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Nuclease-resistant chimeric ribozymes containing deoxyribonucleotides and phosphorothioate linkages

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ABSTRACT

Hammerhead ribozymes are considered to be potential therapeutic agents for HIV virus because of their site-specific RNA cleavage activities. In order to elucidate structure – function relationship and also to hopefully endow ribozymes with resistance to ribonucleases, we firstly synthesized chimeric DNA/RNA ribozymes in which deoxyribonucleotides were substituted for ribonucleotides at noncatalytic residues (stems I, II, and III). Kinetic analysis revealed that (i) DNA in the hybridizing arms (stems I and III) enhanced the chemical cleavage step. (ii) stem II and its loop do not affect its enzymatic activity. Secondly, we introduced deoxyribonucleotides with phosphorothioate linkages to the same regions (stems I, II, and III) in order to test whether such thio-linkages further improve their resistance to nucleases. Kinetic measurements revealed that this chimeric thio-DNA/RNA ribozyme had seven-fold higher cleavage activity (k_{cat} = 27 min^{-1}) than that of the all-RNA ribozyme. In terms of stability in serum, DNA-armed ribozymes gained about 10-fold higher stability in human serum but no increase in stability was recognized in bovine serum, probably because the latter serum mainly contained endoribonucleases that attacked unmodified catalytic-loop regions of these ribozymes. Thirdly, in order to protect them from endoribonucleases, three additional modifications were made at positions U^7, U^4 and C^3 within the internal catalytic-loop region, that succeeded in gaining more than a hundred times greater resistance to nucleases in both serums. More importantly, these catalytic-loop modified ribozymes had the comparable cleavage activity (k_{cat}) to the wild-type ribozyme. Since these chimeric thio-DNA/RNA ribozymes are more resistant to attack by both exonucleases and endoribonucleases than the wild-type all-RNA ribozymes *in vivo* and since their cleavage activities are not sacrificed, they appear to be better candidates than the wild type for antiviral therapeutic agents.

INTRODUCTION

The word 'ribozyme' is derived from the terms ribonucleic acid (RNA) and enzyme, and it denotes a type of RNA molecule with enzymatic properties. It was not until the catalytic actions of RNA by Cech and Altman that RNA was shown to play a biological role other than that of a messenger (1, 2). A number of other ribozymes have been discovered since these initial discoveries (3–6), and they include the hammerhead ribozyme (3, 7–10). The hammerhead-type ribozyme is mainly encoded in some plant viroid and satellite RNA genomes. A comparison of these RNAs allowed a minimal secondary structure for hammerheads to be proposed (8). But from the application standpoint, the most important discovery relating to hammerhead ribozymes was the construction of a *trans*-acting system, which was first described by Uhlenbeck (9) and by Haseloff and Gerlach (10). Self-cleaving ribozymes can be divided into two molecules, 'substrate' and 'enzyme' (ribozyme). Haseloff and Gerlach constructed a *trans*-acting system in which the substrate requires only a trinucleotide sequence (usually GUC) for cleavage (10). In their system, hammerhead ribozymes can cleave various RNAs, an indication that they may be useful for controlling gene expression and as therapeutic agents, such as anti-AIDS drugs. Many studies by mutation and modification analysis have been reported (11–30) and the mechanism of cleavage by the ribozyme, which acts as a metalloenzyme, is gradually being clarified (31–41). At the same time, ribozymes have been used to cleave the HIV genome (RNA) in the test tube (42–44), in *E.coli* (45) and in cultured cells (46,47).

There are basically two ways to use ribozymes as cleavers of undesirable RNAs in the living body: integration of a ribozyme gene into the host chromosome, with resultant endogenous expression of the ribozyme in the cell; and direct introduction of ribozyme RNA into the living cell with the help of a drug-delivery system (DDS). The advantage of the latter strategy is that it is possible to engineer and use ribozymes with improved properties, such as higher cleavage activity (48–50) or higher resistance to nucleases (20,21,43,49,51).

