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Effects of helical structures formed by the binding arms of DNAzymes and their substrates on catalytic activity

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ABSTRACT

As a part of our efforts to clarify structure–function relationships in reactions catalyzed by deoxyribozymes (DNAzymes), which were recently selected *in vitro*, we synthesized various chimeras and analyzed the kinetics of the corresponding cleavage reactions. We focused on the binding arms and generated helices composed of binding arms and substrates that consisted of RNA and RNA, of RNA and DNA or of DNA and DNA. As expected for the rate limiting chemical cleavage step in reactions catalyzed by DNAzymes, a linear relationship between log(k_{cat}) and pH was observed. In all cases examined, introduction of DNA into the binding helix enhanced the rate of chemical cleavage. Comparison of CD spectra of DNAzyme·substrate complexes suggested that higher levels of B-form-like helix were associated with higher rates of cleavage of the substrate within the complex. To our surprise, the enhancement of catalytic activity that followed introduction of DNA into the binding helix (enhancement by the presence of more B-form-like helix) was very similar to that observed in the case of the hammerhead ribozymes that we had investigated previously. These data, together with other observations, strongly suggest that the reaction mechanism of metal-ion-dependent DNAzymes is almost identical to that of hammerhead ribozymes.

INTRODUCTION

Catalytic RNAs have been investigated by many researchers, with the goal of developing gene therapy, of clarifying the origin of the catalytic activity and of gaining clues to the origin of life (1–15). We have investigated the reaction mechanism of hammerhead ribozymes and performed basic studies directed towards the potential application of ribozymes to the treatment of serious diseases (16–27), such as acquired immune deficiency syndrome (AIDS) and chronic myelogenous leukemia (CML). Elucidation of the mechanism of RNA cleavage reactions and the application of nucleic acid enzymes to gene therapy might be accelerated by studies of a novel class of such enzymes, namely deoxyribozymes (DNAzymes). Catalytic DNAs that can cleave RNA (28–30), ligate chemically activated DNA (31), promote the incorporation of a metal atom into porphyrin (32) or cleave DNA (33) have been identified by *in vitro* selection. For application to gene regulation, the DNAzymes isolated by Joyce's group, which can cleave almost any RNA, seem to be especially valuable (30). The first type of DNAzyme that they isolated can cleave the phosphodiester linkage located between adenine and guanine residues and the second type, shown in Figure 1A and used in this study, can cleave a phosphodiester bond located between purine and pyrimidine residues. The catalytic activity of the latter DNAzyme is higher than that of the former and, therefore, we used the latter DNAzyme in this study (26,30). The DNAzyme is similar to hammerhead ribozymes, at least in terms of secondary structure, having two binding arms and a catalytic loop that captures indispensable catalytic metal ions, such as Mg^{2+} and Mn^{2+} ions. The substrate recognition arms bind the RNA substrate via Watson–Crick base pairs (5–27,30). Although the DNAzyme can cleave almost any RNA substrate with high sequence specificity and seems likely to be an effective agent for gene therapy, since it is probably more stable than any ribozyme *in vivo*, structure–function relationships are not yet fully understood. As a part of our efforts to clarify such relationships, and with the eventual goal of further improving the properties of DNAzymes for application *in vivo*, we synthesized a normal DNAzyme (Dz) and a chimeric DNAzyme with binding arms composed of RNA (R-Dz), as well as normal (R-sub) and chimeric (D-sub) substrates (Fig. 1B). Then, we determined the kinetic parameters of the various cleavage reactions and analyzed them in terms of the structure of the binding helix.

