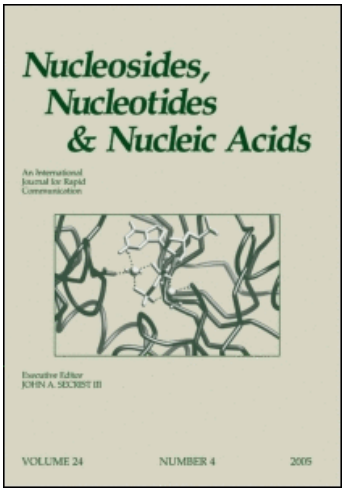

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CLEAVAGE OF SHORT RNAs CONTAINING HIGHER ORDERED STRUCTURES BY HAMMERHEAD RIBOZYMES#

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Abstract: Cleavage of two types of secondary structure-forming substrates by their cognate hammerhead ribozymes were studied by measuring their kinetic parameters. A substrate with a self-complementary structure (GGUCCUAGGA, CL-3) was slowly cleaved by a two-stranded ribozyme. An isomer having no complementary sequence (GGUCGUAGCA, CL-3N) was cleaved more than 10 times faster than the self-complementary substrate. A newly designed ribozyme which contained a stable loop and stem cleaved the self-complementary decamer 40 times faster than the two-stranded ribozyme. A 15 mer which derived from a *ras* mRNA was found to have an intermolecular base pairs and was used to design more efficient ribozymes. Gel mobility shift assay was employed to investigate the binding properties of substrates to ribozymes. Investigations of the thermodynamic stability of the ribozyme-substrate complex are essential in the design of ribozymes that efficiently cleave RNA.

INTRODUCTION

Hammerhead ribozymes have been found in the plus strand of the satellite RNA of tobacco ringspot virus, the plus and minus strands of avocado sunblotch viroids, and a transcript of newt satellite DNA.[1-3] These ribozymes consist of 13 conserved sequences and 3 stems.[4, 5] Ruffner *et al.* and our lab have shown that 11 out of 13 nucleotides are important for cleavage activity.[6, 7] We have also shown that designed ribozymes can act as endonucleases that cleave a targeted RNA with a UX (X= A, C or U) sequence with high specificity.[8-11] Some ribozymes have also been designed to cleave RNAs that are related to diseases, such as HIV and cancer.[12-15]

To design efficient hammerhead ribozymes for a target RNA, using the information described above, we have to consider two important points. The first point is to determine

This paper is dedicated to Professor Y. Mizuno on the occasion of his 75th birthday.

the best site for efficient cleavage of the target RNA. It was shown that the cleavage rates of some ribozymes targeted to the same mRNA molecule were different, probably due to a difference in the structures of the target.[16-18] Multi-targeted ribozymes have been also designed to cleave HIV mRNA.[19, 20] Another point is to find how many base pairs between the ribozyme and the substrate are necessary for an efficient cleavage reaction. Herschlag has shown that a larger number of base pairs was not always advantageous because discrimination between matches and mismatches requires equilibrium binding prior to cleavage.[21] Recently, it has been shown that 12 or 13 bases-length hybridized arms in the ribozyme gave the optimum cleavage rate *in vitro* or *in vivo*.[22] However, in the case of the *tat* gene of HIV mRNA as the target, 33 nucleotides in each hybridized arm of the hammerhead ribozyme were necessary to cleave the substrate *in vivo*.[23]

In this paper, we report the relationship between the secondary structure of substrates and the cleavage rate by cognate ribozymes using two types of substrate. The first target contained partial self-complementarity and was prone to form an intermolecular duplex, and the other type was a hairpin RNA. Both secondary structures are often found in natural RNAs. We wish to discuss strategies in designing ribozymes with high efficiency against targeted RNA, both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

