).

Self-Trimming Reaction of the Ribozymes—The DNA template (2.5 nM), 0.5 mM of each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-3HCl, 5 mM DTT, 1 unit of RNase inhibitor (Takara Shuzo), 0.5 μCi/μl [α-^{32}P]UTP, and T7 RNA polymerase from the AmpliScribe™ T7 kit (A.R. Brown; 2 μl) were incubated in a total volume of 20 μl at 37°C for 1 h. After ethanol precipitation, loading solution (10 M urea, 50 mM Na_2 EDTA, 0.1% bromophenol blue) was added to the aliquots, which were fractionated on an 8% polyacrylamide gel (acrylamide:bisacrylamide, 19:1) containing 8 M urea. The gel was dried and exposed to a Bioimaging analyzer plate (FUJIX BAS2000), and the percentages of the trimming reactions were measured.

The cleavage rate constants for the *cis*-cleavage reaction were determined as described previously (*32, 33*). Aliquots were taken at time intervals, and the uncleaved fraction [$L/(L+S)$] was plotted *versus* time. The cleavage rate constants (k) for *cis*-cleavage were determined by fitting the data by a least-squares method to the following equation:

$$L/(L+S) = (1 - e^{-kt})/kt.$$

where L is the concentration of the full-length transcript, S is the concentration of the cleaved transcript, t is the time, and k is the unimolecular rate constant for the cleavage.

Trans-Cleavage Reaction—Ribozymes processed after transcription were prepared as described previously (*30*). The ribozyme (5′FTriRz, 5′FTriRz-2, or 5′FTriRz-2A) was dissolved in a cleavage buffer [40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine-3 HCl] to a concentration of 80 nM (50 μl). The 5′-end labeled iNOS85 was dissolved in the cleavage buffer (15 μl) to concentrations ranging from 100 to 1,000 nM. The ribozyme (5′FTriRz, 5′FTriRz-2, or 5′FTriRz-2A; 50 μl) was heated at 90°C for 2 min, and was then transferred to an ice bath. The ribozyme solution was preincubated at 37°C for 5 min; and to start the cleavage reaction, an equal volume of the ribozyme solution (15 μl) was added to the substrate solution (15 μl), which had been heated at 90°C for 2 min and then cooled in an ice bath. The final concentrations of ribozymes and substrates were 40 nM and 50-500 nM, respectively. The reaction mixture (30 μl) was incubated at 37°C, and aliquots were taken at time intervals and added to loading solution containing 10 M urea and 50 mM Na_2EDTA to stop the reaction. After electrophoresis on 8% polyacrylamide gels (acrylamide:bisacrylamide, 19:1) containing 8 M urea, the gels were dried, and the cleavage percentages were determined by a Bioimaging analyzer (Fujix BAS2000). The initial velocities were calculated, and the kinetic parameters for 5′FTriRz, 5′FTriRz-2, and 5′FTriRz-2A were obtained from Hanes-Woolf plots.

RESULTS AND DISCUSSION

Self-Trimming Reactions—TriRz is processed during transcription, and 5′- and 3′-fragments (5′FTriRz, 3′FTriRz) are released (Fig. 1b). The new three-domain ribozymes (TriRz-1 and TriRz-2 in Fig. 2) were designed to form hydrogen bonds with the 5′-side of the substrate RNA sequence after the removal of 3′FTriRz-1 and 3′FTriRz-2. The 3′-end sequences of both 5′FTriRz-1 and -2 are complementary to the 5′-side sequences of the substrate RNA, and a three-way junction (TWJ) would be formed between these 5′-TriRz and the substrate RNA. In TriRz-1, domain I′ is conjugated with the 3′-end of domain II by a six-base linker (AAAAAA), whereas a seven-base linker (AAAAAAG) is inserted in TriRz-2. Since both 5′FTriRz-1 and -2 are designed to bind to the substrate RNA by helices 1, 2, and 5, the stems to the internal loop of domain I′ are different in TriRz-1 and TriRz-2.

