Is Your Ribozyme Design Really Correct?:
A proposal of simple single turnover competition assay to evaluate ribozymes

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Today, many nucleic acid enzymes are used in gene therapy and gene regulations. However, no simple assay methods to evaluate enzymatic activities, with which we judge the enzyme design, have been reported. Here, we propose a new simple competition assay for nucleic acid enzymes of different types to evaluate the cleaving efficiency of a target RNA molecule, of which the recognition sites are different but overlapped. Two nucleic acid enzymes were added to one tube to make a competition of these two enzymes for one substrate. The assay was used on two ribozymes, hammerhead ribozyme and hairpin ribozyme, and a DNA-enzyme. We found that this assay method is capable of application to those enzymes, as a powerful tool for the selection and designing of RNA-cleaving enzymes.

Key words: hammerhead ribozyme; hairpin ribozyme; DNA-enzyme; 5I2 antigen mRNA; competition assay

Since the discovery of a self-splicing group I intron, the catalytic RNA molecules have been found, identified, and applied in many cases.1–3 Among them, the properties of the RNA-cleaving enzymes have been frequently analyzed. The catalytic RNAs are divided into two groups; small ribozymes and large ribozymes. Small ribozymes, approximately those less than 100 nucleotides, include the hammerhead ribozyme, hairpin ribozyme, and hepatitis delta virus ribozyme. These ribozymes cleave the target RNAs to produce 2',3'-cyclic phosphate and 5'-hydroxyl groups. Large ribozymes contain ribonuclease P RNA (M1 RNA) and group I and II introns, which cleaves the target RNA to produce 3'-hydroxyl and 5'-phosphate groups.1–3 The hammerhead ribozyme requires a 5' UH 3' sequence (where H can be A, C, or U), and the hairpin ribozyme requires a 5' RYNGUC 3' sequence (where R can be G or A; Y can be C or U; N represents any base). These enzymes are highly designable, therefore applied to cleave many RNA sequences.3 On the other hand, the development of in vitro selection methods enabled us to obtain the RNA-cleaving DNA, the DNA-enzyme. A DNA-enzyme selected in vitro by Joyce et al. is also highly designable to cleave the RNAs.4 This DNA enzyme requires only 5' RY 3' sequence (where R denotes G or A; Y denotes C or U) on the target RNA.

At present, studies on these enzymes are mainly intended for the application of in vivo use, however, reports of successful examples are not so many. Of course, we know that many protein factors are involved in living cells which disturb the ribozyme reaction to cleave the target RNAs. Then, was the ribozyme design we made really correct? Unfortunately, there has been no reports that show the criteria for evaluation of the ribozyme design. In most reports, the RNA-cleaving enzymes have, however, been assayed and analyzed separately under arbitrary conditions mainly optimized to their originated ones. Until now, many studies on kinetic analysis of these RNA-cleaving enzymes have been reported, however the assays were done independently under different conditions. These measurements were done under various conditions; containing various concentration of spermidine, magnesium ion or salt, or at various values of pH or temperature. The \( k_{\text{cat}} \) of these enzymes range from 0.02 to 4.2 min⁻¹, and \( K_{M} \) range from 1 to 300 nM.5–20 The parameters are records of each measurement; however the values of kinetic parameters are easily affected by the assay conditions, including the sequence of the substrate and the enzymes. It is difficult to compare the RNA-cleaving enzymes all at once. Therefore, a criterion for comparative studies on these enzymes is required. So far, no systematic kitchenal analysis of these enzymes, especially from the point of view of evaluation of the ribozyme design, have yet been reported.

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In many protein enzymes, some competition and inhibition assays are applied to evaluate the enzyme activities: for examples, protein protease inhibitors to evaluate substrate specificity of the proteases, fluorescent metal terbium to evaluate the metal binding affinity of calcium- or magnesium-binding protein, etc.\textsuperscript{25,26} We expected that the strategies of the inhibitor assay can be also applied to evaluate the nucleic acid enzyme activities.