Oligoribonucleotides were synthesized by the phosphoramidite method, using 5'-*O*-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl nucleosides 3'-*O*-phosphoramidite (American Bionetic, Inc. or Milligen), on a DNA/RNA synthesizer, Applied Biosystems models 391 and 394. Deprotection and purification of synthetic oligoribonucleotides were performed with modifications of the procedures.[24, 25] In brief, the protected oligoribonucleotide was cleaved from the support (1 μmol scale) using c. NH_4OH-EtOH (3:1 v/v, 2 ml). The solution was evaporated to dryness. The ammonia/EtOH solution (5 ml) was added and the mixture was heated at 55 °C for 16 hr. The reaction mixture was evaporated to dryness, then 1 M tetrabutylammonium fluoride in THF (1 ml, Aldrich) was added, and the solution was stirred for 18 hr. Triethylammonium acetate (0.1 M, 5 ml) was added, and the solution was chromatographed on a preparative C-18 reverse phase column (Waters) with a linear gradient of 5-40% CH_3CN in 50 mM triethylammonium bicarbonate. The fractions containing the 5'-dimethoxytritylated product were evaporated and repeatedly evaporated with H_2O. Hydrochloric acid (0.01 N, pH 2.0) was added, the solution was stirred for 1 hr at room temperature, and then neutralized with 0.1 N NH_4OH. The solution was washed with AcOEt, and was evaporated. The deprotected oligoribonucleotide was purified by reverse phase HPLC (column: YMC A-324, 10 x 300

mm., Yamamura Chemical Laboratories) with a linear gradient of CH_3CN in 0.1 M triethylammonium acetate (pH 7.0), and by anion-exchange HPLC (column: TSK gel DEAE-2SW, 4.6 x 250 mm., Tosoh) with a linear gradient of $HCOONH_4$ in 20% CH_3CN.

Oligoribonucleotides containing 2'-*O*-methylnucleosides were also synthesized by the phosphoramidite method, using 5'-*O*-dimethoxytrityl-2'-*O*-methylnucleoside 3'-*O*-phosphoramidite on a DNA/RNA synthesizer.[26, 27]

RNA Cleavage Reaction *in vitro*

The 5'-labeled substrate was dissolved in 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 20 mM NaCl, and was incubated with the ribozyme in the same solution at 37 °C. The reaction was stopped by the addition of 50 mM EDTA (pH 8.0), and was analyzed by homochromatography using Homo-mix III.[27] Cleavage rates were estimated by quantitating the radioactivity of the products with a Bio-image analyzer BAS-2000 system (Fuji Film).

Measurements of T_m Values of Oligonucleotides

Tm values of oligonucleotides were measured by a Gilford Response II UV-VIS Spectrophotometer. Oligoribonucleotides were dissolved in a buffer of 10 mM sodium cacodylate (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM Na_2 EDTA, or a buffer of 10 mM sodium cacodylate (pH 7.5), 100 mM NaCl, and 1 mM Na_2 EDTA.

Gel Mobility Shift Analysis of Complexes between Ribozyme and Substrate

The ribozyme (1.0 μM), dissolved in a buffer of 20 mM Tris-acetate (pH 7.5), 25 mM $Mg(OAc)_2$, and 20 mM sodium acetate, was mixed with the substrate (1.0 μM), which was substituted with a 2'-*O*-methylnucleoside at the cleavage site, and was dissolved in the same solution. The mixture was heated at 70 °C and cooled to room temperature. Loading buffer was added, and the reactions were analyzed by 15% polyacrylamide gel electrophoresis at either 5 °C or 37 °C. The ratio of complex formation was quantitated with a Bio-image analyzer BAS-2000 system.