Firstly we have synthesized chimeric DNA/RNA ribozymes that contain DNA portions at noncatalytic residues in an attempt to protect them from exoribonucleases (29,30,48). Secondly, we

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have further introduced phosphorothioate linkages in the same noncatalytic region to improve resistance to exonucleases. Kinetic results revealed that these (thio-)DNA-armed chimeric ribozymes have higher k_{cat} values than the all-RNA ribozyme. Thirdly, we have further introduced phosphorothioate linkages, within the catalytic loop, at adjacent to some bases and also exchanged a non-conserved base (U^7) in an attempt to endow the ribozymes with resistance to endoribonucleases. The results showed that these catalytic loop protected ribozymes are more than a hundred times more stable in fetal bovine serum and in human serum than the wild-type ribozyme and they have higher activities or at least similar activities (kcat value) to those of the wild-type ribozyme, indicating that they are actually good alternatives for cleavers of RNAs such as the HIV genome.

MATERIALS AND METHODS

Synthesis of chimeric thio-DNA/RNA ribozymes

All ribozymes were synthesized on an ABI DNA synthesizer (380B; Applied Biosystems, Inc., Foster City, California). DNA or RNA phosphoramidite monomers were purchased from ABN (American Bionetics, Inc., Hayward, California). Phosphorothioate linkages were introduced by the use of the TETD reagent, purchased from ABI. Synthesized oligonucleotides, with 5′-trityl groups removed, were automatically cleaved from CPG columns (ABN) with 2 ml of concentrated ammonia/ethanol (3:1) and heated at 55°C for 8 h to remove some protecting groups. The solutions were evaporated to be aqueous solutions, and then the yields were checked by measuring UV absorbance. The aqueous solutions were lyophilized in a Speed-Vac concentrator and the resultant residues were dissolve in 1 M tetrabutylammonium fluoride/THF (10 μl per OD_{260} unit) and were kept at room temperature for 12 h to remove 2′-protecting *tert*-butyldimethlysilyl groups. After adding the same amount of 0.1 M triethylamine acetate, the mixture was evaporated in a Speed-Vac concentrator. The viscous liquid of the crude deprotected oligonucleotides were diluted with 1 ml of 0.1 M triethylamine acetate, and then purified by a fast desalting column, reverse-phase HPLC, and 20% polyacrylamide/7M urea denaturing gel electrophoresis followed by extraction from the gel with extraction buffer (0.3 M ammonium acetate, 0.1 mM EDTA, 20 mM Tris·HCl (pH 8.0)).

Preparation of fetal bovine serum and human serum

Fetal bovine serum was purchased from Irvine Scientific (Irvine, California). Human serum was prepared by collecting 25 ml of blood from each of five healthy male volunteers. Immediately after collection of 25 ml blood from an antecubital vein of each volunteer, samples were mixed and kept at room temperature for 1 h. The resulting blood-clot was pelleted by centrifugation at 3,000 rpm for 15 min at 25°C. The fresh serum was collected and stored at −80°C until use.

Stability of synthetic ribozymes in two kinds of serum

The stabilities of the synthetic ribozymes were examined by incubating ribozymes at 2.7 μM in fetal bovine serum and human serum at several concentrations at 37°C. Incubations were stopped by removing aliquots from the serum solutions at appropriate intervals and mixing with 1 equivalent volume of a solution containing 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. Samples were directly loaded onto 20% polyacrylamide gels that contained 7 M urea and the results were analyzed by use of an Image Analyzer (BA 100; Fuji Film, Tokyo).

Digestion of RNA

For digestion analysis, HPLC-purified ribozymes were used. Alkaline hydrolysis and digestion with ribonucleases from *B.cereus* were carried out with an RNA Sequencing Kit (Pharmacia, Uppsala, Sweden). For alkaline hydrolysis, 1 μl of a 27 μM solution of ^{32}P-5′-end-labeled ribozyme, 150 mM $NaHCO_3/Na_2CO_3$ (pH 9.2), and carrier tRNA in solution were mixed and heated at 90°C for 6 minutes and then returned to an ice bath. For digestion with ribonucleases from *B.cereus*, 1 μl of a 27 μM solution of ^{32}P-5′-end-labeled ribozyme, 3 μl of buffer III [33 mM sodium citrate (pH 5.0), 1.7 mM EDTA, 1 mg/ml carrier tRNA], 1 μl of a solution of tRNA, and the U- and C-specific ribonuclease from *B.cereus* were mixed and incubated at 55°C for 12 minutes and then returned to the ice bath. Ribozymes were also digested by incubation in 0.1% fetal bovine serum at 37°C for 30 min. Samples were loaded onto 20% polyacrylamide gels that contained 7 M urea for analysis.

