Published in final edited form as:

Arch Biochem Biophys. 2009 October 15; 490(2): 163–170. doi:10.1016/j.abb.2009.08.020.

Rational optimization of the DSL ligase ribozyme with GNRA/receptor interacting modules

Junya Ishikawa¹, Shigeyoshi Matsumura², Luc Jaeger³, Tan Inoue^{4,5}, Hiroyuki Furuta¹, and Yoshiya Ikawa^{1,6,*}

¹Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka 819-0395, Japan

²Institut de Science et d'Ingenierie Supramoleculaires (ISIS), Université Louis Pasteur, 8 Allée Gaspard Monge, B. P. 70028, 67083 Strasbourg Cedex, France.

³Department of Chemistry and Biochemistry, Biomolecular Science and Engineering Program, University of California at Santa Barbara, Santa Barbara, CA 93106-9510, USA.

⁴Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan.

⁵ICORP, Japan Science and Technology Agency (JST), Tokyo 102-0075, Japan.

⁶PRESTO, Japan Science and Technology Agency (JST), Tokyo 102-0075, Japan

Abstract

The DSL ribozyme is a class of artificial ligase ribozymes with a highly modular architecture, which catalyzes template-directed RNA ligation on a helical substrate module that can be either covalently connected (*cis*-DSL) or physically separated (*trans*-DSL) from the catalytic module. Substrate recognition by the catalytic module is promoted by one or two sets of GNRA/receptor interactions acting as clamps in the *cis* or *trans* configurations, respectively. In this study, we have rationally designed and analyzed the catalytic and self-assembly properties of several *trans*-DSL ribozymes with different sets of natural and artificial GNRA-receptor clamps. Two variants newly designed in this study showed significantly enhanced catalytic properties with respect of the original *trans*-DSL construct. While this work allows dissection of the turnover and catalytic properties of the *trans*-DSL ribozyme, it also emphasizes the remarkable modularity of RNA tertiary structure for nano-construction of complex functions.

Keywords

Ribozyme; RNA motifs; RNA self-assembly; RNA tertiary structure; self-folding; tectoRNA

© 2009 Elsevier Inc. All rights reserved.

*Corresponding author. Tel: +81-92-802-2866; Fax: +81-92-802-2865; yikawa@cstf.kyushu-u.ac.jp.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.XXXX.XX.XXX.

Introduction

Recent progress in the structural biology of functional RNAs and RNA – protein complexes revealed that stable RNAs have hierarchical modular architectures in which limited sets of small “RNA motifs” are commonly employed to build up large and complex 3D-structures [1-3]. While RNA modularity may be an important factor for RNA structure evolution [3-8], it also has potential for facilitating biochemical analyses and technological engineering of naturally occurring RNAs [9,10].

In parallel with the accumulation of structural knowledge of RNA structures, the emergence of a constitutive approach in bioscience has facilitated the bottom-up construction of artificial RNA structures by assembling RNA motifs in a rational manner [11-15]. Some of the earliest examples of rationally designed RNAs took advantage of GNRA/receptor and triple helix interactions to promote either self-folding or self-assembly of RNA molecules [12,15]. For instance, a self-folding RNA (termed type-B RNA) was computationally designed to direct the folding of three helices through these structural motifs in a manner reminiscent of the P4-P6 domain of self-splicing group I introns [15]. Subsequently, the type-B RNA was used as a structural scaffold for isolating modular catalytic RNAs and RNA aptamers through *in vitro* evolution [16-18].

The *cis*-DSL ribozyme, an artificial ribozyme generated by installing a catalytic module into type-B RNA, promotes the template-dependent RNA ligation reaction with a rate 10^6 -fold faster than the corresponding non-catalyzed reaction [18,19]. Interestingly, due to its modularity, the catalytic module of the DSL ribozyme is structurally well defined and could be engineered further by taking advantage of another class of designed RNA with self-dimerizing ability (tectoRNA) [11,12,20]. In the resulting ribozyme (*trans*-DSL-1 ribozyme), the catalytic module can assemble non-covalently through two sets of GNRA/receptor interactions with a substrate module that is comprised of two RNA termini aligned by a template strand [16,21]. More recently, the modular characteristics of the DSL ribozyme were used to improve its catalytic activity through *in vitro* selection and continuous evolution [22], to convert it into allosteric ribozymes [23], and to design an *in vitro* selection system for isolating novel RNA – RNA interaction motifs [24].

In naturally occurring ribozymes, fine-tuning of the modular structure motifs that promote the folding and assembly of functionally important domains has been employed as a strategy to engineer variants that exhibit superior catalytic ability [25-28]. For example, group I intron ribozymes with improved activity or thermostability have been constructed by changing their original GNRA-receptor interactions to those with greater thermodynamic stability [25-27]. Here, we report the rational modular design of new *trans*-DLS ribozymes by taking advantage of similar strategies to improve their catalytic activity and facilitate the characterization of their enzymatic properties.