RESULTS

Secondary Structures of Two Types of Substrates

We have reported a designed ribozyme that could sequence-specifically cleave a substrate with a partial sequence of a self-cleavage domain of newt satellite DNA transcripts (CL-3; 5'GGUCCUAGGA3', Fig. 1a).[9, 29] The cleavage site is shown by an

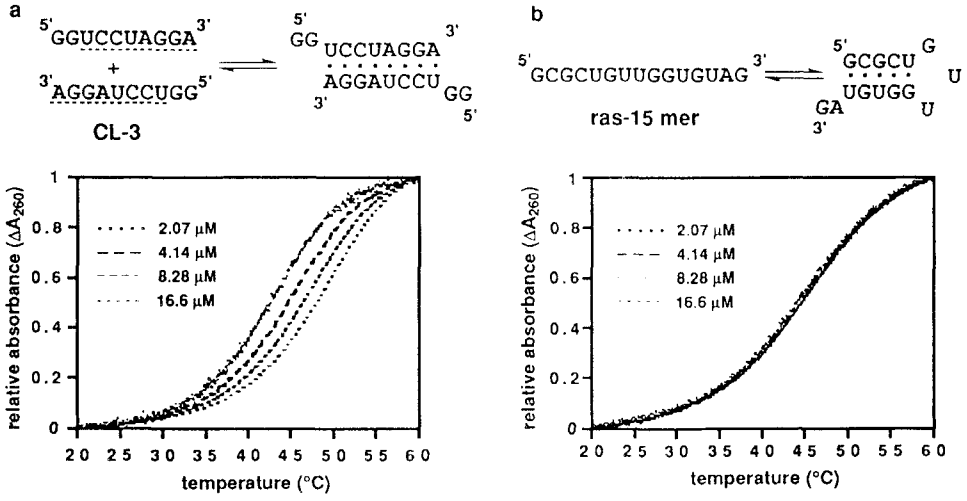

FIG. 1. Putative Secondary Structures of Two Substrates and the Profiles of Their Melting Temperatures. a) UV-temperature profile of CL-3, which forms an intermolecular duplex. b) UV-temperature profile of a ras-15 mer, which forms a hairpin structure. T_m values were measured in a solution of 10 mM sodium cacodylate, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM Na_2 EDTA.

arrow (Fig. 2a). Because CL-3 contains a partial self-complementarity, CL-3 might form an 8 base-pair intermolecular duplex or an intramolecular hairpin structure. When the melting temperature (T_m) of CL-3 was measured in a solution of sodium cacodylate and $MgCl_2$, a biphasic curve was observed (Fig. 1a). The T_m value for CL-3 depended on its concentration, from 2.07 μM to 16.6 μM. Thermodynamic parameters of CL-3 were also obtained from T_m data at different concentrations of CL-3 as shown in Table 1. These data indicate that CL-3 formed a stable intermolecular duplex, which probably had base-pairing within UCCUAGGA (Fig. 1a). To disrupt the complementary structure of CL-3, a decamer (5'GGUCGUAGCA3', CL-3N) was synthesized that replaced the two underlined nucleotides as shown in Fig. 2b. The melting profile of the new decamer, CL-3N, was not a biphasic curve (data not shown), indicating that this oligoribonucleotide did not form a duplex.

We have also shown that an oligoribonucleotide (ras-15mer; 5'GCGCUGUUGGUGUAG3', Fig. 1b, derived from a mutated *ras* mRNA) was cleaved by a designed ribozyme containing a stable C(UUCG)G loop.[11] The T_m value for this ras-15mer, measured under the same conditions described above, was 44.3 °C (Fig. 1b), and those measured with the different concentrations (from 2.07 μM to 8.29 μM) were found

TABLE 1. Thermodynamic Parameters of CL-3 and ras-15 mer.

substrate	Mg^{2+}*	$\Delta G_{37}°$ (kcal/mol)	ΔH (kcal/mol)	ΔS (cal. deg/mol)
CL-3	-	-10.3	-69.5	-191
	+	-14.2	-94.2	-258
ras-15 mer	-	-1.48	-50.8	-159
	+	-1.28	-44.7	-140

* with Mg^{2+}; 10 mM sodium cacodylate (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM Na_2 EDTA, without Mg^{2+}; 10 mM sodium cacodylate (pH 7.5), 100 mM NaCl, and 1 mM Na_2 EDTA.