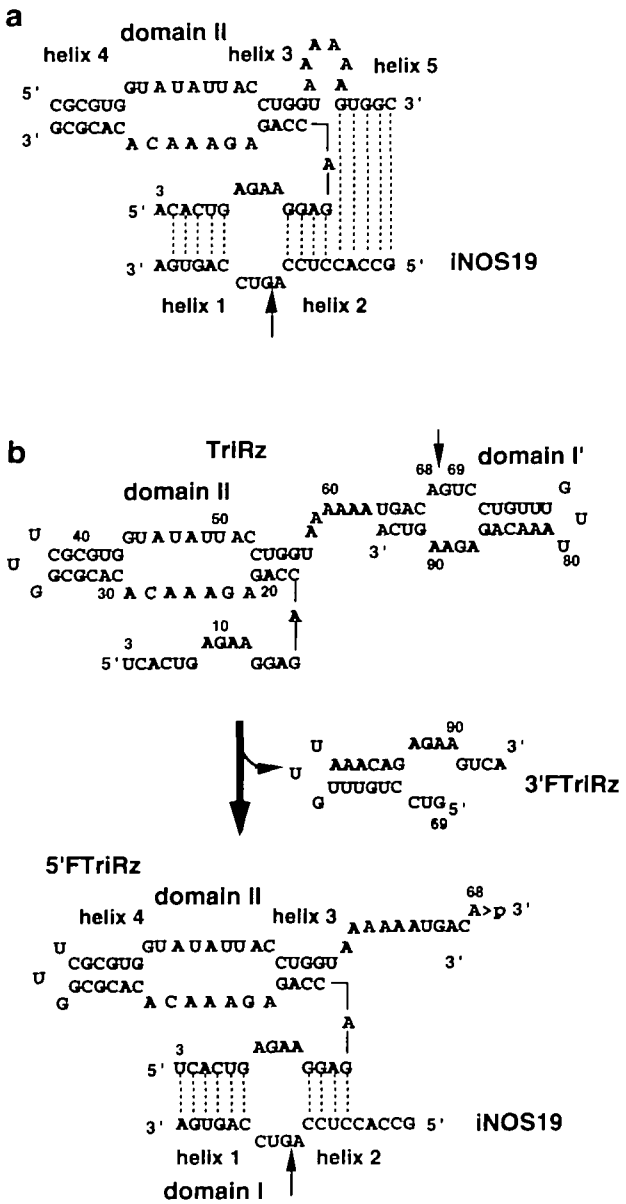

Fig. 1. **(a) Three-way hairpin ribozyme targeted to a 19-mer mRNA of inducible nitric oxide synthase (iNOS19), which consists of two pieces of RNA strands (*29*).** The ribozyme binds with the substrate RNA at helices 1, 2, and 5. The base pairs and the sequence connecting the two domains are represented by broken and continuous lines, respectively. The arrow indicates cleavage site. **(b) Hairpin ribozyme with three-domains (TriRz) (*30*).** TriRz can be self-processed during transcription, and produces 5′FTriRz, which is able to cleave the target RNA in *trans* through binding with the substrate RNA at helices 1 and 2. Arrows indicate cleavage sites.