Materials and methods

Materials

DNA oligonucleotides for PCR were purchased from Sigma-Genosys Japan (Hokkaido, Japan) and Fasmac (Kanagawa, Japan). A DNA/RNA chimeric oligonucleotide and 5'-FAM-labeled RNA oligonucleotides for the *cis*- and *trans*-acting ribozyme reactions were purchased from Japan BioService (Saitama, Japan) and IDT (Coralville, IA), respectively. Alexa488 fluorophore possessing a hydrazide moiety was purchased from Molecular Probes (Eugene, OR).

RNA preparation

In this study, all RNAs except the 5'-FAM-labeled substrate-2 were prepared by *in vitro* transcription with T7 RNA polymerase. As the template for *in vitro* transcription, PCR products bearing T7 promoter sequences were amplified from plasmids encoding the sequences of the respective ribozyme RNAs and the precursors of the substrate-1 RNAs possessing a *cis*-acting hammerhead ribozyme in the 3'-ends [21]. Transcripts of the *cis*-DSL ribozymes, the catalytic unit RNAs of the *trans*-DSL-1 ribozymes were purified by electrophoresis on 5% and 12% denaturing polyacrylamide gels, respectively. The mature substrate-1 RNAs were prepared by autoprocessing of their precursor RNAs with a *cis*-acting hammerhead ribozyme in the 3'-ends and purified by electrophoresis on 12% denaturing polyacrylamide gels [21].

Labeling of the 3'-termini of RNAs with fluorophores

For activity assays and folding analysis of the *cis*-DSL ribozymes, the 3'-ends of the ribozymes were labeled with BODIPY fluorophore bearing a primary amine [29]. Labeling reaction was performed as described [30].

For gel mobility shift assays of the *trans*-DSL ribozymes, the 3'-ends of the enzyme unit RNAs were labeled with Alexa488 fluorophore possessing a hydrazide moiety. Labeling reaction was performed as described [31].

Activity assay of the *trans*-acting DSL ribozymes

Catalytic unit RNA and substrate-1 RNA were dissolved in water and denatured by heating at 80°C for 2 min. A 10× concentrated reaction buffer was added to the mixture followed by incubation at 37°C for 5 min. The ligation reaction was started by addition of FAM-labeled substrate-2. Final concentrations of catalytic unit, substrate-1, and substrate-2 RNAs in respective assays are described in the figure legends. Ligation reactions were performed in the presence of 30 mM Tris-Cl adjusted to appropriate pH, 25 mM KCl, and an appropriate concentration of MgCl₂. Reactions were stopped by adding an equal volume of 75% formamide containing 100 mM EDTA. Samples were separated on 12% polyacrylamide denaturing gels and quantified using a Pharos FX FluoroImager (BioRad, Hercules, CA). The rate constants for formation of the ligated product (k_{obs}) were determined by fitting the data to the following equation:

$$\text{Fraction reacted} = F_a (1 - e^{-kt}),$$

where t is time, k is the observed rate constant for formation of the ligated product (k_{obs}), and F_a is the calculated final yield that corresponds to the fraction of the substrate – ribozyme complex in an active conformation. All experiments were repeated at least twice. The mean values are shown in the figures, and error bars indicate the minimal and maximal values. For multiple turnover reactions, turnover frequency per hour (abbreviated as TOF) was determined from the initial linear phase in the reaction time course, where the product yield was less than 20%.

Kinetic assays of the *cis*-acting DSL ribozymes

The *cis*-DSL ribozymes the 3'-ends of which were labeled with BODIPY fluorophore were dissolved in H₂O and then denatured by incubation at 80°C for 5 min, followed by incubation at 37°C for 5 min. RNA folding was initiated by adding 5× concentrated reaction buffer at 37°C. After incubation of the mixture for 10 min, 10× concentrated substrate (5'-CGTACACGTACTCACGCGTATACA-rGrUrCrCrArC-3' where the first 24 and the last 6 nucleotides were DNA and RNA, respectively) was added to initiate the reaction. The reaction

conditions are described in the Results and Discussion section as well as the figure legends. At various time points, aliquots were treated with an equal volume of stop solution consisting of 80% formamide, 90 mM EDTA, and 0.1% xylene cyanol. Samples were separated on 5% denaturing polyacrylamide gels. Quantification of the products and calculation of the rate constants were performed as described for the *cis*-acting ribozymes.

Gel mobility shift assay of *trans*-DSL ribozymes

Aqueous solutions containing the product-form of the substrate unit at a given concentration (0 – 400 nM) and the Alexa488-labeled enzyme unit (25 nM) were heated at 80°C for 3 min. A 10× concentrated folding buffer was added to adjust the mixture to 50 mM Tris-OAc (pH 7.5) and 25 mM Mg(OAc)₂. The resulting mixture was incubated at 4°C for 30 min. After adding 6× concentrated gel loading buffer consisting of glycerol and bromophenol blue (0.1%), the samples were loaded onto a 10% native polyacrylamide gel (39:1 acrylamide:bisacrylamide) containing 50 mM Tris-OAc (pH 7.5) and 25 mM Mg(OAc)₂. Electrophoresis was carried out at 4°C with 17 V/cm for the initial 2 min and then 5 V/cm. The resulting gels were analyzed with a Pharos FX FluoroImager.