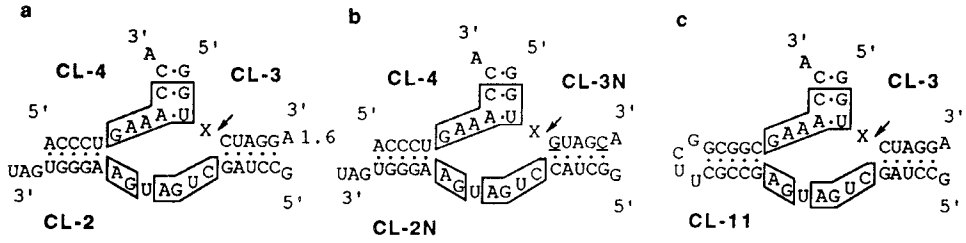

FIG. 2. Secondary Structures of Hammerhead Ribozymes with CL-3 and Related Substrates. a) Two-stranded ribozyme (CL-2, 4) with a duplex-forming substrate (X=C; CL-3, X=2'-*O*-methylcytidine; CL-3-Cm) (see ref. 9). b) Two-stranded ribozyme (CL-2N,4) with a substrate that could not form a duplex (X=C; CL-3N, X=2'-*O*-methylcytidine; CL-3N-Cm). c) Ribozyme containing the unusually stable loop 5'C(UUCG)G3' (CL-11) with CL-3. The cleavage site is indicated by an arrow. The consensus sequences in the ribozyme are boxed. Numbering is according to ref. 37.

to be similar. These data indicate that the ras-15mer contains intramolecular base pairs and probably forms a hairpin loop structure, as shown in Fig. 1b. Thermodynamic parameters were measured and calculated for two conditions, with or without $MgCl_2$, according to the method of Marky and Breslauer.[30] The $\Delta G_{37}°$ value of the ras-15 mer shown in Table 1 is larger than that of CL-3, suggesting that this proposed hairpin structure is not very stable.

Cleavage of Oligoribonucleotides (CL-3 and CL-3N) by a Hammerhead Ribozyme Containing a Stable C(UUCG)G Loop

We have shown that CL-3 was cleaved by the two-stranded ribozyme (CL-2 ,4)[9, 29] and that the cleavage rate of this small complex (Fig. 2a) was slower than that of the other

one-strand hammerhead ribozymes.[5] When CL-3N, which did not form a duplex as described above, was used as the substrate (Fig. 2b), the rate of the cleavage reaction between CL-3N and CL-2N,4 at 37 °C was about 12-fold faster than that between CL-3 and CL-2,4 as shown in Table 2. This indicates that the duplex structure of CL-3 interferes with the cleavage of RNA by a ribozyme.

Since a loop in a hammerhead ribozyme could be substituted with a thermodynamically stable loop C(UUCG)G that had a high T_m value, over 90 °C (data not shown),[11, 31, 32] a one-stranded ribozyme (CL-11) with this stable loop targeted to CL-3 was designed, as shown in Fig. 2c, to avoid unfavorable secondary structures within the ribozyme as shown in ref. 11. The cleavage rate of CL-3 by CL-11 in the presence of 25 mM Mg^{2+} at 37 °C is shown in Table 2. The $t_{1/2}$ value of this substrate by CL-11 was about 43-fold smaller than that by the two-stranded ribozyme (CL-2,4). The difference of the cleavage activity between CL-11 and CL-2,4 might be due to the dissociation of the active CL-2,4 complex.

Gel Mobility Shift Assay for Non-cleavable Derivatives of CL-3 and CL-3N with Ribozymes