Since self-trimming reactions occur during transcription from chemically synthesized double-stranded DNAs containing the T7 promoter, T7 RNA polymerase was used for transcription to investigate the self-trimming activities of these three-domain ribozymes. As a consequence, both ribozymes were processed in domain I′ during transcription and the rate constants of intramolecular cleavage were calculated, as shown in Table I. The rate constant of TriRz-1 was about 20-fold lower than that of TriRz-2. Since the rate constant of TriRz (Fig. 1b), which releases 5′FTriRz, was previously reported to be 0.07 min^{-1} (*30*), the self-trimming activity of TriRz-1 was about 7-fold lower than that of TriRz. Since the 5′-side base of the cleavage site can be varied in the wild-type hairpin ribozyme (*4, 6*), the slow trimming reaction of TriRz-1 might be derived from the sequence of the stem to the internal loop of domain I′. The stem to the internal loop of domain I′ contains a G67•C91 base pair in TriRz-1, in contrast to C67•G91 in TriRz and C68•G92 in TriRz-2. Introduction of the G•C pair into the corresponding site in helix 2 (domain I) of the wild type has been reported to decrease the *trans*-cleavage activity (*7, 34*). Since three-domain ribozymes are trimmed by interaction between domain II and domain I′, similar to the domain I-II interaction that occurs in the wild type, the low trimming activity of TriRz-1 seems to be attributable to the G67•C91 base pair in domain I′. On the other hand, TriRz-2 showed about 3-fold higher trimming activity than TriRz. In TriRz-2, domain I′ is connected with domain II by a linker with seven bases (AAAAAAG), in contrast to six bases in TriRz. It is thought that the longer linker of TriRz-2 may enable domain I′ to interact with domain II more efficiently than TriRz-1.

Trans-Cleavage of a Larger Substrate RNA—Although the three-way hairpin ribozyme binding with the target RNA at helices 1, 2, and 5 yields higher cleavage activity toward a short substrate RNA than ribozymes with helices 1 and 2 (*29*), its cleavage activity against a longer substrate RNA has not been studied. Thus, we investigated whether the ribozymes processed from the three-domain ribozymes would also cleave a longer substrate RNA efficiently. Although both 5′-products (5′FTriRz-1 and 5′FTriRz-2) trimmed from TriRz-1 and -2 were designed to hybridize to the target RNA by three helices (1, 2, and 5), TriRz-1 showed little autoprocessing activity. Therefore, the *trans*-cleavage activities of the 5′-trimmed 5′FTriRz (Fig. 1b) and 5′FTriRz-2 were measured. Another 5′FTriRz-2 was then constructed (5′FTriRz-2A, shown in Fig. 2b) so as to have fewer helix 1 base pairs (X3=A), since the turnover activity of 5′FTriRz-2 would be affected, by the binding of the three helices with the substrate. 5′FTriRz, 5′FTriRz-2, and 5′FTriRz-2A were recovered from the trimming reactions of TriRz, TriRz-2, and TriRz-2A, respectively. We previously described the *trans*-cleavage activity of the three-way hairpin ribozyme against a 19-mer RNA re-

presenting part of the mRNA of the inducible nitric oxide synthase (iNOS) from human glioblastoma cell line (*29*). The *trans*-cleavage activities of these self-trimmed ribozymes were tested using an 85 base target RNA from the mRNA of the iNOS, because a longer substrate has not hitherto been studied (*35*) (iNOS85, shown in Fig. 3).

The kinetic rate constants were measured under multiple turnover conditions, and are listed in Table II. 5′FTriRz, 5′FTriRz-2, and 5′FTriRz-2A cleaved iNOS85 at a specific site (Fig. 4). 5′FTriRz-2, with helix 5, had about 80% of the catalytic efficiency of 5′FTriRz without helix 5. This result indicates that the presence of helix 5 did not increase the catalytic efficiency toward such a long target RNA. Although 5′FTriRz-2 showed the highest k_{cat} among the three ribozymes, the K_m value of this ribozyme was also the largest. The reason for the reduced catalytic efficiency in TriRz-2 is unclear; however, 5′FTriRz-2 with helix 5 might fold into a form that is unfavorable for binding with the target RNA. 5′FTriRz-2A, with a helix 1 that is one base shorter than that of 5′FTriRz-2, had about 50% of the catalytic efficiency of 5′FTriRz. This suggests that helix 1 is important for *trans*-cleavage against this substrate, and the