Results

Optimization of two sets of GNRA-receptor clamps in the *trans*-DSL ribozyme

Prior to this study, two types of *trans*-DSL-1 ribozyme, designated *trans*-1a [16] and *trans*-1b [21] in Table 1, were designed and their activities were investigated. The association between the substrate and enzyme unit in the *trans*-1a ribozyme is stronger than that in *trans*-1b because the interaction between GAAA and R(11nt) is much stronger than that between GUAA and R(B7.8) [20,32]. However, the molecular design of *trans*-1a has a serious defect because its enzyme and substrate unit can form a homosubstrate dimer and homoenzyme unit dimer [11, 12,21]. These dimers should reduce the overall catalytic efficiency of the ribozyme. As direct comparison of *trans*-1a and *trans*-1b had not been performed, we first compared their activities under common reaction conditions. In the presence of 4.0 μM substrate-1, 1.0 μM FAM-labeled substrate-2, and 0.1 μM enzyme unit, *trans*-1a and *trans*-1b showed similar activities (Fig. 2A, Table 2), indicating that both the affinity in formation of the substrate – enzyme complex and the selectivity of hetero-dimerization (substrate – enzyme complexation) against homosubstrate- or homoenzyme-dimerizations are important factors for the overall efficiency of multiple turnover reaction. This result predicts that turnover reaction can be improved by increasing the affinity between substrate and enzyme unit of *trans*-1b without sacrificing its binding selectivity.

To fulfill the above requirements, we prepared two new variant ribozymes designated as *trans*-1c and *trans*-1d. The two variants both possess a GAAA-R(11nt) unit and a GGAA-R(1) unit at different positions (Fig. 1C and Table 1). The R(1) motif, which was isolated recently by *in vitro* selection, selectively recognizes the GGAA loop, and the binding affinity of the GGAA-R(1) pair was markedly stronger than that of the GUAA-R(B7.8) pair and close to that of the GAAA-R(11nt) pair [20].

Before investigation of the catalytic activities of *trans*-1c and *trans*-1d, we evaluated the physical affinities between the enzyme and substrate units. Gel mobility shift assays of the post-reaction complexes of *trans*-1c and *trans*-1d were carried out in the presence of 25 mM Mg²⁺ (Figs. 2B and 2C). The assay results indicated that the approximate concentrations of enzyme RNA where half of the fluorescently labeled product RNA formed a complex with the enzyme RNA was 200 – 250 nM for *trans*-1c (Fig. 2B) or 75 – 100 nM for *trans*-1d (Fig. 2C). Thus, the substrate – enzyme complexes of *trans*-1c and *trans*-1d have similar stabilities, with a difference within two- or threefold. (note: we could not determine the precise *K_d* values

because of the limited sensitivity of fluorescently labeled product RNA with a lower detection limit of 25 nM).

We then investigated the activities of several variants of the *trans*-DSL-1 ribozyme involving *trans*-1c and *trans*-1d under multiple turnover conditions (Fig. 2A, see also Fig. S1 in Supplementary Data). Consistent with our rational prediction, *trans*-1c and *trans*-1d were more active than *trans*-1a and *trans*-1b (Fig. 2A). We also determined the turnover frequency per hour (abbreviated as TOF) from the initial linear phase in the reaction time course. TOF values of *trans*-1c and *trans*-1d were also higher than those of *trans*-1a and *trans*-1b (Fig. 2D). These results clearly indicated that rational modular optimization of affinity and selectivity of the substrate – enzyme complex was effective for improving the turnover ability of the *trans*-DSL ribozyme.

The rate constant of product formation (k_{obs}) of the *trans*-1c ribozyme with the GGAA-R(1) unit as the upper clamp module ($2.3 \times 10^{-2} \text{ min}^{-1}$) was slightly (1.4-fold) higher than that of *trans*-1d with the GAAA-R(11nt) unit ($1.7 \times 10^{-2} \text{ min}^{-1}$) (Table 2). The TOF value of *trans*-1c was also slightly higher than that of *trans*-1d (Fig. 2D). With 10 nM enzyme unit, the k_{obs} values were $2.6 \times 10^{-3} \text{ min}^{-1}$ and $1.7 \times 10^{-3} \text{ min}^{-1}$ for *trans*-1c and *trans*-1d, respectively (Table 2, see also Figs. S2A and S2B in Supplementary Data). While k_{obs} values were approximately 10-fold smaller than those with 100 nM enzyme, TOFs with 10 nM enzyme were similar to those with the enzyme at 100 nM (Fig. 2D).