Kinetic measurements

Since HPLC-purified ribozymes still contained some shorter fragments, the HPLC-purified ribozymes were further gel-purified for kinetic use. Concentrations of ribozymes were determined by measuring UV absorbance at 260 nm. Reactions were run at 37°C, 25 mM $MgCl_2$ and 50 mM Tris−HCl (pH 8.0). Both 32P-5′ end-labeled substrate (ranged from 1 nM to 15 μM) and ribozyme (ranged from 0.03 nM to 15 nM) in 25 mM $MgCl_2$, 50 mM Tris−Cl (pH 8.0) were separately preincubated at 37°C. The reaction was initiated by adding the substrate to solution of the ribozyme. Reaction was stopped by removing aliquots from the reaction mixture at appropriate intervals and mixing with 1 equivalent volume of a solution containing 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue. Substrate and 5′-cleaved product were separated by 20% polyacrylamide/7M urea denaturing gel electrophoresis and were detected by autoradiography. The extents of cleavage were determined by measurement of radioactivity in the substrate and product bands by using FUJI Bio-Image Analyzer BA100. We used the simple Michaelis−Menten equation for kinetic analysis according to Scheme I:

$$S+R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S{:}R \xrightarrow{k_2} SP_1P_2 \xrightarrow{k_3} S+P_1+P_2$$

Scheme 1

Calculations of K_m and V_{max} were derived from both Eadie−Hofstee and Lineweaver−Burk plots. k_{cat} values were calculated from V_{max}/[ribozyme].

RESULTS

Synthesis of (thio-)DNA-armed ribozymes and their stability in bovine serum

We firstly constructed four 32-mer chimeric hammerhead ribozymes that have modifications at non-catalytic sites (stems I and III in Figure 1) to investigate the effects on catalytic activiy and stability. Figure 1 shows structures of such ribozymes, that include the wild-type all-RNA (R32), two chimeric DNA/RNA ribozymes in which deoxyribonucleotides were substituted for ribonucleotides at noncatalytic residues [stems I and III (DRD32)