In this paper, we propose a new simple competition assay, in combination of the single turnover kinetics and the competitive inhibition kinetics. This assays is aided to evaluate the nucleic acid enzyme activities using three kinds of enzyme, hammerhead ribozyme, hairpin ribozyme, and DNA-enzyme. These enzymes are designed to cleave neighboring sites, and to bind to the overlapping site of the target RNA to compete in the substrate-binding step. Previous to in vivo applications, all nucleic acid enzymes should be examined, at least once, under in vitro conditions. Our assay method will give a criterion to evaluate the enzymes.

**Materials and Methods**

**Preparation of RNA substrate and RNA-cleaving enzymes.** The 63-mer substrate RNA, 5'-gggc gc aauc\textsuperscript{2} GAGUG CAUCU GGCGG UCCUG CUGCU GUUUC UGUCG CCAUC UACUU UGGaa gcu-3', where small letters indicate the sequences from the plasmid pGEM-3Z (Promega), and also containing the partial sequence of rat S12 antigen mRNA (corresponding to the sequence numbered 123-170)\textsuperscript{3,6} was prepared by \textit{in vitro} transcription. The hammerhead ribozymes, 5'-gggc gc aauc ACAGCA GCAGC UGAGU AGUCC UUUUG GACGA A UACCU CAGA Uaaaa u-3' (the underlined bases 'G' and 'A' were replaced by 'A' and 'G' for the inactivated mutant, respectively), and the hairpin ribozyme, 5'-gggac acaac cuaAC AGCAG CAGAG AACCA ACCA GAGAA GCAAC ACGACUUG UGUUA UAUUA CCUGG Uguau u-3', were also transcribed in \textit{in vitro} using T7 RNA polymerase from a linearized pGEM-derived ribozyme-coding plasmid DNAs as described (ref. 7-9; see Fig. 1). The RNAs were labeled internally with [\textalpha\textsuperscript{32}P]UTP for the multiple turnover kinetics or labeled at the 5'-end with [\textgamma\textsuperscript{32}P]ATP for the single turnover competition kinetics, respectively. The DNA-enzymes, 5'-CAGCA GGAGG CTAGC TACAA CGAGG CCGA T-3' (the underlined base 'G' was replaced by 'A' for the inactivated mutant), were chemically synthesized. DNAs used were purchased from Funakoshi Corporation (Japan). These DNAs and RNAs were purified on 20% PAGE. The concentration of RNAs were measured by radioactivities, and the concentration of DNA was measured spectrophotometrically at 260 nm.

![Fig. 1. Designing of RNA-Cleaving Enzymes.](image-url)

Substrate RNA is shown by a line with the target sequence for the enzymes. Arrows indicate the target cleavage sites. The hammerhead ribozyme (designed to cleave between C\textsuperscript{197}C\textsuperscript{198}), the hairpin ribozyme (designed to cleave between C\textsuperscript{196}G\textsuperscript{197}), and the DNA-enzyme (designed to cleave between C\textsuperscript{197}T\textsuperscript{198}) are shown. The substrate binding sequences of the enzymes are italicized. The numbers (130 and 140) are nucleotide numbers of S12 antigen mRNA according to Sakurada et al.\textsuperscript{6} The enzyme binding sites on the substrate RNA are summarized in the box (below right).
Analysis of the kinetic parameters of RNA-cleaving enzymes. The multiple turnover assay was done obeying the normal Michaelis-Menten kinetics. The concentration of each enzyme was adjusted to 1 nm. The concentrations of the substrate were 10–250 nm. Reactions were started by adding the enzyme to the reaction mixture. Assays were done at 37°C, pH 8.0 (50 mM Tris-HCl, 20 mM MgCl2). The reaction mixtures were then developed on 20% PAGE (containing 8 M urea), and were analyzed using a PhosphorImager (Molecular Dynamics). Each experiment was done in triplicate. Spontaneous hydrolysis of the enzyme was small enough within experimental error. Kinetic parameters, $k_{cat}$ and $K_m$, were calculated from the initial rate measurements for hydrolysis of the RNA substrates, by fitting to the Michaelis-Menten equation using a nonlinear regression algorithm.