There may be two reasons for the fact that CL-3 was cleaved more slowly than CL-3N by the ribozyme (CL-2,4). The substrate formed a stable self-duplex under the cleavage conditions. Consequently, the ribozyme might not bind to the substrate. The other reason might be that their cleaved products could not dissociate from the hybridizing arms of the ribozyme at 37 °C, and the ribozyme did not act catalytically. Therefore, we analyzed the binding abilities of the ribozymes to the substrates using the gel mobility shift assay. In order to measure the formation of the complex under the condition in the presence of Mg^{2+}, each ribozyme was mixed with the uncleavable substrate strand, which had a 2'-*O*-methylcytidine (Cm) instead of cytidine at the cleavage site (X=Cm, in Fig. 2a-c), then the mixture was subjected to native PAGE at 5 °C (Fig. 3). Complexes between CL-3N-Cm and the two-stranded ribozyme (CL-2N, 4) were detected under these conditions (lane 3 in Fig. 3). However, when CL-3-Cm was used as the substrate, neither complexes between CL-3-Cm and CL-2, 4 nor between CL-3-Cm and CL-11 were detected (lane 6 and 8 in Fig. 3). Since the mobility shift of the labeled CL-3-Cm (lane 4 in Fig. 3) was slower than that of the labeled CL-3N-Cm (lane 1 in Fig. 3), the CL-3-Cm would be a duplex form. These results show that CL-3 formed a very stable self-duplex that could not easily hybridize with the ribozyme, indicating that the binding step was rate limiting in the cleavage reaction.

TABLE 2. Half-life of Substrates with Ribozymes.

substrate	ribozyme	$t_{1/2}$(min)*	relative activity
CL-3	CL-2 , 4	260	1.0
CL-3N	CL-2N , 4	21	12.3
CL-3	CL-11	6	43.3

*The substrate (1.0 μM) was treated at 37 °C with the ribozyme (0.4 μM) in the buffer described in the Materials and Methods.

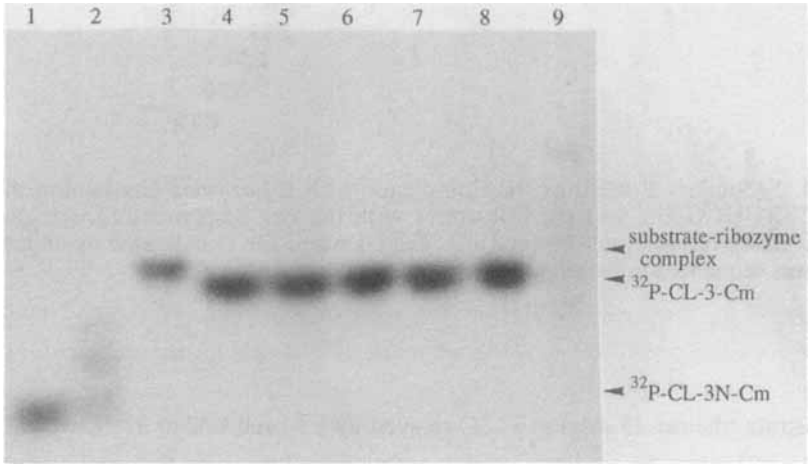

FIG. 3. Analysis of the Binding Abilities of the Ribozymes to the Duplex-formed Substrates Using a Gel Mobility Shift Assay at 5 °C. Details of the method are described in the Materials and Methods. Lane 1; ^{32}P-labeled CL-3N-Cm, lane 2; ^{32}P-labeled CL-3N-Cm with CL-2N, lane 3; ^{32}P-labeled CL-3N-Cm with CL-2N and CL-4, lane 4; ^{32}P-labeled CL-3-Cm, lane 5; ^{32}P-labeled CL-3-Cm with CL-2, lane 6; ^{32}P-labeled CL-3-Cm with CL-2 and CL-4, lane 7; ^{32}P-labeled CL-3-Cm, lane 8; ^{32}P-labeled CL-3-Cm with CL-11, lane 9; ^{32}P-labeled CL-11.