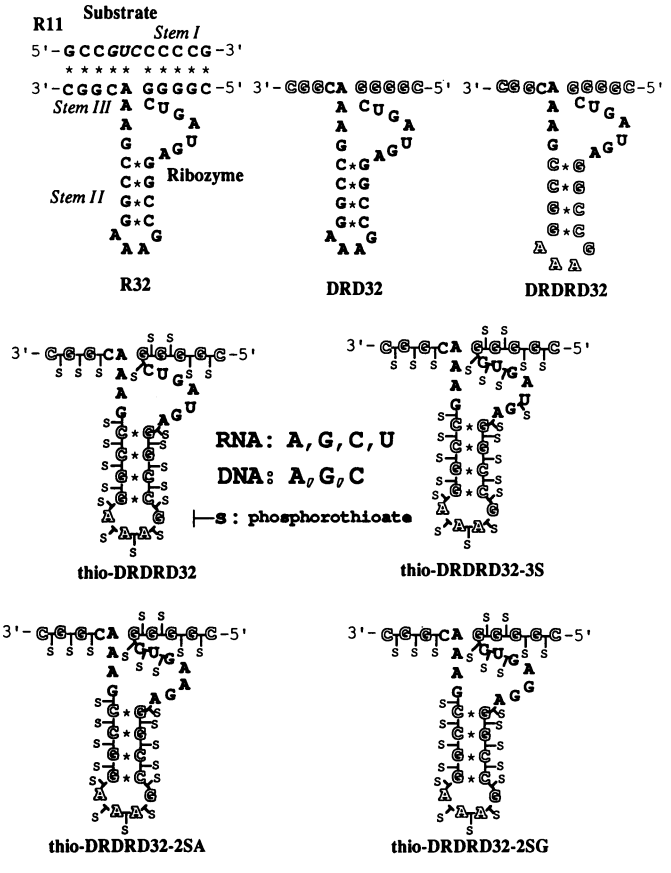

Figure 1. A *trans*-acting system with hammerhead-type ribozymes. R32, which is an all-RNA 32-mer ribozyme, and R11, which is an all-RNA substrate, are shown as a complex by base paring. DRD32 is a chimeric DNA/RNA ribozyme with DNA in the substrate-binding site (stem I and stem III). DRDRD32 has additional substitutions at stem II and loop,which is also a non-catalytic site. Thio-DRDRD32 has phosphorothioate linkages in the DNA portion of DRDRD32. Thio-DRDRD32-3S has three additional phosphorothioate linkages at the catalytic site, which are susceptible to degradation in serum. Thio-DRDRD32-2SA has two additional phosphorothioate linkages and replacement of U^7 by A^7. Thio-DRDRD32-2SG has two additional phosphorothioate linkages and replacement of U^7 by G^7. DNA regions are indicated by outlined letters and phosphorothioate linkages are indicated by '-s'.

and stems I, II and III (DRDRD32)], and the chimeric thio-DNA/RNA ribozyme (thio-DRDRD32) in which phosphorothioate linkages were introduced at DNA domains of DRDRD32. It is to be noted that the thio-DRDRD32 consists of 2^{21} (= 2,097,152) isomers because each thio-linkage produces Rp and Sp diastereomers. Thus, the property of thio-DRDRD32 represents the average of total molecules.

It has been reported that substitutions by deoxyribonucleotides in the substrate-binding regions (stems I and III) increase the stability of ribozymes *in vivo* [in H9 lymphocytes, (49)] but the increase in stability was marginal in our hands in fetal bovine serum. The stability depends not only on the concentration of the serum but also on that of the ribozyme because ribozymes at higher concentrations survive much longer in a fixed concentration of serum (data not shown). Since the relative stabilities of various ribozymes are the same under any set of conditions employed, we diluted the bovine serum to 0.1% instead of increasing the concentration of the ribozymes, and the half-life of the R32 ribozyme (2.7 μM) was about 5 min under

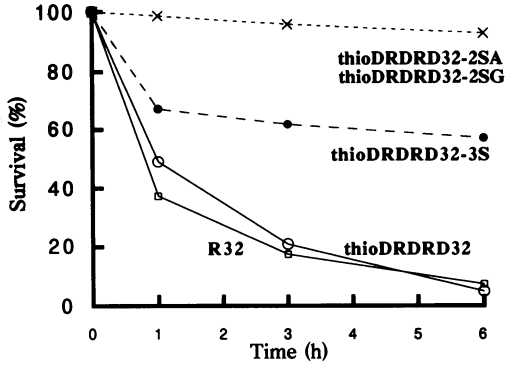

Figure 2. Stabilities of various ribozymes (at 2.7 μM) in 0.1% fetal bovine serum. Surviving ribozymes were quantitated after 5 min, 1 h, 3 h and 6 h.

these conditions. Then, under the identical conditions, we compared the stabilities of these four ribozymes (R32, DRD32, DRDRD32, and thio-DRDRD32), and no significant differences in stability were observed at least in the bovine serum. Specifically, as shown in Figure 2, all-RNA R32 and the chimeric thio-DRDRD32 ribozymes were degraded at the same rate within the range of experimental error, probably because the serum that we used mainly contained endoribonucleases.

In all these ribozymes, the internal catalytic-loop region, composed of RNA, was very sensitive to the endoribonuclease(s). Figure 3 shows the patterns of degradation of R32 and thio-DRDRD32 by alkaline hydrolysis (lane 2), fetal bovine serum (lane 3) and the pyrimidine-specific endonuclease from *B. cereus* (lane 4). Since thio-DNA portions are resistant to alkaline hydrolysis, ladders corresponding to those sections are missing from lane 2 for the thio-DRDRD32 ribozyme. According to our analysis of the patterns of degradation in this serum, in both cases, the 3′ phosphodiester linkages at U^7, U^4 and C^3 were attacked except for A^6 of the all-RNA ribozyme [the numbering system follows the rules for hammerhead ribozymes (52)]. These results suggest that there were mainly RNase A-like endoribonuclease(s) in the serum because U^7, U^4 and C^3 are all pyrimidine bases. Moreover, it was clear that there were scarcely any exoribonucleases in fetal bovine serum because the degradation occurred in these internal positions just after addition of the ribozymes to the serum and no other degradation products accumulated with time (data not shown).