Simulation analysis on the anti-sense effect of the enzymes. Under the enzyme-excess competing conditions, the normal Michaelian assay method cannot be applied to the analysis. The catalytic efficiency of one enzyme should be drastically affected by the ratio of the enzyme in the equilibrium of three components, $E_1$, $E_2$, and $S$ (see Fig. 2A). The cleavage efficiency of the enzyme is influenced by the concentration of the Michaelis-complex (E=S). When the substrate concentration ([S]) is fixed to be constant, the ratio of E1,S formation to S0 (=[E1,S]/[S]) can be described by the concentrations of the enzyme $E_1$ (=[E1]), the enzyme $E_2$ (=[E2]), the substrate (=[S]), and the magnitudes of the dissociation constants, $K_{m}$ and $K_{S}$. Under enzyme-excess conditions ([E1,E2] >> [S]), the scheme shown in Fig. 2A can be described by Eq. (1) with the following presumptions:

$$[E_{1,10}] + [E_{1}] + [E_{2}] + [E_{2}, S], [S_0] = [S] + [E_1] + [E_2] + [E_2, S], K_{m} = [E_1][S]/[E_2][E_2, S],$$

$K_{S} = [E_2][S]/[E_2]$ (when the presumptions are developed concerned with $[E_2]S$)

$$= 1/([E_{1,10}] + [E_1] + [E_{2, total}])/K_{m}[E_1][E_2]$$

$$= K_{S}([E_{1,10}]/[E_2]).$$

(1)

The Eq. (1) is developed with respect to $[E_2]$, and the following equation is obtained:

$$K_{m}K_{S}([E_1][S] + 1 - (K_{m} - K_{S})([E_{1,10}] + [S_0] + K_{S} + [E_1][E_{1,10}] + K_{m}[E_2][E_{2, total}])/[E_1][S]$$

$$[E_2, total] + (K_{m} - K_{S})([E_{1,10}] + [S_0] + K_{S} + [E_1][E_{1,10}] + K_{m}$$

$$- K_{m}[E_{2, total}] + K_{S}[S_0] + [E_1][E_{1,10}] + K_{S}[S_0] + [E_1][E_{1,10}] + K_{m}$$

$$+ K_{S}[E_2, total]^2[S_0] = 0.$$  

(2)

Using Eq. (2), the value of $[E_2]S$ can be calculated at various values of $[E_1]/([E_{1,10}] + [E_{2, total}])$ (i = 1 or 2), where the value of $[E_{1,10}] + [E_{2, total}]$ is constant in this case (= 100 nm). And the ratio of occupation of the substrate by each enzyme (=[E_1,S]/[S_0]) can be calculated. The occupancy ratio can be more simply compared when the values are evaluated relatively. In this paper, we used the $K_m$ values of the enzymes instead of the dissociation constants for the simulation. All calculations were done by fitting the parameter to Eq. (2) using a nonlinear regression algorithm.

Single turnover competition analysis of RNA-cleaving enzymes. The hammerhead ribozyme, the hairpin ribozyme, and the substrate RNA were transcribed in vitro as above described. The DNA-enzymes were chemically synthesized. The substrate was labeled at 5' end with [32P]ATP and T4 polynucleotide kinase, and purified on 20% PAGE as described. The concentration of the substrate was measured by radioactivity, and the concentration of the RNA-cleaving enzymes were measured spectrophotometrically at 260 nm.