Cleavage Reaction of the Hairpin Oligonucleotide (ras-15 mer) Using Hammerhead Ribozymes with Hybridized Arms of Different Lengths

We have shown that a ras-15 mer was cleaved by the designed ribozyme (E38) with 7 nucleotides in each hybridizing arm.[11] Since the 15 mer was found to have a hairpin structure, disruption of the intermolecular base pairs was thought to be advantageous for the ribozyme reaction. In this study, we designed four ribozymes that had hybridized arms of different lengths, as shown in Fig. 4 (E30, E32, E34, and E36). The half-lives ($t_{1/2}$) of

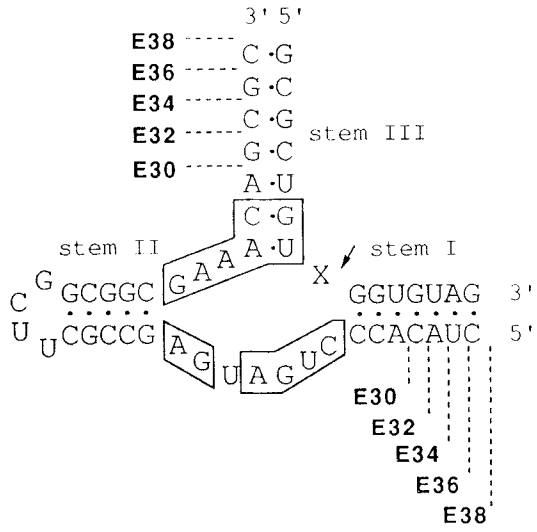

FIG. 4. Secondary Structures for Complexes with Ribozymes Containing the Stable Loop 5'C(UUCG)G3' and the Substrates with the *ras* Sequences (X=U; ras-15mer, X=2'-*O*-methyluridine; ras-15mer-Um). The cleavage site is indicated by an arrow. The consensus sequences in the ribozyme are boxed.

the substrate (the ras-15 mer) at 37 °C cleaved by E30 and E32 at 37 °C were 2.7 x 10^3 and 1.0 x 10^3 min, respectively, which were estimated from the cleavage rate constants. The cleavage rates for the substrate by E34 and E36 at 37 °C were about a 2-fold greater than that by E38. The cleavage rates of the ribozymes (E34, E36 and E38) that had the hybridizing arms longer than 10 base-pairs were about two-order faster than those of the ribozymes (E30 and E32) having the arms shorter than 10 base-pairs at 37 °C. Then, we investigated the cleavage rates for the substrate by three ribozymes (E34, E36 and E38) at different temperatures. However, in the case of E30 and E32 as the ribozymes, the optimum temperature was not exactly obtained, because the cleavage rates of these ribozymes were very slow. The optimum temperature, at which the $t_{1/2}$ value became the smallest, was obtained from Fig. 5 and shown in Table 3. The optimum temperature depended on the length of the hybridized arms of the ribozyme.

Gel Mobility Shift Assay for Complexes with the 2'-*O*-methyl ras-15 mer

Since the lengths of the hybridized arms in the five ribozymes were different, we analyzed the binding abilities of these ribozymes to the substrate using the gel mobility shift assay. Each ribozyme was mixed with the uncleavable substrate which had 2'-*O*-

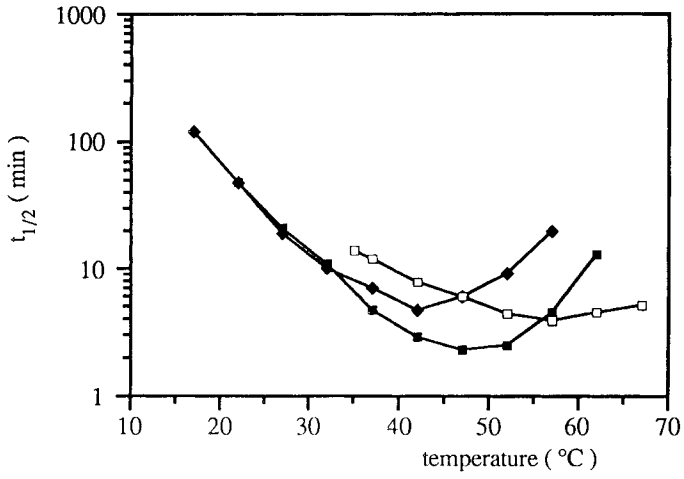

FIG. 5. Plots of Temperature vs. $t_{1/2}$ of the Substrate Cleaved by the Ribozymes. The substrate (1.0 μM) was treated with the ribozyme (0.4 μM) in the buffer described in the Materials and Methods. The $t_{1/2}$ values of the substrates cleaved by E34, E36, and E38 are shown as diamonds, solid squares, and open squares, respectively.