Comparative analysis of the *trans*-1c and *trans*-1d ribozymes

To compare the activities of *trans*-1c and *trans*-1d systematically, we first examined the effects of pH of the reaction buffer. In the presence of 4.0 μM substrate-1, 1.0 μM FAM-labeled substrate-2, 10 nM enzyme, and 25 mM Mg^{2+} , the reactions of *trans*-1c and *trans*-1d were examined at 37°C under conditions of different pH between 6.5 and 8.5 (Fig. S2A and S2B in Supplementary Data). TOF values of the two ribozymes increased with increasing pH and reached 43 h^{-1} and 46 h^{-1} for *trans*-1c and *trans*-1d, respectively, at pH 8.5 (Fig. 3A). The logarithmic plots of k_{obs} vs. pH exhibited linear relationships between pH 7.0 and 8.0 with a slope of 1.2 for both (Fig. S2C in Supplementary Data). This was similar to the reported value of 1.4 for *cis*-DSL-1S [19].

To examine the effects of Mg^{2+} ion concentration and reaction temperature, we measured the TOF values of the reaction at three different temperatures in the presence of 25, 50, and 100 mM Mg^{2+} (Figs. 3B – 3D). TOF values of the two ribozymes both increased with increasing Mg^{2+} ion concentration. At 25 and 50 mM Mg^{2+} ions, no distinct relationship was observed between TOF and reaction temperature, presumably because reaction temperature had inverse effects on the substrate binding step and chemistry step. At 100 mM Mg^{2+} ions, however, TOF values of *trans*-1c and *trans*-1d increased with increasing reaction temperature, suggesting that a Mg^{2+} ion concentration of 100 mM is sufficient to ensure efficient association between the substrate-1/-2 complex and the enzyme even at 42°C. To determine the ability of total turnover of the enzyme unit, reactions were performed under conditions where the substrate unit was present in 1000-fold excess over the enzyme unit (4.0 μM substrate-1, substrate-2, 4.0 nM enzyme). In the presence of 50 mM Mg^{2+} , one enzyme unit joined over 400 substrate units during a 24-h reaction (Fig. 4). These turnover abilities are much higher than those of the previous ligase ribozymes [21]. Under these conditions, TOF values of *trans*-1c and *trans*-1d were 37 h^{-1} and 30 h^{-1} , respectively. Comparison of these TOF values with those in Fig. 3C (17 h^{-1} and 13 h^{-1} for *trans*-1c and *trans*-1d with 50 mM Mg^{2+} at 37°C, respectively) suggested that the initial concentration of the substrate-1/-2 complex (which is limited by the concentration of the substrate-2) affects the turnover efficiency of the ribozyme.

Comparative analysis of the *cis*-DSL and *trans*-DSL ribozymes

To dissect the catalytic ability of *trans*-1c and *trans*-1d ribozymes, we carried out the ligation reaction with an excess amount of the enzyme unit over the substrate-1/-2 complex and compared the results with those of the *cis*-DSL ribozyme (Fig. 5). In the presence of 10 μM ribozyme unit in 2.5- and 10-fold excess over the substrate-1 (4.0 μM) and FAM-labeled substrate-2 (1.0 μM), respectively, both *trans*-1c and *trans*-1d exhibited efficient ligation ability, and the k_{obs} value of *trans*-1c (0.54 min^{-1}) was slightly (1.4-fold) higher than that of *trans*-1d (0.39 min^{-1}) (Fig. 5A, Table 2). Similar results were also obtained in the activity assay of the *cis*-DSL ribozyme possessing GAAA-R(11nt) or GGAA-R(1) as upper clamp module. Under reaction conditions identical to those employed for *trans*-ribozyme (30 mM Tris-Cl, pH 7.5, 25 mM KCl, and 25 mM MgCl_2 , 37°C), the reaction between *cis*-DSL-G2A2 possessing the GGAA-R(1) unit (1.0 μM) and the substrate RNA (4.0 μM) gave a k_{obs} value of 0.12 min^{-1} , which was also slightly (1.3-fold) higher than the value of 0.09 min^{-1} for the reaction of the parental *cis*-DSL-1S possessing the GAAA-R(11nt) unit (Fig. 5B, Table 3). These results suggested that the identity of the upper clamp module yielded a slight but distinct difference in the activity of the *trans*- and *cis*-ribozymes. These results were also consistent with the recent finding that the DSL ribozyme accelerates the ligation reaction more efficiently in the *trans* configuration than in the *cis* configuration [21].

Modulating the ability of the catalytic unit

To gain additional insight into the reaction by the *trans*-DSL ribozyme, we attempted to modulate the ability of the catalytic unit by preparing a variant of *trans*-1d (termed *trans*-1d-old) with a less active catalytic unit extracted from the *cis*-DSL-01 ribozyme (Fig. 1D and Table 1) [16].