The competition kinetics was done obeying the scheme (Fig. 2A). The concentration of the substrate was adjusted to 10 nm. The RNA-cleaving enzyme(s) was/were added to the reaction mixture to give a final concentration of 100 nm. Reactions were started by adding magnesium ion to the reaction mixture. Assays were done at 37°C, pH 8.0 (50 mM Tris-HCl, 20 mM MgCl2; incubation of each solution was done at 37°C for ten minutes, and the reaction mixture was incubated at 37°C for five minutes). Reactions were stopped by addition of 3 μl of 0.5 M EDTA solution. The reaction mixtures were then developed on 20% PAGE (containing 8 M urea), and were analyzed using a PhosphorImager (Molecular Dynamics). The cleaved 5'-fragments were detected and the cleavage efficiencies were measured. Each experiment was done in triplicate.

Results and Discussion

Design of nucleic acid enzymes

For comparison of catalytic efficiencies of three nucleic acid enzymes, an RNA substrate which contains the common target sequence should be prepared. The hammerhead ribozyme requires a 5' UH 3' sequence (where H can be A, C, or U) in the target RNA, the hairpin ribozyme requires a 5' RNYGUC 3' sequence (where R can be G or A; Y can be C or U; N represents any base), and the DNA-enzyme requires a 5' RY 3' sequence (where R can be G or A; Y can be C or U). The RNA sequence required for the comparative studies of three RNA-cleaving enzymes is 5'-RNYGUC-3' (where R represents for A or G, Y for U or C), the base sequence required for the hairpin ribozyme; the required bases of the hammerhead ribozyme and the DNA-enzyme are included within the sequence. We used an mRNA of a rat 512 anti-
**Fig. 2.** Theoretical Evaluation of the Competitive Assay Based on the Antisense Effect.

(A) Schematic representation of the competition kinetics. Two enzymes (designated as ‘Ei’ and ‘Ej’) compete in the binding to the substrate ‘S’. Each enzyme cleaves the substrate at different sites to produce the product ‘Pi’ or ‘Pj’, respectively. The Michaelis complex, the Michaelis constant, the catalytic rate constant, of each enzyme are shown as EiS, K\text{M}_i, k_{\text{cat}}^i, where ‘i’ corresponds to ‘1’ for the enzyme Ei and ‘2’ for the enzyme Ej. The area in the box made of a dashed line was used for the evaluation of the antisense effect of each enzyme as a competitor.

(B, C) The value of relative occupancy of the substrate by each enzyme was plotted against the ratio of two enzymes in the mixture under enzyme-excess conditions. The competition of the hammerhead ribozyme (dashed line) with the hairpin ribozyme (solid line) (in B), and the competition of the hammerhead ribozyme (dashed line) with the DNA-enzyme (solid line) (in C) are shown. The substrate concentration was fixed to 10 nM, and the sum of the concentration of two enzymes ([Ei]_\text{total} + [Ej]_\text{total}) was fixed to 100 nM (the values correspond to the experimental conditions; see Fig. 3). The value of occupancy of the substrate by each enzyme ([EiS]/[S]) was calculated according to the equation 2 using the K\text{M}_i values in Table 1 instead of the dissociation constant. The relative values of the occupancies were normalized by the value in the absence of the competitor (when the ‘ratio’ of the enzyme is equal to 1.0). The simulated values of occupancy of the substrate by each enzyme alone were 0.79 (by the hammerhead ribozyme), 0.75 (by the hairpin ribozyme), and 0.21 (by the DNA-enzyme), respectively.

gen, a rat membrane inhibitor complement protein, as a common substrate for the enzymes.\textsuperscript{5,6} The computational secondary structure prediction showed no structural obstacle to the ribozyme reaction around the target site (data not shown). Three RNA-cleaving nucleic acids, hammerhead ribozyme, hairpin ribozyme, and DNA-enzyme, were designed to cleave the same substrate at adjacent sites (summarized in Fig. 1). The substrate RNA contains the sequence required for these enzymes at around position 140. The hammerhead ribozyme was designed to cleave the phosphodiester bond between C\textsuperscript{139}-C\textsuperscript{140}, with 10 + 10 bases for substrate binding. The hairpin ribozyme was designed to cleave the C\textsuperscript{136}-G\textsuperscript{137} bond, with 4 + 10 bases for substrate binding. The DNA-enzyme was designed to cleave the G\textsuperscript{137}-U\textsuperscript{138} bond, with 8 + 8 bases for substrate binding. The length of base pairing in the substrate binding site was determined according to previous reports.\textsuperscript{4,9-24}