Synthesis of thio-DRDRD32-3S, thio-DRDRD32-2SA and thio-DRDRD32-2SG ribozymes and their stability in bovine serum

In order to protect the internal catalytic-loop from degradation at susceptible sites, we synthesized three derivatives of thio-DRDRD32. One has three phosphorothioate linkages at C^3, U^4 and U^7, and is named thio-DRDRD32-3S. The other two derivatives have phosphorothioate linkages on the 3′ sides of C^3 and U^4 and have one exchanged base, namely, A^7 or G^7 (thio-DRDRD32-2SA and thio-DRDRD32-2SG, respectively). Because U^7 is the only non-conserved residue at the catalytic site, that is to say, it is the only potentially replaceable base, we changed U to either A or G, which would not be recognized by RNase A, rather than introducing a phosphorothioate linkage at this site. Figure 2 shows the stability of these ribozymes in fetal bovine serum. In the case of thio-DRDRD32-3S, about 70% of

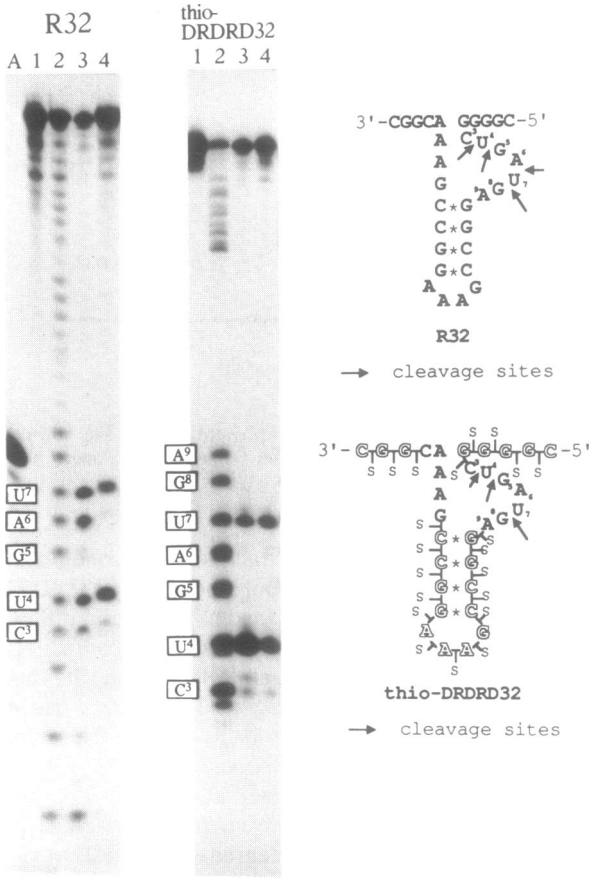

Figure 3. Degradation of the all-RNA ribozyme (R32) and the chimeric thio-DRDRD32 ribozyme. Lanes, A, marker for an RNA 11-mer (R11); 1, intact ribozyme; 2, alkaline hydrolysis; 3, treatment with fetal bovine serum; 4, treatment with pyrimidine-specific RNase from *B.cereus*. Thio-DRDRD32 was cleaved in the serum at the three phosphodiester bonds at the catalytic site, as indicated by arrows, on the 3′ side of U^7, U^4, and C^3, whereas R32 was also degraded on the 3′ side of A^6. Migration of the chimeric ribozyme was faster than that of the all-RNA ribozyme.

this ribozyme remained intact after 6h incubation at 37°C. Moreover, in the case of thio-DRDRD32-2SA and thio-DRDRD32-2SG, there was hardly any degradation at all after a 6-h incubation. These results indicate that: (i) endoribonuclease(s) in this serum virtually failed to recognize the A^7 and G^7 bases, which suggests that they are pyrimidine-specific; and (ii) U^7 may be the most susceptible base under these conditions due to the tertiary structure of the ribozyme. All in all, modifications at only three positions, namely, C^3, U^4 and U^7, in the internal catalytic-loop region were sufficient to make the chimeric thio-DRDRD32 ribozyme more than a hundred times more resistant to nucleases in serum than the wild-type.