TABLE I. **Intramolecular cleavage rate constants.**

Ribozyme	k_{cis} (min^{-1})
TriRz-1	0.01
TriRz-2	0.21

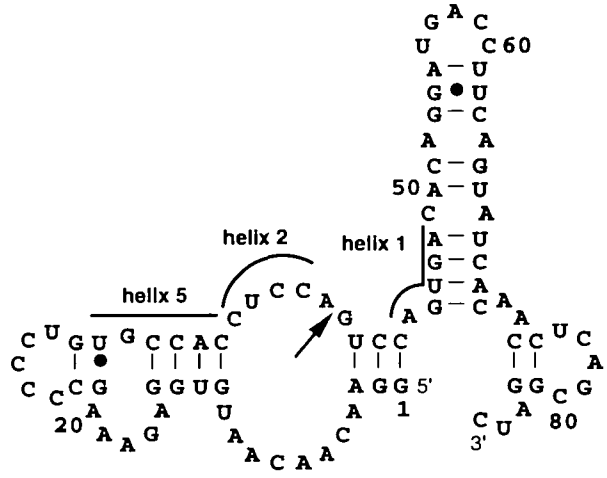

Fig. 3. **Secondary structure model of iNOS85 generated by the MFold program (*36*).** The binding regions of the ribozymes are indicated by solid lines. The arrow indicates the cleavage site.