Please remark that our enzyme design in this paper is advantageous to the hairpin ribozyme, because the target sequence was chosen for this enzyme. The
results of the multiple turnover kinetics showed this tendency (summarized in Table 1): the hairpin ribozyme and the DNA-enzyme were superior to the hammerhead ribozyme in the view of \( k_{\text{cat}} \), and the two ribozymes were superior to the DNA-enzyme in the view of \( K_M \). The \( K_M \) of two ribozymes were almost equal to each other, and the \( k_{\text{cat}} \) of the hairpin ribozyme and the DNA-enzyme were almost the same. Using these enzymes, the following experiments were done.

**Evaluation of enzymes by the single turnover competition assay**

Normally, the assays of nucleic acid enzymes are done obeying both/either the multiple turnover kinetics and/or the single turnover kinetics. These assays, however, cannot evaluate the enzyme design well enough, because most RNA-cleaving enzymes are examined in and applied to more complicated conditions than those of multiple turnover and single turnover kinetics. The assay mixture for these assays, based on the elegant and pure assay system, is ‘heaven’ for the enzymes: there is no protein which might interact with the ribozymes, and there is no nucleic acid competitor which might exist and be located beside the enzyme or the target nucleic acids or which might affect as the antisense nucleotide. Our assay method provides more crucial conditions for the enzymes for evaluation. The assay system is made up by the combination of the single turnover kinetics and the inhibitory competition kinetics, but is more simple than each of these assay protocols.

Two enzymes are used for competition under the single turnover conditions, mixed in one tube, also with the substrate, under the following conditions (see Fig. 2A): (1) the substrate concentration ([S]) is constant, (2) each enzyme can behave as a competitive inhibitor for the other enzyme, (3) the sum of the concentration of the two enzymes \( \left( = [E_{1,\text{total}}] + [E_{2,\text{total}}] \right) \) is kept constant, (4) the reactions are done with excess enzyme \( \left( [E_{1,\text{total}}] + [E_{2,\text{total}}] > [S] \right) \), and (5) the product of each enzyme can be independently measured \( (P_1 \neq P_2) \). Using this assay system, the activities of two enzymes can be compared exactly under the same conditions. The 63-base RNA was used as substrate. The substrate was labeled at the 5'-end. The hammerhead ribozyme, the hairpin ribozyme, and the DNA-enzyme produce 27-base, 24-base, and 25-base labeled fragments (5'-product fragments), respectively. These 5'-labeled fragments were to be detected separately on the same gel. In this case, we used three different type enzymes that had overlapping substrate binding sites, and also different cleavage sites (Fig. 1).

Additionally, as the single turnover competition assay should be greatly affected by the magnitude of the dissociation constant between the enzyme and the substrate, the anti-sense effect of the enzyme\(^{29} \) was also expected to be evaluated by this assay system.

At first, the occupancies of the substrate by the enzymes were evaluated by simulation analysis using the \( K_M \) obtained from the multiple turnover kinetics under the same temperature and pH. By comparison with the experimental results shown in the following section, the dissociation constant between the enzyme and the substrate under these single turnover conditions can be evaluated, which cannot be easily determined. The results of simulation are shown as Fig. 2. Two different combinations were tested: a combination of the hammerhead ribozyme with the hairpin ribozyme (Fig. 2B), and the combination of the hammerhead ribozyme with the DNA-enzyme (Fig. 2C). By showing the relative values of occupancy of the substrate, the plots indicated vertical symmetry in both cases. As the \( K_M \) of the hammerhead and the hairpin ribozymes were almost identical, the plots for these enzymes in Fig. 2B were almost linear and horizontally symmetric. On the other hand, when the \( K_M \) of the DNA-enzyme greatly differs from that of the hammerhead ribozyme (about 16-fold larger), the plots were not linear: the plots of the hammerhead with smaller \( K_M \) curved to the upper side, and the plots of the DNA-enzyme with larger \( K_M \) curved below (Fig. 2C). In this case, the simulation showed that the hammerhead ribozyme monopolized the substrate under competing conditions with the DNA-enzyme.