We first compared the effect of the catalytic unit by employing *cis*-ribozymes. The *cis*-DSL-01 ribozyme ($k = 7.3 \times 10^{-3} \text{ min}^{-1}$ obs) was 12-fold less active than *cis*-DSL-1S (0.09 min^{-1}) (Fig. 5B, Table 3). This result was consistent with the findings for *trans*-ribozymes under conditions of enzyme excess where k_{obs} of *trans*-1d-old (0.025 min^{-1}) was 16-fold smaller than that of *trans*-1d (0.39 min^{-1}) (Fig. 5A, Table 2).

To determine whether the identity of the catalytic unit affects the physical affinity between the substrate and enzyme units, we performed mobility shift assay of the post-reaction complex of *trans*-1d-old in the presence of 25 mM Mg^{2+} (Fig. 5C). The approximate concentration of the enzyme RNA where half of the fluorescently labeled RNA product formed a complex with the enzyme RNA was 75 – 100 nM for *trans*-1d-old (Fig. 5C). This value was similar to that of *trans*-1d (Fig. 2C), indicating that the thermodynamic stability of the product – enzyme complex was unaffected by replacement of the catalytic unit.

The catalytic activity of *trans*-1d-old was then investigated in the presence of a 100-fold excess substrate; the TOF value of *trans*-1d-old (TOF = 1.3 h^{-1}) was 6.8-fold smaller than that of *trans*-1d (TOF = 8.8 h^{-1} , see Fig. 2D), whereas the k_{obs} value of *trans*-1d-old ($4.2 \times 10^{-4} \text{ min}^{-1}$) was 4.0-fold smaller than that of *trans*-1d ($1.7 \times 10^{-3} \text{ min}^{-1}$) (Fig. 5D, Table 2).

Discussion

In this study, we have rationally designed and analyzed the catalytic properties of several *trans*-DSL ribozymes with different sets of natural and artificial GNRA-receptor clamps. We identified particular sets of GNRA-receptor pairs that improved turnover activity for the *trans*-DSL ribozyme. Using these improved ribozymes, we also analyzed the multiple turnover reaction catalyzed by the *trans*-DSL ribozyme.

Effects of substrate binding and product release in the turnover reaction

Comparison of *trans*-1d and *trans*-1d-old provided insight into the rate-limiting step of the multiple turnover reaction of the *trans*-DSL-1 ribozyme. Under the single turnover conditions, *cis*- and *trans*-ribozyme with the prototype catalytic unit (extracted from the *cis*-DSL-01) were 12- and 16-fold less active than the corresponding ribozymes with the most active catalytic unit (extracted from *cis*-DSL-1S), respectively (Figs. 5A and 5B). Under multiple turnover conditions, the TOF value of *trans*-1d-old ribozyme was also 6.8-fold smaller than that of *trans*-1d (Fig. 5D). However, the ability of the catalytic module affected the multiple turnover reaction of the *trans*-DSL ribozyme less significantly than the single turnover reaction with excess enzyme where the difference between *trans*-1d and *trans*-1d-old was 16-fold (Table 2).

On the other hand, the gel shift analyses of *trans*-1d and *trans*-1d-old showed no marked differences in complex formation of the product and enzyme unit (Figs. 2C and 5C), suggesting that product release is unlikely to determine the efficiency of the turnover reaction.

While dissociation of the product RNA is unlikely to limit the turnover efficiency, the binding step of the substrate-1/-2 complex to the enzyme unit seems to affect the turnover efficiency because: i) comparison of *trans*-1b and *trans*-1d suggested that affinity between the substrate and enzyme positively affects turnover efficiency (Fig. 2D), and ii) comparison of Figs. 3B and 4 suggested that TOF values of *trans*-1c and *trans*-1d were improved by increasing the concentration of substrate-2 that governs the concentration of substrate-1/-2 complex. Currently, this discussion is one of possible explanations and the kinetic parameters of substrate binding and product dissociation needs to be determined to fully elucidate the reaction mechanism of the *trans*-DSL ribozyme.

Effects of the chemistry step (catalysis) in the turnover reaction

While several observations suggested that substrate binding affects the turnover ability of *trans*-DSL ribozymes, another line of evidence suggested that the chemical step (catalysis) also influenced the turnover reaction. The pH dependency of the reaction rates of multiple turnover reactions of *trans*-DSL ribozymes (slope = 1.2, Fig. S1) was similar to that of the single turnover reaction by *cis*-DSL-1S ribozyme (slope = 1.4, [19]), suggesting that the common step participates in the rate-limiting of the two reactions. This is also consistent with the observation that the slight but distinct differences in k_{obs} value between *trans*-1c and *trans*-1d were similar between substrate-excess reaction (1.4-fold or 1.6-fold, Table 2) and ribozyme-excess reaction (1.4-fold, Table 2). These differences were also similar to those for *cis*-DSL ribozyme (1.3-fold, Table 3). These data suggest that the differences in substrate-excess (multiple turnover) reactions and enzyme-excess reactions may originate from the common step.