Stabilities of several ribozymes in human serum

Although even the above-mentioned ribozymes with a protected internal catalytic-loop (at 2.7 μM each) were not stable in 10% fetal bovine serum (data not shown), we assumed that this purchased serum was likely to have been prepared with rather less than maximum care, with greater resultant cell lysis. With this possibility in mind and to ensure that our findings with fetal bovine serum would be applicable to human serum, we checked

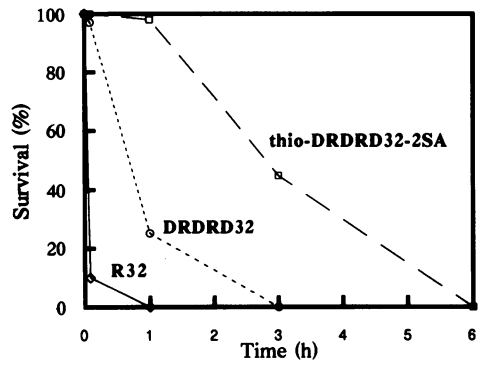

Figure 4. Stabilities of several ribozymes (at 2.7 μM) in 20% human serum. Surviving ribozymes were quantitated after 5 min, 1 h, 3 h and 6 h.

Table 1. Kinetic parameters for reactions catalyzed by chimeric ribozymes

Ribozyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
R32	0.020	4.0	200
DRD32	1.3	13	10
DRDRD32	1.2	16	13
thio-DRDRD32	4.4	27	6.1
thio-DRDRD32-3S	3.4	6.7	2.0
thio-DRDRD32-2SA	0.41	5.5	13
thio-DRDRD32-2SG	0.43	2.0	4.7

*All measurements were made in 25 mM $MgCl_2$, 50 mM Tris−HCl (pH 8.0) at 37° C.

the stability of the ribozymes in human serum that we prepared ourselves. We found that human serum contained much less ribonuclease activity in general than fetal bovine serum. In 0.1% human serum, only 20% of the wild-type all-RNA R32 ribozyme was degraded after a 6-h incubation (data not shown) whereas more than 90% of R32 was degraded at the same concentration of fetal bovine serum (Figure 2). The patterns of degradation of these ribozymes in human serum were almost the same as those in the fetal bovine serum. We also checked the stabilities of the three kinds of ribozymes, namely, R32, DRDRD32 and thio-DRDRD32-2SA, in 20% human serum. As shown in Figure 4, although about 90% of R32 was degraded within 5 min of incubation, about 20% of DRDRD32 remained intact after a 1-h incubation and about half of the thio-DRDRD32-2SA remained intact after a 3-h incubation. These results indicate that the ribozymes with a protected internal catalytic-loop, in particular, thio-DRDRD32-2SA and thio-DRDRD32-2SG, are good reagents for use *in vivo*, given their long half lives in human serum. The difference in stability between R32 and DRDRD32 suggests that the human serum most probably contains some exoribonucleases.

Determination of kinetic parameters of the chimeric ribozymes

Kinetic measurements with these ribozymes revealed that all of them were active and their kinetic parameters are listed in Table 1. Regarding kinetics of DRD32 and DRDRD32, both of them have three to four times greater k_{cat} values than that of wild-type with an increase in K_m. Furthermore, comparison of DRD32 and DRDRD32 reveals that, since their kinetic parameters are nearly identical, stem II and its flanking loop regions do not affect its

enzymatic activities. This finding is in agreement with the 'miniribozyme' found by Jennings' group (28). Thio-DRDRD32 possesses the greatest k_{cat} value among all chimeric ribozymes, which is about seven times greater than that of wild-type R32 ribozyme, which is also accompanied by an increase in K_m. This k_{cat} value (>20 min^{-1}) is even one of the highest values ever reported for hammerhead ribozymes. On the other hand, by phosphorothioate modification in the internal catalytic-loop (thio-DRDRD32-3S, thio-DRDRD32-2SG and thio-DRDRD32-2SA), k_{cat} values decreased as compared to that of thio-DRDRD32, but they were still almost the same k_{cat} values as that of the wild-type R32 ribozyme. K_m values were relatively insensitive to the introduction of phosphorothioate linkages at these three internal positions: the K_m values of thio-DRDRD32 and DRDRD32-3S were almost identical. However, K_m values were affected by changing the U^7 base: K_m values of thio-DRDRD32-2SG and thio-DRDRD32-2SA were about ten times lower than that of thio-DRDRD32. Therefore, all the thio-DNA modifications resulted in either an increase in or maintenance of k_{cat} values, with an accompanying increase in the K_m values. In principal, these catalytic loop protected chimeric thio-DNA/RNA ribozymes may potentially be better therapeutic agents than the wild-type ribozyme.