The comparison of the theoretical data and the experimental data emphasizes the difference between the Michaelis constant from the multiple turnover kinetics and the dissociation constant of the enzyme and the substrate. The results showed the RNA-cleaving enzymes cleaved the substrate at the expected sites (data not shown). The experimental results are summarized in Fig. 3. The comparison of relative cleavage efficiencies of the hammerhead ribozyme and the hairpin ribozyme (Fig. 3A), and the comparison of those of the hammerhead ribozyme and the DNA-enzyme (Fig. 3B) are shown. The tendency of the plots for the hammerhead ribozyme and the DNA-enzyme (Fig. 3B) is similar to those of the simulated data (Fig. 2C). The results showed that the hammerhead ribozyme in smaller \( K_M \) monopolized the substrate in the competition with the DNA-en-

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**Table 1. Kinetic Parameters of Cleavage Reactions Governed by Different RNA-cleaving Enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{\text{cat}} )</th>
<th>( K_M )</th>
<th>( k_{\text{cat}}/K_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hammerhead ribozyme</td>
<td>0.07 ± 0.004</td>
<td>25 ± 2.9</td>
<td>2.8 × 10^6</td>
</tr>
<tr>
<td>Hairpin ribozyme</td>
<td>0.15 ± 0.009</td>
<td>28 ± 3.5</td>
<td>5.2 × 10^6</td>
</tr>
<tr>
<td>DNA-enzyme</td>
<td>0.18 ± 0.025</td>
<td>390 ± 76</td>
<td>4.6 × 10^5</td>
</tr>
</tbody>
</table>
zyme in larger $K_M$. The resemblance of two plots (Fig. 2C and Fig. 3B) suggests that the ratio of $K_M$ of two enzymes are almost same as the ratio of the dissociation constants of the enzymes, because the plots were normalized relatively, neglecting the effects of the catalytic efficiencies. On the other hand, the experimental results of the competition of the hammerhead ribozyme and the hairpin ribozyme differed (Fig. 3A). The results indicate that the affinity between the hammerhead ribozyme and the substrate was lower than that between the hairpin ribozyme and the substrate, although the $K_M$ of these enzymes were of almost the same magnitude. We have also done the same experiment for the competition of two ribozymes under slightly different conditions (containing spermidine, but at the same pH and temperature). The results are also plotted as circle data within the same graph (Fig. 3A), which showed no difference.

Preliminarily we also did same the experiments using internally labeled substrate, which showed same results as above described (data not shown). This shows that the labeling point of the substrate does not affect our competition assay.

Above theoretical and experimental data indicate that the hairpin ribozyme was superior to the other enzymes, especially under competing conditions, although the substrate binding length of the hairpin ribozyme was the shortest in these enzymes tested. The competitive conditions were disadvantageous to the hammerhead ribozyme and the DNA-enzyme. The results suggest that longer substrate binding sites are required for these enzymes to raise their affinity to their substrate.

The data of cleavage efficiencies had another piece of information: the data obtained in the absence of the competitor represent the cleavage efficiencies in the single turnover kinetics. The cleavage of the substrate by each enzyme was about 60% (by the hairpin ribozyme), 15-30% (by the hammerhead ribozyme), and about 65% (by the DNA-enzyme), respectively. When compared with the multiple turnover results,
Fig. 4. Evaluation of Anti-sense Effect of RNA-cleaving Enzymes using Inactivated Mutant of the Hammerhead Ribozyme and the DNA-enzyme.

(A) Design of the inactivated hammerhead ribozyme (above) and the inactivated DNA-enzyme (below). The boxed bases represent the replaced bases of the enzymes. The substrate binding sequence of each enzyme (shown in italics) is the same as the active enzyme as shown in Fig. 1.