Conclusions

Taken together, the experimental data obtained in this study suggested that the turnover efficiency of *trans*-DSL ribozymes seems to be governed by both the substrate-binding step and chemistry step. The dominant factor limiting the reaction may alter depending on reaction conditions. The multiple turnover ability is not only one of the fundamental properties of modern protein enzymes but is also a crucial property of RNA replicase ribozyme that would be a central issue in the hypothetical RNA world [33,34]. This study suggested that the DSL ribozyme is a promising model to understand the structure, function, and evolution of *trans*-acting ribozymes because its modularity enables us to employ a rational approach to elucidate how RNA catalysts acquired and optimized their turnover activity. By employing *trans*-1c and *trans*-1d, advanced forms of the *trans*-DSL ribozyme with self-replicating ability or allosteric function are under development. This study also emphasizes the suggestion that modularity of

RNA tertiary structures would be crucial for both natural evolution and rational design of functional RNAs with complex 3D structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was partly supported by Grant-in-Aids on Innovative Areas “Emergence in Chemistry” (No.21111518 to Y.I.), for Exploratory Research [No.19657071 to Y.I.], and the Global COE program, “Science for Future Molecular Systems” [to H.F.] from The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and also by a grant from the National Institutes of Health [R01-GM079604 to L.J.]. L.J., H.F., and Y.I. also thank to a joint program between Kyushu University Global COE (Science for Future Molecular Systems) and the California NanoSystems Institute (CNSI) for supporting this research.

Abbreviations used

BODIPY	boron-dipyrromethene
DSL	designed and selected ligase
FAM	carboxyfluorescein
R(11nt)	11-nucleotides receptor
THS	triple helical scaffold
TOF	turnover frequency per hour

References

1. Batey RT, Rambo RP, Doudna JA. *Angew. Chem. Int. Ed* 1999;38:2326–2343.
2. Hendrix DK, Brenner SE, Holbrook SR. *Q. Rev. Biophys* 2005;38:221–243. [PubMed: 16817983]
3. Jaeger L, Verzemnieks EJ, Geary C. *Nucleic Acids Res* 2009;37:215–230. [PubMed: 19036788]
4. Kazantsev AV, Pace NR. *Nat. Rev. Microbiol* 2006;4:729–740. [PubMed: 16980936]
5. Vicens Q, Cech TR. *Trends Biochem. Sci* 2006;31:41–51. [PubMed: 16356725]
6. Ikawa Y, Shiraishi H, Inoue T. *Nat. Struct. Biol* 2000;7:1032–1035. [PubMed: 11062558]
7. Ikawa Y, Sasaki K, Tominaga H, Inoue T. *J. Biochem* 2003;133:665–670. [PubMed: 12801919]
8. Y., Ikawa; T., Shiohara; S., Ohuchi; T., Inoue. *J. Biochem* 2009;139:429–435.
9. Jaeger L L, Chworos A A. *Curr. Opin. Struct. Biol* 2006;16:531–543. [PubMed: 16843653]
10. Saito H, Inoue T. *Int. J. Biochem. Cell. Biol* 2009;41:398–404. [PubMed: 18775792]
11. Jaeger L, Leontis NB. *Angew. Chem. Int. Ed* 2000;39:2521–2524.
12. Jaeger L, Westhof E, Leontis NB. *Nucleic Acids Res* 2001;29:455–463. [PubMed: 11139616]
13. Chworos A, Severcan I, Koyfman AY, Weinkam P, Oroudjev E, Hansma HG, Jaeger L. *Science* 2004;306:2068–2072. [PubMed: 15604402]
14. Severcan I, Geary C, Verzemnieks E, Chworos A, Jaeger L. *Nano Lett* 2009;9:1270–1277. [PubMed: 19239258]
15. Ikawa Y, Fukada K, Watanabe S, Shiraishi H, Inoue T. *Structure* 2002;10:527–534. [PubMed: 11937057]
16. Ikawa Y, Tsuda K, Matsumura S, Inoue T. *Proc. Natl. Acad. Sci. USA* 2004;101:13750–13755. [PubMed: 15365187]
17. Fujita Y, Furuta H, Ikawa Y. *RNA* 2009;15:877–888. [PubMed: 19307294]
18. Shiohara T, Saito H, Inoue T. *Nucleic Acids Res* 2009;37:e23. [PubMed: 19136470]
19. Horie S, Ikawa Y, Inoue T. *Biochem. Biophys. Res. Commun* 2006;339:115–121. [PubMed: 16298344]