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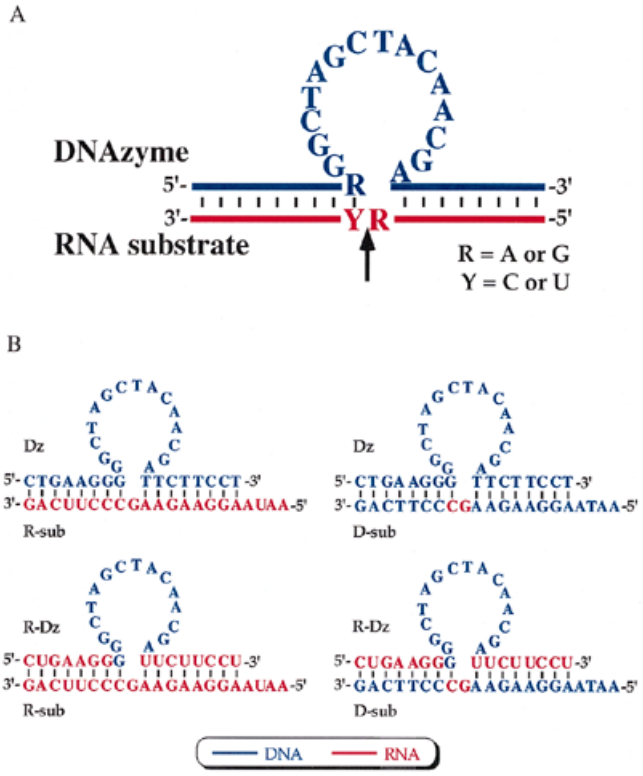

Figure 1. (**A**) The general purpose RNA-cleaving DNAzyme developed by Joyce *et al.* (30). The arrow indicates the cleavage site. (**B**) The combinations of DNAzymes and substrates investigated in this study. The DNAzyme with a substrate-binding region composed of RNA and the substrate with a DNAzyme-binding region composed of DNA were synthesized chemically, as were the normal DNAzyme and its substrate. The blue lines and letters indicate DNA and the red RNA.

DNA substitutions in the binding arms of hammerhead ribozymes have been made by several research groups (34–42). Replacement of the substrate-binding region (so-called stems I and III) by DNA generally enhanced the rate of the cleavage reaction (35,37,38). Such enhancement was reported both in cases where the rate limiting step was the product release step and in cases where it was the chemical cleavage step. In the former case, replacement of the binding region by DNA might reduce affinity for the product (and, thus, also for the substrate), with a resultant increase in catalytic activity (38,43). The rate limiting step in the reaction catalyzed by the ribozyme used in our laboratory is the chemical cleavage step, because the substrate-binding region is relatively short (16–22,35,44–46). Even when the chemical cleavage step was the rate limiting step, we observed enhancement of the rate of the reaction and, moreover, we found that DNA arms that generated more B-form-like helix made a ribozyme·substrate complex that was more similar to the structure of the transition state than was a complex composed exclusively of RNA. Therefore, with a DNA-armed ribozyme, a smaller conformational change was required to reach the transition state and, thus, enhancement of the cleavage reaction was shown to be driven entropically (47).

Since we are interested in the similarities between the DNAzyme selected by Joyce's group and hammerhead ribozymes and, in particular, in the structure–function relationships of DNAzymes as they relate to the structure of the binding helix, we synthesized chimeric DNAzymes and characterized the structures of the binding helices by CD spectroscopy. Our analysis revealed the relationship between the helical structure and the catalytic activity of the DNAzymes. We found not only that the mechanism of action of DNAzymes is very similar to that of hammerhead ribozymes, but also that the relationship between the structure of the binding helix and the catalytic activity of a DNAzyme was very similar to that of hammerhead ribozymes. More specifically, we found that the activity of DNAzymes increased as the amount of B-form-like helix in the complex between the DNAzyme and its substrate was increased.

MATERIALS AND METHODS

Synthesis of chimeric DNAzymes and substrates

All DNAzymes and substrates were synthesized chemically on a DNA/RNA synthesizer (ABI 394; Perkin-Elmer, Foster City, CA) by application of the methods known collectively as phosphoramidite chemistry. Reagents were purchased from Glen Research (Starling, VA). Oligonucleotides were purified as described in the *ABI User Bulletin* (no. 53, 1989) with minor modifications. In brief, each synthesized oligonucleotide was incubated in 2 ml of a mixture of concentrated ammonia and ethanol (3:1 v/v) at 55°C for 8 h to remove protecting groups from bases. The solution was lyophilized with a freeze dryer (Nihon Freezer; Tokyo, Japan) and the residue was incubated with 1 ml 1 M tetrabutylammonium fluoride in tetrahydrofuran (THF) at room temperature for 15 h to remove 2′-protecting *t*-butyldimethylsilyl groups. After addition of 1 ml 0.1 M triethylamine acetate, the mixture was lyophilized again. Each crude preparation of deprotected oligonucleotides was then purified on a fast desalting column and by electrophoresis on a 20% polyacrylamide–7 M urea denaturing gel with subsequent extraction from the gel with 0.3 M sodium acetate and ethanol precipitation. The concentration of each purified substrate and DNAzyme was determined from the absorbance at 260 nm.