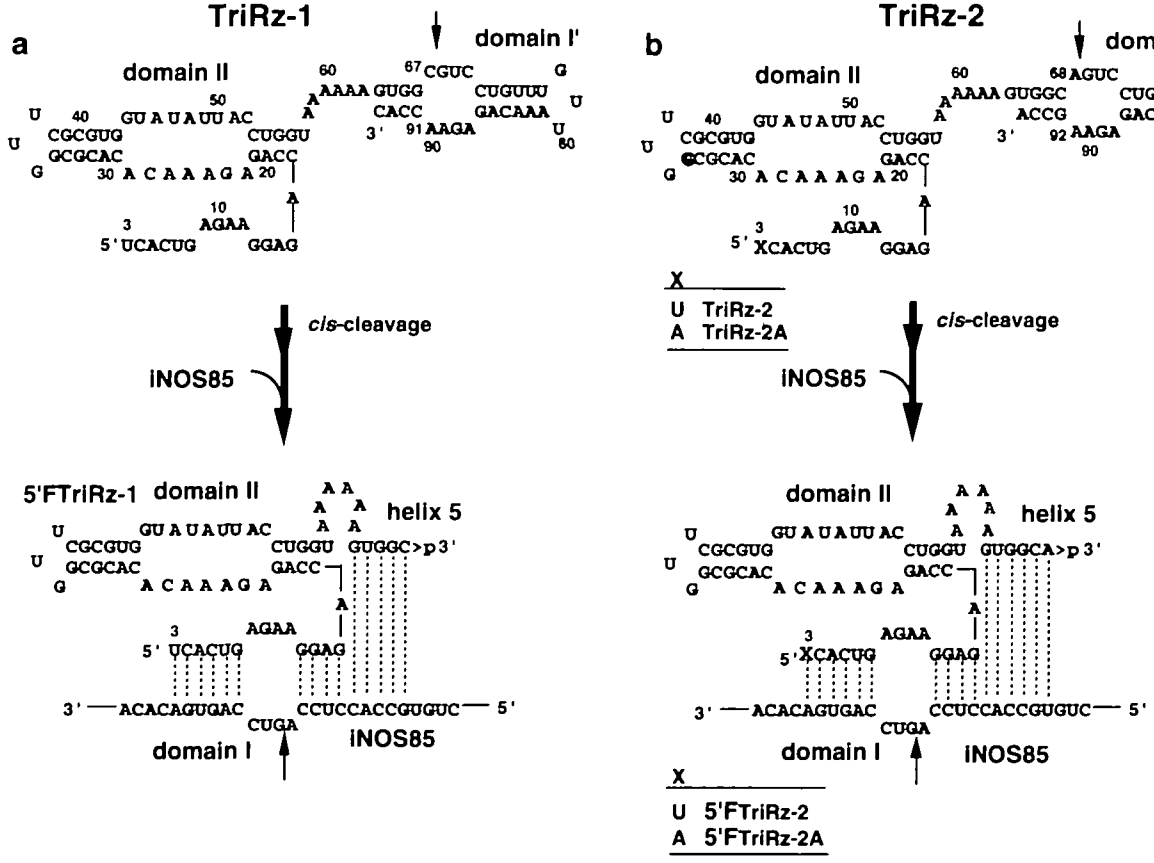

Fig. 2. **Scheme of self-trimming and *trans*-cleavage reactions of three-domain hairpin ribozymes producing three-way hairpin ribozymes.** (a) TriRz-1 is trimmed during transcription, and 5′FTriRz-1 is designed to cleave the substrate RNA (iNOS85) through binding with helices 1, 2, and 5. (b) Both TriRz-2 and TriRz-2A have a linker that is one base longer than TriRz-1, and 5′FTriRz-2 and 5′FTriRz-2A are able to cleave the substrate RNA (iNOS85). Helix 5 of both 5′FTriRz-2 and 5′FTriRz-2A consists of six bases. The base pairs and the sequence connecting the two domains are represented by broken and continuous lines, respectively. Arrows indicate cleavage sites.

TABLE II. **Kinetic parameters for trimmed ribozymes against 5′-end labeled iNOS85.**

Ribozyme	$k_{cat} \times 10^2$ (min^{-1})	K_m (μM)	k_{cat}/K_m (rel)	k_{obs}[a] $\times 10^2$ (min^{-1})
5′FTriRz	1.3	0.11	1.0	3.7
5′FTriRz-2	3.1	0.37	0.75	2.6
5′FTriRz-2A	0.57	0.097	0.54	1.0

[a] k_{obs} was determined from a single-turnover condition containing 20 nM iNOS85 and 0.8 μM ribozyme.

catalytic efficiency of the three-way ribozyme was not alleviated by the instability of helix 1. 5′FTriRz-2A also had the lowest cleavage activity toward the substrate RNA under single turnover conditions (Table II).

It is unclear why the introduction of helix 5 did not increase the cleavage activity of the long substrate RNA. One reason may arise from the secondary structure of iNOS85. Intramolecular folding of the target RNA is thought to decrease the ribozyme activity, because the formation of a ribozyme-substrate complex is inhibited. It is likely that iNOS85 has a more complicated structure than the 19-base substrate. In the predicted secondary structure of iNOS85, shown in Fig. 3, the bases complementary to helix 2 are located in a single-stranded region, whereas the sequences pairing to helices 1 and 5 form intramolecular base pairs within the substrate. Thus, these ribozymes with three helices would have difficulty binding at both helix 1 and 5. Since the formation of helices 1 and 2 is essential for the cleavage activity of the hairpin ribozyme (*37*), the instability of helix 1 might directly influence the cleavage activity, rather than the introduction of helix 5. Helix 5 increased the catalytic efficiency toward the shorter substrate RNA; however, this suggests that helix 5 might not increase the cleavage activity of a substrate, in which the region corresponding to helix 1 is intramolecularly folded. If the region of the substrate corresponding to helix 2 forms a structure that interferes with ribozyme binding, then helix 5 may augment the catalytic activity of the ribozyme, because helix 5 is adjacent to helix 2. It may be important to change the lengths of both helix 1 and helix 5 to facilitate pairing to each target RNA. Two guanine bases from the template (G+1G+2) were added to the 5′-end of these ribozymes, and G+2 can form a base pair with the target RNA. These extra base pairs might be responsible for the difference in the cleavage activity toward the long substrate RNA compared with that reported previously toward a short substrate RNA (*29*).