(B) Relative cleavage efficiencies of the DNA-enzyme in the presence of the hammerhead ribozyme (closed squares) or the inactivated hammerhead ribozyme (open squares). The value '100%' corresponds to the cleavage of 64.7% or 61.5% of the RNA substrate by the DNA-enzyme in the presence of the active or inactivated hammerhead ribozyme, respectively. The data of the active hammerhead ribozyme are from Fig. 3B. The substrate concentration was adjusted to 10 nM. The concentration of each enzyme is shown below or above the graph. The concentration of the sum of two enzymes was also kept to 100 nM (see the legend of Fig. 3).

(C) Relative cleavage efficiencies of the hammerhead ribozyme in the presence of the DNA-enzyme (closed squares) or the inactivated DNA-enzyme (open squares). The value '100%' corresponds to the cleavage of 27.9% or 21.0% of the RNA substrate by the hammerhead ribozyme in the presence of the active or inactivated DNA-enzyme, respectively. The data of the active DNA-enzyme are from Fig. 3B.
our hammerhead ribozyme was less active under the single turnover conditions. Interestingly, the results showed that the DNA-enzyme was more active under the single turnover conditions than the multiple turnover conditions, if in the absence of the competitor.

As these results displayed, our competition assay is simpler than traditional assays when the comparison of two enzymes is intended. If you want to compare the activities of two enzymes by traditional ways, you have to perform complicated experiments to raise the accuracy of the measurements. Our competition assay, however, requires fewer experiments, because the measurements of two enzymes will be done in exactly the same tube. Moreover, theoretical simulation of the competition assay will support the experimental results.

Evaluation of the competition effect of the inactivated enzyme

Like in many protein enzymes, the competition assay can be used to evaluate the binding strength of the inhibitor. In our case, two inactivated mutants of nucleic acid enzymes, the inactivated hammerhead ribozyme and the inactivated DNA-enzyme, were prepared and examined (see Fig. 4A). All experiments were done under the same conditions as those in Fig. 3 except for using the inactivated mutants. The results of the inactivated hammerhead ribozyme are shown in Fig. 4B, and the results of the inactivated DNA-enzyme are shown in Fig. 4C (see data in open squares). For the comparison, the data with the active enzyme are also shown within the graph (closed squares). The data of the inactivated hammerhead ribozyme with the DNA-enzyme showed interesting results: the DNA-enzyme won the inactivated ribozyme in the competition (Fig. 4B). This result suggested that the anti-sense effect of the hammerhead ribozyme was not very strong, although the corresponding affinity of the enzyme (=Km of the active enzyme) was greater than that of the DNA-enzyme. On the other hand, the anti-sense effect of the DNA-enzyme seems to be different. The effects of the inactivated DNA-enzyme were greater than those of the active DNA-enzyme (Fig. 4C). The inactive DNA-enzyme caught the substrate molecules equivalently as the competitor ribozyme. This tendency was similar to the simulation plot in Fig. 2B, in the case that the affinity of each of competing enzymes toward the substrate is of the same magnitude. This also suggests that the anti-sense effect of the DNA-enzyme greatly affects its apparent activity.

Conclusion

Here, we have designed a new type of competition assay system for nucleic acid enzymes. From this competition assay, we can obtain new characteristics of enzymes that may not be seen from the ordinary assay. The results suggested that our hammerhead ribozyme and our DNA-enzyme required longer substrate binding sites to achieve efficient binding to the target site of the substrate when competitors exist. When the DNA-enzyme has longer substrate binding site, anti-sense effects are also expected. To cleave the target site, our hairpin ribozyme was the best in this case. In general, there is no forecast to tell the best enzyme from our results. However, since the competition assay described here is not troublesome, it may be advantageous to try this assay during each step of designing of nucleic acid enzymes. The single turnover competition assay, we propose here, can be a powerful tool to evaluation of designing and selection of the nucleic acid enzymes.

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