Measurements of kinetic parameters

Reactions were performed in 50 mM Tris–HCl (pH 8.0, except for measurements of dependence on pH) and 25 mM $MgCl_2$ at 37°C. Substrates were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Takara Shuzo, Kyoto, Japan). Reactions were started by addition of a solution that contained the substrate to the complete reaction mixture prepared without the substrate. They were stopped at appropriate intervals by mixing aliquots from the reaction mixture with an equal volume of stop solution, which contained 100 mM EDTA, 9 M urea, 0.1% xylene cyanol and 0.1% bromophenol blue. Substrates and 5′-cleaved products were separated by electrophoresis on a 20% polyacrylamide–7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA2000; Fuji Film, Tokyo, Japan) and k_{cat} and K_m values were calculated from Eadie–Hofstee plots. For the measurements under saturating (k_{cat}) conditions (one example of such results is shown in Fig. 2), each partially ^{32}P-labeled DNAzyme (Dz or R-Dz) and a fully ^{32}P-labeled substrate (R-sub or D-sub) were incubated in 50 mM Tris–HCl (pH 8.0) and 25 mM $MgCl_2$ at 37°C. The concentrations of DNAzyme and substrate were 2 µM and 10 nM respectively. All reactions were started and stopped as

described above and subsequent procedures were also as described above.

Profiles of log(k_{cat}) versus pH were obtained under conditions similar to saturating conditions. The concentrations of DNAzyme and substrate were 500 and 5 nM respectively. The reactions were examined of pH 7.0, 7.5 and 8.0.

Measurements of melting temperatures (T_m)

The T_m of the complex formed by a DNAzyme (1 μM) and a substrate (1 μM) was determined in a solution that contained 10 mM Tris–HCl (pH 8.0) and 100 mM NaCl. Before measurement, the complex was allowed to form by heating the solution at 95°C for 2.5 min and gradual cooling. Absorbance was monitored in a quartz cuvette (1 cm path length) with a spectrophotometer (model UV-2100PC; Shimadzu, Kyoto, Japan) at 260 nm. The temperature of the solution was raised by 1°C/min from 5 to 80°C with a temperature controller (model SRP-8; Shimadzu). Measurements were made in triplicate at least. The data shown in Figure 5 were obtained by smoothing the derivative curves of averaged absorbance plots.

Analysis of circular dichroism

The circular dichroism spectrum (CD) of the complex formed by a DNAzyme (1 μM) and a substrate (1 μM) was measured in a solution of 10 mM Tris–HCl (pH 8.0) and 1 M NaCl at 15°C. Before measurement, the complex was allowed to form by heating the solution at 95°C for 2.5 min and gradual cooling. Measurements were made in a quartz cuvette (5 mm path length) with a CD spectrophotometer (model J-600; JASCO, Tokyo, Japan) from 340 to 200 nm in triplicate at least. The spectra shown in Figure 6 were obtained by smoothing the averaged spectra with a calculator.

RESULTS AND DISCUSSION

Comparison of the kinetic parameters of the all-DNA deoxyribozyme (DNAzyme) and the chimeric DNA/RNA DNAzyme

In order to examine the effect of substitution of DNA arms by RNA on the catalytic activity of DNAzymes, we synthesized two DNAzymes, Dz and R-Dz, and two substrates, R-sub and D-sub. Figure 1B shows the respective sequences and secondary structures with DNA and RNA portions indicated in blue and red respectively. Dz was an all-DNA 31mer DNAzyme and R-Dz was a chimeric DNAzyme consisting of a DNA catalytic core flanked by two substrate-binding RNA arms. R-sub and D-sub had the same RNA cleavage site, rG-rC, flanked by enzyme-binding regions composed of RNA and DNA respectively. These DNAzymes and substrates should make complexes with 8 bp helices on both sides of the catalytic core.