The three-domain ribozymes described here exhibited about three-fold higher trimming activity than the previous one (TriRz). Considering the overall reactions, from self-trimming to *trans*-cleavage of the substrate, the new three-domain ribozyme (TriRz-2) is potentially more efficient than TriRz. The extra sequences derived from the vector can be trimmed by a hammerhead ribozyme inserted into the 3′-side sequence of the transcripts (*38-40*). However, in the hammerhead ribozyme, the availability of cleavage sites is limited. Since the 5′-side of the cleavage site in the hairpin ribozyme lacks this constraint, the three-domain ribozyme with self-trimming activity may be applied not only to release the *trans*-acting ribozyme with helix 5 but also to add useful sequences with the 3′-end of the hairpin ribozyme.

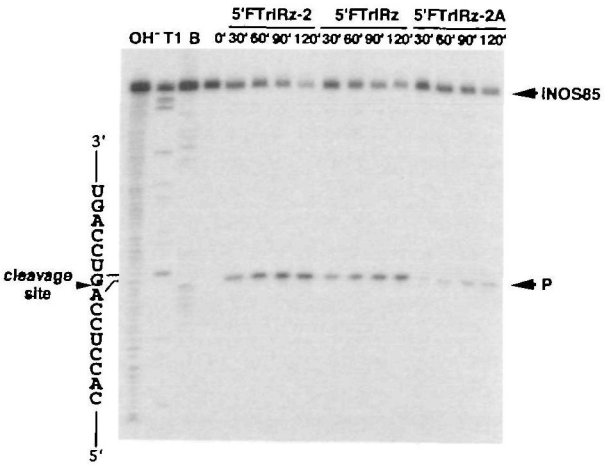

Fig. 4. **Analysis of *trans*-cleavage reactions of iNOS85 by 5′FTriRz, 5′FTriRz-2, and 5′FTriRz-2A, using 15% denaturing polyacrylamide gels.** The 5′-end labeled iNOS85 was partially digested by RNase T1 (lane T1), RNase from *B. cereus* (lane B), and limited alkaline hydrolysis (lane OH^-). Ribozymes and reaction times are indicated at the top of the gel. P indicates 5′-cleavage products. iNOS85 (100 nM) was treated with these ribozymes (100 nM), respectively in the cleavage reactions.

CONCLUSION

Three-domain hairpin ribozymes were designed to release three-way hairpin ribozymes after the self-trimming reaction. TriRz-1, which had a G67·C91 base pair, showed sevenfold lower trimming activity than that previously reported with TriRz for the C67·G91 base pair (*29*). This result proves that the base pair C67·G91, adjacent to the internal loop of domain I′, is as important as the corresponding base pair in domain I. In contrast, TriRz-2, with one more base in its connecting linker, provided threefold higher trimming activity than TriRz.

We investigated whether the trimmed three-way ribozyme could cleave a long substrate RNA efficiently. Both 5′-products trimmed from TriRz and TriRz-2 could cleave a substrate RNA consisting of 85 bases, forming a part of the mRNA of human inducible nitric oxide synthase. The three-way hairpin ribozyme 5′FTriRz-2 had about 80% of the *trans*-cleavage activity of TriRz without a third hybridizing arm (helix 5). 5′FTriRz-2A, with a mis-matched base pair in helix 1, had about 50% of the catalytic efficiency of TriRz. Shortening helix 1 did not compensate for the loss of catalytic efficiency. Since the region corresponding to helix 1 in the substrate RNA is predicted to be a stem region from the secondary structure, it may be difficult for the ribozyme to bind with the substrate RNA. Thus, the stabilization of helix 1 might be important for the efficient cleavage of the longer substrate RNA studied here. However, in terms of the overall reaction from self-trimming to *trans*-cleavage, TriRz-2 is better than TriRz, and applications of the three-domain ribozymes are now being investigated.

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