DISCUSSION

When ribozymes are to be used as antiviral agents *in vivo*, they can either be produced endogenously from the corresponding DNA template in a cell or they can be delivered exogenously by means of a drug-delivery system (DDS). Although, unmodified all-RNA ribozymes appear to be relatively stable in H9 lymphocytes (49), exogenously prepared ribozymes may still encounter several ribonucleases before reaching their target. So it seems important to change ribonuclease-sensitive ribozymes into resistant ones. We firstly constructed chimeric DNA/RNA ribozymes in which RNA portions of non-catalytic sites were replaced by DNA (DRD32 and DRDRD32 in Figure 1). This type of chimeric DNA/RNA ribozymes are expected to be more stable than an all-RNA ribozyme to attack by exoribonucleases because the binding arms at both the 5′ and 3′ ends have been replaced by deoxyribonucleotides.

At first, kinetic measurements were performed in order to examine the effects of those modifications. To our surprise, these constructs had higher k_{cat} values than the corresponding all-RNA ribozyme (48). According to ribozyme reaction (scheme 1), the k_{cat} value includes two factors; cleavage step (k_2) and dissociation step (k_3). To make clear that which is the rate-limiting step in our system, we tried to ascertain whether there exist burst kinetics with our ribozymes or not. If dissociation step is the rate-limiting, much faster cleavage reaction would be observed at the early stage (single turnover stage). The result was that no burst kinetics were observed at both wild-type R32 and chimeric DRD32 reaction (data not shown), indicating that cleavage step is the rate-limiting in our ribozyme reaction. Thus, DNA arms actually improved cleavage activity. This finding was in agreement with the reports by Hendry et al. (50). Further experiments are necessary to clarify why the DNA hybridizing arms enhance the rate of cleavage step. Rossi's group also found an increase in k_{cat} by DNA armed ribozymes. However, in their case, dissociation step rather than the cleavage step was the rate-limiting (49). The K_m value of our DRD32 was totally different from that of Hendry et al. (although those values for the corresponding wild-types are nearly identical). In their case, the increase in K_m value by DNA arms was subtle. In contrast, the K_m value of DRD32 was about sixty times higher than that of wild-type R32 in our case. These results suggest that the change in K_m values of DNA-armed ribozymes were sequence dependent. Thus, the observed increase in K_m value for the DRD32 is not a general phenomenum.

Next, we introduced phosphorothioate linkages into the DNA portion of DRDRD32 (thio-DRDRD32; Figure 1) to examine the effects of the phosphorothioate substitutions and also to improve the resistance toward exonucleases since the phosphorothioate bond is more resistant to nucleases than the normal phosphodiester bond (53). However, the test of stability for the thio-DRDRD32, in fetal bovine serum, revealed that the modification of non-catalytic residues alone (stems I, II and III) was not sufficient to stabilize the ribozyme against endoribonucleases (Figure 2 and ref. 50). To enhance the endoribonuclease resistance of thio-DRDRD32, in addition to the already achieved potential resistance to exonucleases, we constructed ribozymes in which 3′-linkages to pyrimidines within the internal catalytic loop of thio-DRDRD32 were protected by introducing phosphorothioate linkages and, in the other case, the U^7 base was also exchanged by A or G (thio-DRDRD32-3S, thio-DRDRD32-2SA and thio-DRDRD32-2SG; Figure 1). These modifications were adopted because, according to the analysis of the degradation of ribozymes in human serum and fetal bovine serum (Figure 3), the majority of endoribonucleases appeared to be of the RNase A type, such as ribozymes are expected to encounter in the living body. Thus, for protection from endoribonucleases, modifications of only three pyrimidine bases in the internal catalytic-loop that are susceptible for RNase A type ribonulceses may be required. In general, the least modification possible of the internal catalytic-loop is desirable for the maintenance of catalytic activity; the introduction of phosphorothioate linkages at some purine bases within the internal catalytic loop, for example, greatly reduced cleavage activity (17). As we expected, modifications of only three pyrimidine bases, namely, at C^3, U^4 and U^7 (thio-DRDRD32-2SA and thio-DRDRD32-2SG) gave ribozymes that were much more resistant to both exoribonucleases and endoribonucleases, without any sacrifice of catalytic activity, as we discuss below. As we mentioned, U^7 is very susceptible to endoribonucleases for two reasons: (i) it is targeted by RNase A type ribonucleases, and (ii) it may be situated somewhat on the outside of the catalytic site. Most researchers base their studies on a the hammerhead structure that contains U^7 and is derived from the satellite RNA of tobacco ringspot virus. However, according to our results, if ribozymes are to be used in the cell to cleave RNAs, A^7 or G^7 are much more desirable, even for the endogenous expression of a wild-type ribozyme from a ribozyme gene.