Since we are interested in the cleavage step rather than in the product release step of DNAzyme-catalyzed reactions, we performed our analyses of kinetics under single turnover (enzyme-saturated) conditions, where the product release step is not observed. The difference in catalytic activity of Dz and R-Dz, examined under single turnover conditions, can be seen in the autoradiogram shown in Figure 2. In this experiment, we used partially ^{32}P-labeled DNAzymes (Dz and R-Dz) and fully ^{32}P-labeled substrates (R-sub and D-sub). The ratio of DNAzyme to substrate was 200:1, to ensure that the concentration of each DNAzyme was well above the K_m of the respective DNAzyme·substrate complex (measurement of k_{cat}, the rate constant for the cleavage step). Only a small fraction of the DNAzyme molecules was ^{32}P-labeled, so that both the DNAzyme and the substrate could be visualized on the same gel. Therefore, the relative intensities of the bands of DNAzyme and substrate do not reflect the actual molar ratio. Comparison of reaction rates between the combinations of Dz and R-sub and R-Dz and R-sub, or of Dz and D-sub and R-Dz and D-sub revealed that replacement of the binding regions of the DNAzyme with RNA suppressed the catalytic activity, regardless of whether the substrate used was R-sub or D-sub. It was also apparent that replacement of the binding regions of the substrate by DNA increased the reaction rate. These results suggested that DNA within the binding regions of both the DNAzyme and the substrate increased the reaction rate, as is also observed with hammerhead ribozymes (47–50).

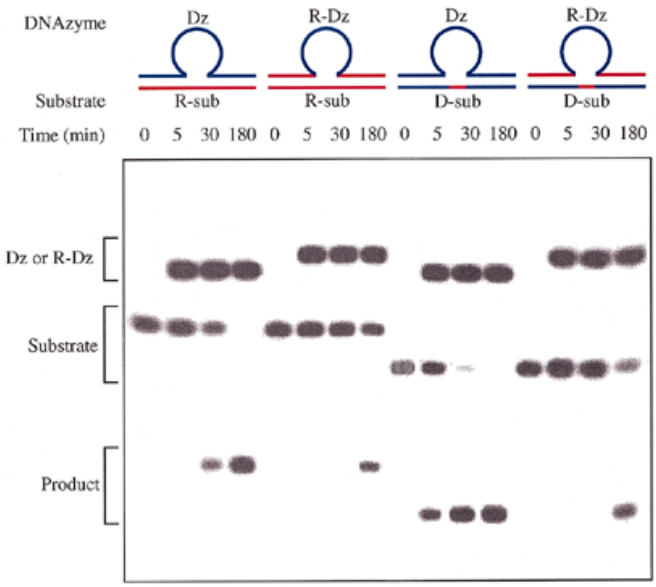

Figure 2. Typical autoradiogram obtained after reactions under single turnover conditions. A solution containing 50 mM Tris–HCl (pH 8.0), 25 mM $MgCl_2$ and 2 mM partially ^{32}P-labeled DNAzyme was supplemented with a small amount of fully ^{32}P-labeled substrate to start each reaction. The molar ratio of DNAzyme to substrate was 200 (single turnover condition). The blue and red lines indicate DNA and RNA respectively. The autoradiogram shows that Dz had higher activity than R-Dz and that D-sub was a more reactive substrate than R-sub.

In order to quantitate the extent of enhancement due to the DNA-containing helices within DNAzyme·substrate complexes, we determined kinetic parameters for reactions in 50 mM Tris–HCl (pH 8.0, except in the case of analysis of dependence on pH) and 25 mM $MgCl_2$ at 37°C. The kinetic parameters that we obtained are summarized in Figure 3. The k_{cat} value for the combination of Dz and D-sub was 8.6×10^{-2}/min, which was ~10 times higher than that for Dz and R-sub, namely 9.9×10^{-3}/min. The k_{cat} value for R-Dz and R-sub was 1.9×10^{-3}/min, which was ~20% of that for Dz and R-sub. These results indicate that DNA in the binding regions of both the DNAzyme and the substrate increased the reaction rate. The maximum difference (~50-fold) was observed between the Dz and D-sub combination, which should form an almost complete DNA·DNA helix, and the R-Dz and R-sub