TABLE 3. Cleavage Rate and Optimum Temperature of Reaction of a Hairpin-forming Substrates by Ribozymes

ribozyme	number of base pairs	$t_{1/2}$[a] (min)	relative activity	optimum temperature[b] (°C)
E30	6	2.7×10^3	4.4×10^{-3}	-
E32	8	1.0×10^3	1.2×10^{-2}	-
E34	10	7.0	1.7	42
E36	12	4.7	2.6	47
E38	14	12	1.0	57

a) The substrate (1.0 μM) was treated at 37 °C with the ribozyme (0.4 μM) in the buffer described in the Materials and Methods.
b) The optimum temperature of the cleavage reaction was obtained from the data of Fig. 5.

methyluridine instead of uridine at the cleavage site, in a buffer containing Mg^{2+}, and then the mixture was subjected to native PAGE at 5 °C (Fig. 6a). Complexes between the substrate and the two ribozymes (E30 and E32) were not detected under these conditions (lane 2 and 3 in Fig. 6a). E36 and E38 almost formed complexes with the substrate (lane 5 and 6 in Fig. 6a). In the case of E34 as the ribozyme, about one-half of E34 paired with the substrate to form the hammerhead complex (lane 4 in Fig. 6a). When the electrophoresis was performed at 37 °C, E36 and E38 almost formed ribozyme-substrate complexes (lane 5 and 6 in Fig. 6b). However, although no band derived from complexes between E34 and the substrate was visible, we observed a smeared substrate, probably due to partial binding of the substrate to the ribozyme (lane 4 in Fig. 6b). These results show that E36 was the most compromised of the five ribozymes, from the viewpoint of hybridization to the substrate and release of its products at 37 °C.

DISCUSSION

We investigated relationships between the secondary structure of substrates and their cleavage rates in the ribozyme reaction. We assumed that the binding of the substrate (CL-3) that formed a stable self-duplex ($\Delta G_{37}°$= -14.2 kcal/mol, Table 1) to the ribozyme (CL-2,4 and CL-11) was the rate-limiting step, from the data of the cleavage reaction with the mutated substrate and using the gel mobility-shift analysis of the substrate-ribozyme complex (Fig. 3). On the other hand, when an oligoribonucleotide (the ras-15 mer) with a comparatively unstable hairpin structure ($\Delta G_{37}°$= -1.28 kcal/mol, Table 1) was used as a substrate, more than ten base pairs in the arms hybridized with the ribozyme were required for efficient cleavage. (Table 3 and Fig. 5). In the case of the ribozymes having the arms shorter than ten base-pairs, the gel mobility shift analysis showed that the initial binding step against the substrate with the hairpin structure was the rate-limiting in the cleavage reaction (Fig. 6). However, the ribozymes having the hybridizing arms with longer than ten base-pairs had a higher optimum temperature than physiological temperature, and it was shown that release of the products from these ribozymes becomes rate-limiting under physiological conditions. And then, the cleavage rates of the ribozymes (E34, E36 and E38) that had the hybridized arms longer than 10 base-pairs were about a two-order faster than those of the ribozymes (E30 and E32) having the arms shorter than 10 base-pairs at 37 °C. We calculated the association constant (K_a) between the hybridized arms of the ribozymes and the substrate according to the Turner's rules[33] (Table 4). The value of K_a that was about 10^6 might be a threshold for the binding between the ribozyme and the substrate under this condition, because the K_a of E34 was about a two-order larger than that of E32, whose ratio was comparable to that of the cleavage rate between E32 and E34.