With respect to the activities of phosphorothioate-containing ribozymes, thio-DRDRD32 had the largest k_{cat} value among our constructs which is also accompanied by a slight increase of K_m compared to DRD32 and DRDRD32. This k_{cat} value of >20 min^{-1} in 25 mM $MgCl_2$, pH 8.0 is one of the largest values ever reported for the hammerhead-type ribozymes examined under similar conditions (54). With respect to the kinetic parameters of thio-DRDRD32-3S, thio-DRDRD32-2SA and thio-DRDRD32-2SG, their k_{cat} values were similar to that of the wild-type all-RNA R32 ribozyme, although the values were lower than that of thio-DRDRD32 (Table I). The modifications in the internal catalytic-loop apparently changed the most favorable

active-site structure of thio-DRDRD32, resulting in the reduction of its catalytic activity, as compared with that of thio-DRDRD32, to the level of the wild-type ribozyme. K_m values were not affected by introduction of phosphorothioate linkages at the above three internal positions. Similar to DRD32 and DRDRD32, we believe that the increase in the k_{cat} value of thio-DRDRD32 by the introduction of phosphorothioate linkages is due to the enhancement of the chemical-cleavage step rather than the accelaration of dissociation of cleaved products. In short, a rate-limiting step is invariably a chemical cleavage step in the reactions of all our ribozymes. In support of this statement, for example, the k_{cat} value of thio-DRDRD32-3S is about 4 times smaller than that of thio-DRDRD32 in spite of the fact that both of them have identical thio-DNA hybridizing arms. If the dissociation step were the rate-determining step, such difference in k_{cat} values would not be expected.

Ribozymes can, in general, discriminate between correctly matched and mismatched substrates upon association. However, differences in base-pairing energies do not always ensure good cleavage-specificity when the competing matched and mismatched substrates are very similar in sequence (55,56). Under conditions where substrate association is the rate-limiting step and where the ribozyme cleaves essentially every substrate, slightly mismatched substrates are cleaved at rates that approach the rate of cleavage of a completely matched substrate. Thus, there is only limited specificity, even when there is a large difference in equilibrium binding constants between the matched and mismatched substrates. One way to improve the substrate specificity is to weaken the substrate binding without sacrificing the cleavage activity (48,55,56). Engineered ribozymes in which substrate binding has been weakened can achieve high specificity because mismatched substrates have the opportunity to dissociate from the ribozyme rather than remaining to be cleaved, whereas matched substrates continue to be selected for cleavage. Introduction of DNA-binding arms with phosphorothioate linkages may be suited for the engineering of substrate specificity because, as can be seen in Table I, this type of modification weakens substrate binding (larger K_m) without sacrificing cleavage activity (larger k_{cat}). Furthermore, additional mutations at positions C^3, U^4 and U^7 within the internal catalytic-loop region significantly improve resistance to nucleases (thio-DRDRD32-2SA and thio-DRDRD32-2SG in Figures 2 and 4), again without sacrificing cleavage activities. Therefore, these engineered ribozymes may be considered better candidates for therapeutic agents than other similar ribozymes.

Lastly, it should be noted that the various thio-ribozymes we have discussed in this work consist of $2^{21}-2^{24}$ isomers since each thio-linkage produces Rp and Sp diastereomers. It is, thus, possible that some isomers have even higher cleavage activities than the rest. It will be of interest to examine some thio-ribozymes that are prepared stereospecifically by the recently developed method of Stec *et al.* (57).

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