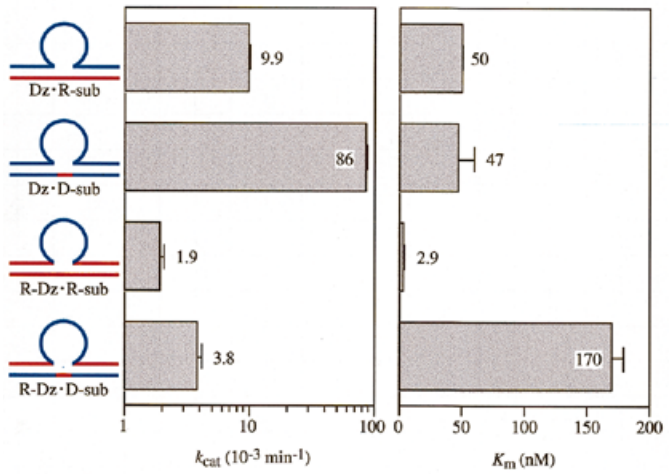

Figure 3. The k_{cat} and K_m values. All kinetic measurements were made in triplicate, at least, and the indicated parameters are average values. The blue and red lines indicate DNA and RNA respectively. The k_{cat} for the combination of Dz and D-sub was ~10 times higher than that for Dz and R-sub, whereas the k_{cat} for R-Dz and R-sub was ~20% of that for Dz and R-sub. The DNAzyme and substrate with binding arms composed of DNA had higher activity and reactivity respectively than those with binding arms composed of RNA.

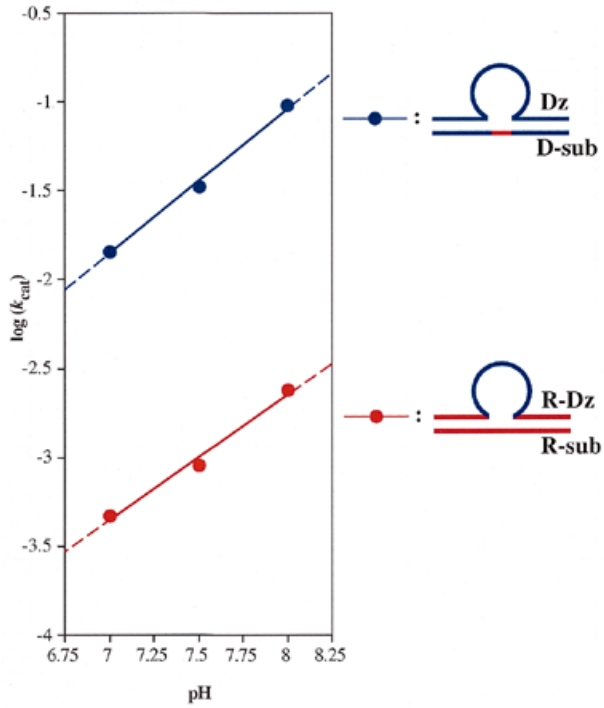

Figure 4. Dependence on pH of k_{cat}. For a discussion of the differences in k_{cat} values among four combinations (shown in Fig. 3) it was important to determine whether the cleavage step or a conformational change (if it occurred) was the rate limiting step. The dependence of k_{cat} on pH was determined for the combinations with the largest k_{cat} (Dz and D-sub, blue line) and with the smallest k_{cat} (R-Dz and R-sub, red line) between pH 7.0 and 8.0. Both for Dz and D-sub and for R-Dz and R-sub, log(k_{cat}) increased linearly with increasing pH, with slopes of 0.8 and 0.7 respectively, indicating that the chemical cleavage step, rather than a conformational change prior to the chemical step, was the rate limiting step.

combination, which should form an almost complete RNA·RNA helix. This result suggests the importance of the formation of a B-form-like helix in establishment of the activated complex (see below).