20. Geary C, Baudrey S, Jaeger L. *Nucleic Acids Res* 2008;36:1138–1152. [PubMed: 18158305]
21. Matsumura S, Ohmori R, Saito H, Ikawa Y, Inoue T. *FEBS lett* 2009;583 in press.
22. Voytek SB, Joyce GF. *Proc. Natl. Acad. Sci. USA* 2007;104:15288–15293. [PubMed: 17878292]
23. Kuramitsu S, Ikawa Y, Inoue T. *Nucleic Acids Symp. Ser* 2005;49:349–350.
24. Ohuchi SP, Ikawa Y, Nakamura Y. *Nucleic Acids Res* 2008;36:3600–3607. [PubMed: 18460545]
25. Costa M, Michel F. *EMBO J* 1995;14:1276–1285. [PubMed: 7720718]
26. Tanner MA, Cech TR. *RNA* 1996;2:74–83. [PubMed: 8846298]
27. Ikawa Y, Naito D, Shiraishi H, Inoue T. *Nucleic Acids Res* 2000;28:3269–3277. [PubMed: 10954594]
28. Qin H, Sosnick TR, Pan T. *Biochemistry* 2001;40:11202–11210. [PubMed: 11551219]
29. Ikawa Y, Moriyama S, Furuta H. *Anal. Biochem* 2008;378:166–170. [PubMed: 18455995]
30. Oh BK, Pace NR. *Nucleic Acids Res* 1994;22:4087–4094. [PubMed: 7524035]
31. Murakami H, Bonzagni NJ, Suga H. *J. Am. Chem. Soc* 2002;124:6834–6835. [PubMed: 12059198]
32. Costa M, Michel F. *EMBO J* 1997;16:289–302.
33. Szostak JW, Bartel DP, Luisi PL. *Nature* 2001;409:387–390. [PubMed: 11201752]
34. Ellington AD, Chen X, Robertson M, Syrett A. *Int. J. Biochem. Cell. Biol* 2009;41:254–265. [PubMed: 18775793]

Figure 1. Secondary structure of DSL-1S and its derivatives

(A) Secondary structure of the *cis*-DSL-1S. Regions enclosed with solid lines indicate the catalytic module, and the upper and lower clamp modules. The upper and clamp modules are GAAA-11nt receptor interaction and triple helical scaffold (THS motif). GAAA loop, its receptor, and THS motif are shown in red, green, and blue, respectively. The last 5 nucleotides of the substrate hybridize with DSL-1S.

(B) Secondary structure of *trans*-DSL-1 (also designated as *trans*-1a in this study) that consists of the substrate unit and enzyme unit. The substrate unit is composed of two RNA fragments (substrate-1 and substrate-2). Regions enclosed with solid lines indicate the catalytic module,

and the upper and lower clamp modules. The upper and clamp modules are both GAAA-11nt receptor interactions (GAAA loop and its receptor are shown in red and green, respectively). (C) Secondary structures of the three classes of GNRA tetraloops (red) and their receptors (green) employed as clamp modules of the DSL ribozymes. (D) Secondary structures of the catalytic modules employed in this study.

Figure 2. Multiple turnover reactions of the four *trans*-DSL ribozymes

(A) Time courses of the ligation reactions of *trans*-1a, *trans*-1b, *trans*-1c, and *trans*-1d.

Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM $MgCl_2$ and 25 mM KCl. The final concentrations of the substrate-1, FAM-labeled substrate-2, and the catalytic unit were 4.0 μ M, 1.0 μ M, and 0.10 μ M, respectively. “P” and “sub2” indicate the amounts of the ligated product and the FAM-labeled substrate-2, respectively.

(B and C) Gel mobility shift assays of *trans*-1c (B) and *trans*-1d (C) in the presence of 25 mM Mg^{2+} ions at pH 7.5. The product form of the Alexa488-labeled substrate unit (25 nM) was titrated with various concentrations of the catalytic unit RNA (0 – 400 nM). Upper and lower bands correspond to the product – ribozyme complex and free product unit, respectively.

(D) Turnover frequency of the ligation reaction of the four *trans*-DSL ribozymes. Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl.

Figure 3. Effects of pH, Mg²⁺ ion concentration, and reaction temperature on the turnover efficiencies of the *trans*-1c and *trans*-1d ribozymes

(A) Effects of pH on the turnover frequency of the ligation reaction of *trans*-1c and *trans*-1d. Reactions were performed at 37°C in the presence of 30 mM Tris-Cl, 25 mM MgCl₂, and 25 mM KCl. The final concentrations of substrate-1, FAM-labeled substrate-2, and the catalytic unit were 4.0 μM, 1.0 μM, and 0.010 μM, respectively.

(B – D) Effects of temperature on the turnover frequency of the ligation reactions of *trans*-1c and *trans*-1d. Reactions were performed in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM KCl, 25 mM (B), 50 mM (C), or 100 mM (D) MgCl₂. The final concentrations of substrate-1,

FAM-labeled substrate-2, and the catalytic unit were 4.0 μM , 1.0 μM , and 0.010 μM , respectively.

Figure 4. Multiple turnover reactions of the *trans*-DSL ribozymes in the presence of a 1000-fold excess of substrate

Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 50 mM MgCl₂, and 25 mM KCl. The final concentrations of substrate-1, FAM-labeled substrate-2, and the catalytic unit were 4.0 μM, 4.0 μM, and 4.0 nM, respectively. “P” and “sub2” indicate the amounts of the ligated product and the FAM-labeled substrate-2, respectively.