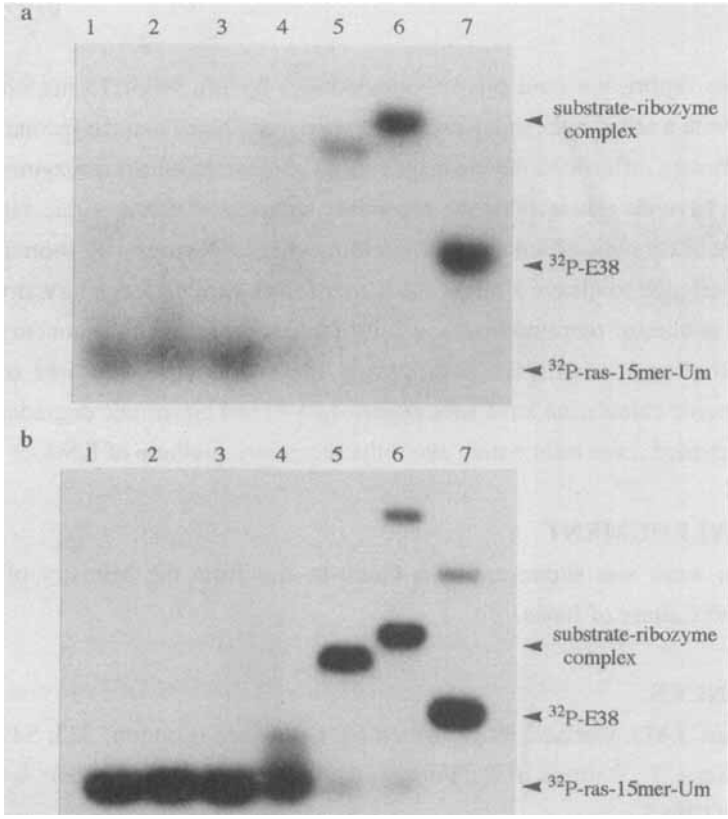

FIG. 6. Analysis of Binding Abilities of the Ribozymes to the Hairpin-forming Substrates, Using the Gel Mobility Shift Assay at (a) 5 °C and (b) 37 °C. Details of the method are described in the Materials and Methods. Lane 1; ^{32}P-labeled ras-15mer-Um, lane 2; ^{32}P-labeled ras-15mer-Um with E30, lane 3; ^{32}P-labeled ras-15mer-Um with E32, lane 4; ^{32}P-labeled ras-15mer-Um with E34, lane 5; ^{32}P-labeled ras-15mer-Um with E36, lane 6; ^{32}P-labeled ras-15mer-Um with E38, lane 7; ^{32}P-labeled E38.

Table 4. Association Constant (K_a) for Complexes between Substrate and Ribozymes

substrate-ribozyme	K_a of stem I (mol/l)	K_a of stem III (mol/l)
ras-15 mer-E30	3.3×10^3	5.6×10^2
ras-15 mer-E32	6.2×10^4	8.8×10^3
ras-15 mer-E34	1.9×10^6	2.2×10^6
ras-15 mer-E36	9.4×10^6	5.6×10^7
ras-15 mer-E38	1.5×10^8	1.4×10^{10}

The free energy of the two stems (stem I which is a 5'-side of the ribozyme and stem III which is a 3'-side of the ribozyme) that formed between the ribozyme and the substrate was predicted by ref. 33. And the association constants (K_a (mol/l)) were calculated from $\Delta G^\circ = -RT \ln K_a$.

In this report, we used oligoribonucleotides having 10 or 15 nucleotides as the substrates with a self-duplex or a hairpin structure and showed that the secondary structure of the substrates influenced the cleavage rate by the hammerhead ribozymes. Fedor and Uhlenbeck have also shown that the secondary structure of the substrate can be a major determinant of catalytic efficiency.[34] Therefore, when ribozymes with shorter hybridizing arms are designed to cleave a target RNA that forms various secondary structures, it is necessary to choose thermodynamically unstable regions of the secondary structures. Some prediction methods for determining the secondary structures of RNAs by thermodynamic calculation have been reported,[33, 35] and enzymatic degradation of RNA has also provided some information about the secondary structure of RNA.[36]

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