The k_{cat} value for R-Dz and D-sub was 3.8×10^{-3}/min, which was about one third of that for the normal combination of Dz and R-sub, although, in both cases, the helix was an RNA·DNA heteroduplex. This result suggested that the increase in the reaction rate did not originate from the constituent nucleotides alone, but might involve other factors. We also determined the K_m value and the order of K_m values was R-Dz and D-sub > Dz and R-sub = Dz and D-sub > R-Dz and R-sub. The measured K_m most probably reflects the stability of each respective complex, because T_m values of the complexes showed the same tendency (see below).

The tendency exhibited by the various k_{cat} values was the same in the case of hammerhead ribozymes and the DNAzyme: in both cases, DNA substitution increased the reaction rate (47–50). In our previous studies (47,48), we also determined kinetic parameters for a chimeric DNA/RNA ribozyme. The k_{cat} values for the combinations of the normal ribozyme and an RNA substrate and the DNA-armed ribozyme and an RNA substrate were 4.0 and 13 per min respectively, indicating that the DNA in the substrate-binding region enhanced the catalytic activity. DNA in the enzyme-binding region of the substrate also increased the reactivity from 4.0 to 11 per min when the normal ribozyme was used (48). The present data indicate that, in the case of DNAzymes, the structure of the binding helix can change the activity by as much as 50-fold (Fig. 3).

The dependence on pH of k_{cat}: evidence that the rate limiting step is the chemical cleavage step

As mentioned above, in order to confirm that the chemical cleavage step is the rate limiting step, we carried out reactions under single turnover conditions, where the product release step is irrelevant. However, we could not completely exclude the possibility that the measured values of k_{cat} in Figure 3 might have reflected the step in which a conformational change occurred. When the origin of differences in k_{cat} values is discussed, it is important to ensure that the determined k_{cat} does indeed reflect the chemical cleavage step and not the putative conformational change. If the rate limiting step is the putative conformational change, the value of log(k_{cat}) would not be expected to be directly dependent on pH, whereas the value of log(k_{cat}) should increase linearly with increasing pH if the rate limiting step is the chemical cleavage step. Figure 4 shows the pH–rate profiles for the combination of Dz and D-sub, which had the highest k_{cat}, and the combination of R-Dz and R-sub, which had the lowest k_{cat}.

As is clear from Figure 4, the graph of log(k_{cat}) versus pH was linear, with slopes of 0.8 and 0.7 for Dz and D-sub (blue line) and for R-Dz and R-sub (red line) respectively, indicating that the rate limiting step was indeed the chemical cleavage step, at least between pH 7.0 and 8.0. This conclusion was also supported by other experiments, including the analysis of burst kinetics (Q.-C.He and K.Taira, unpublished data; S.W.Santro and G.F.Joyce, personal communication). Similar dependence of k_{cat} on pH was observed in the case of hammerhead ribozymes (16,40,47). Thus, for both the DNAzyme and hammerhead ribozymes, the rate limiting step is the chemical cleavage step under our experimental conditions. It is to be noted that, although