Figure 5. Effects of the ability of catalytic module on the DSL ribozyme reactions

(A) Time courses of the ligation reactions of *trans*-1c, *trans*-1d, and *trans*-1d-old under enzyme-excess conditions. Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl. The final concentrations of substrate-1, FAM-labeled substrate-2, and the catalytic unit were 4.0 μM, 1.0 μM, and 10 M, respectively. “P” and “sub2” indicate the amounts of the ligated product and FAM-labeled substrate-2, respectively.

(B) Time courses of the ligation reactions of *cis*-DSL-G2A2, *cis*-DSL-1S, and *cis*-DSL-01. To ensure the structure of the receptor motif, the sequences enclosing the receptor motif were 5'-GCAAACGC-3' for R(11nt) in *cis*-DSL-1S and *cis*-DSL-01 (Fig. 1A), and 5'-

CGCUUCGGCG-3' for R(1) in *cis*-DSL-G2A2. Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl. The final concentrations of the substrate and BODIPY-labeled ribozyme were 4 μM and 1 μM, respectively. "P" and "Rz" indicate the amounts of the ligated product and BODIPY-labeled precursor ribozyme, respectively.

(C) Gel mobility shift assay of the *trans*-1d-old ribozyme in the presence of 25 mM Mg²⁺ ions at pH 7.5. The product form of the Alexa488-labeled substrate unit (25 nM) was titrated with various concentrations of the catalytic unit RNA (0 – 400 nM). Upper and lower bands correspond to product – ribozyme complex and free product unit, respectively.

(D) Time courses of the ligation reactions of *trans*-1d-old and *trans*-1d. Reactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl. The final concentrations of substrate-1, FAM-labeled substrate-2, and the catalytic unit were M, 1.0 μM, and 0.010 μM, respectively. "P" and "sub2" indicate the amounts of the ligated product and the FAM-labeled substrate-2, respectively.

Table 1Modular units of the *cis*- and *trans*-DSL ribozymes used in this study

Ribozyme	Upper clamp module	Lower clamp module	Catalytic module ^a
<i>trans</i> -1a	GAAA - R(11nt)	GAAA - R(11nt)	most active type
<i>trans</i> -1b	GAAA - R(11nt)	GUAA - R(B7.8)	most active type
<i>trans</i> -1c	GGAA - R(1)	GAAA - R(11nt)	most active type
<i>trans</i> -1d	GAAA - R(11nt)	GGAA - R(1)	most active type
<i>trans</i> -1d-old	GAAA - R(11nt)	GGAA - R(1)	prototype
<i>cis</i> -DSL-G2A2	GGAA - R(1)	THS	most active type
<i>cis</i> -DSL-1S	GAAA - R(11nt)	THS	most active type
<i>cis</i> -DSL-01	GAAA - R(11nt)	THS	prototype

THF: triple helical scaffold motif

Table 2

Reactions of the *trans*-DLS ribozymes under standard conditions^a

Ribozyme	Sub-1 (μ M)	Sub-2 (μ M)	Enzyme (μ M)	k_{obs} (min^{-1})	Calculated final yield ^b (%)
<i>trans</i> -1a	4.0	1.0	0.1	7.3×10^{-3}	78
<i>trans</i> -1b	4.0	1.0	0.1	6.1×10^{-3}	70
<i>trans</i> -1c	4.0	1.0	0.1	2.3×10^{-2}	72
<i>trans</i> -1d	4.0	1.0	0.1	1.7×10^{-2}	81
<i>trans</i> -1e	4.0	1.0	0.01	2.6×10^{-3}	75
<i>trans</i> -1d	4.0	1.0	0.01	1.7×10^{-3}	80
<i>trans</i> -1d-old	4.0	1.0	0.01	4.2×10^{-4}	51
<i>trans</i> -1c	4.0	1.0	10	5.4×10^{-1}	71
<i>trans</i> -1d	4.0	1.0	10	3.9×10^{-1}	76
<i>trans</i> -1d-old	4.0	1.0	10	2.5×10^{-2}	86

^aReactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl.

^bDetermination of calculated final yield was described in Materials and Methods.

Table 3Reactions of the *cis*-DLS ribozymes under standard conditions^a

Ribozyme	Substrate (μM)	Ribozyme (μM)	k_{obs} (min ⁻¹)	Calculated final yield ^b (%)
<i>cis</i> -DSL-G2A2	4.0	1.0	1.2×10^{-1}	72
<i>cis</i> -DSL-1S	4.0	1.0	9.0×10^{-2}	79
<i>cis</i> -DSL-01	4.0	1.0	7.3×10^{-3}	68

^aReactions were performed at 37°C in the presence of 30 mM Tris-Cl (pH 7.5), 25 mM MgCl₂, and 25 mM KCl.^bDetermination of calculated final yield was described in Materials and Methods.