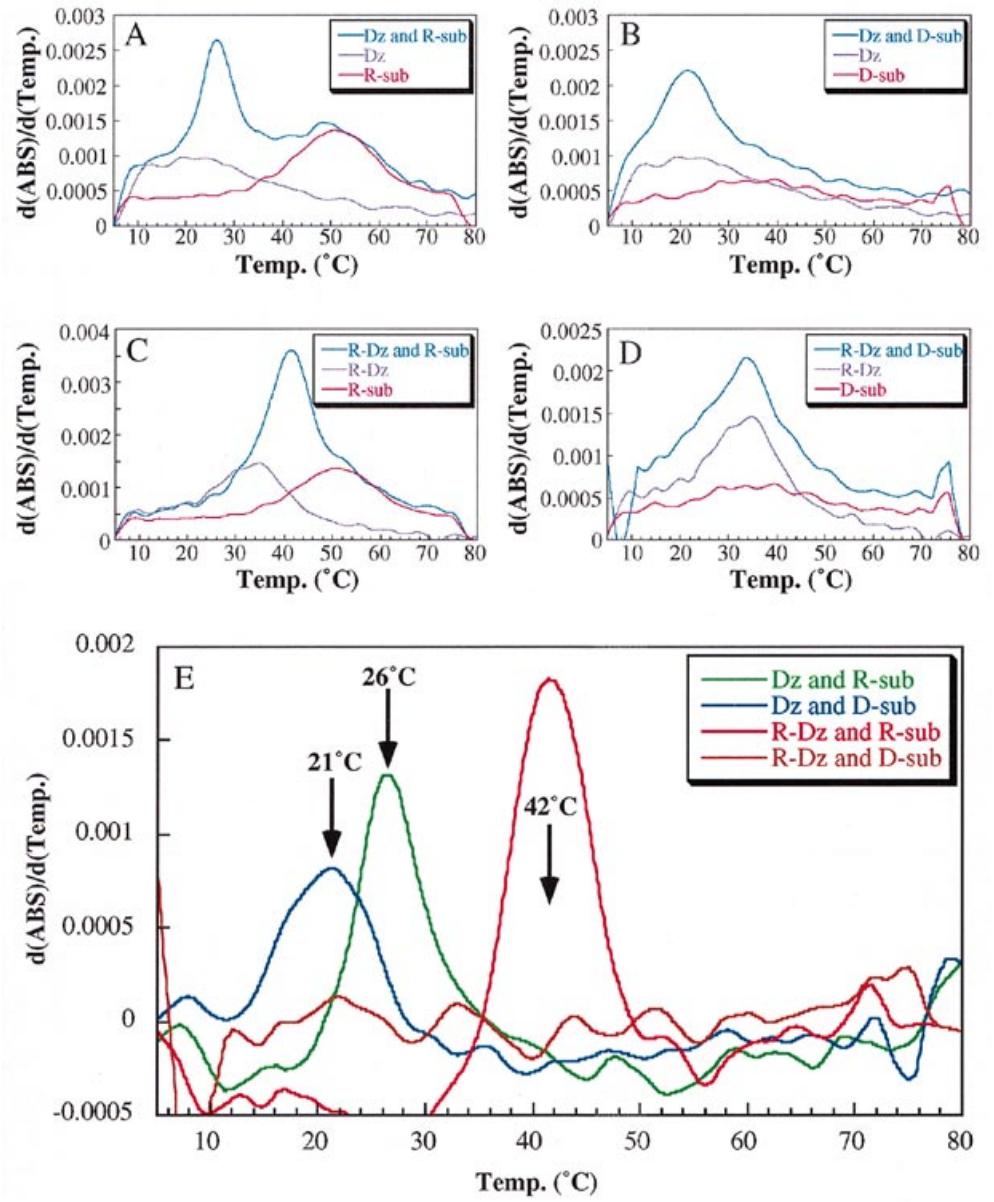

Figure 5. T_m values of the various complexes. (**A–D**) The derivative curves of the absorbance at 260 nm versus temperature for the combinations of Dz and R-sub, Dz and D-sub, R-Dz and R-sub and R-Dz and D-sub respectively. Blue, purple and red lines are the curves of complex, the DNAzyme only and the substrate only respectively. (**E**) The curves obtained by subtraction of the curves for the DNAzyme and the substrate from that for the complex. The T_m values of the complexes of R-Dz·R-sub, Dz·R-sub and Dz·D-sub were 42, 26 and 21°C respectively. The T_m value of R-Dz·D-sub was too low to be determined.

we made a considerable effort to detect the conformational change in reactions catalyzed by hammerhead ribozymes, such a conformational change did not appear to be the rate limiting step under any tested conditions (47).

Structures of binding helices: explanation of the acceleration of reactions by DNA helices

Having confirmed that the k_{cat} represented the chemical cleavage step, we next asked what factor(s) related to the DNA regions might have accelerated cleavage? In the case of hammerhead ribozymes, DNA-binding arms enhanced catalytic activity by making the structure of the DNA-armed ribozyme·substrate complex closer to the structure of the transition state and, therefore, reactions with DNA-armed ribozymes were entropically favored (47). This conclusion, namely that the enhancement of RNA cleavage by the DNA-armed ribozyme was driven entropically, was based on kinetic as well as thermodynamic parameters. It appeared possible that the hybrid helices of the DNA-armed ribozyme·substrate complex might have created a slightly different structure that more closely resembled a B-form-like helix which was responsible for the higher activity of the DNA-armed ribozyme as compared with the all-RNA ribozyme. However, no structural analysis of the DNA-armed complex was undertaken in our previous study.

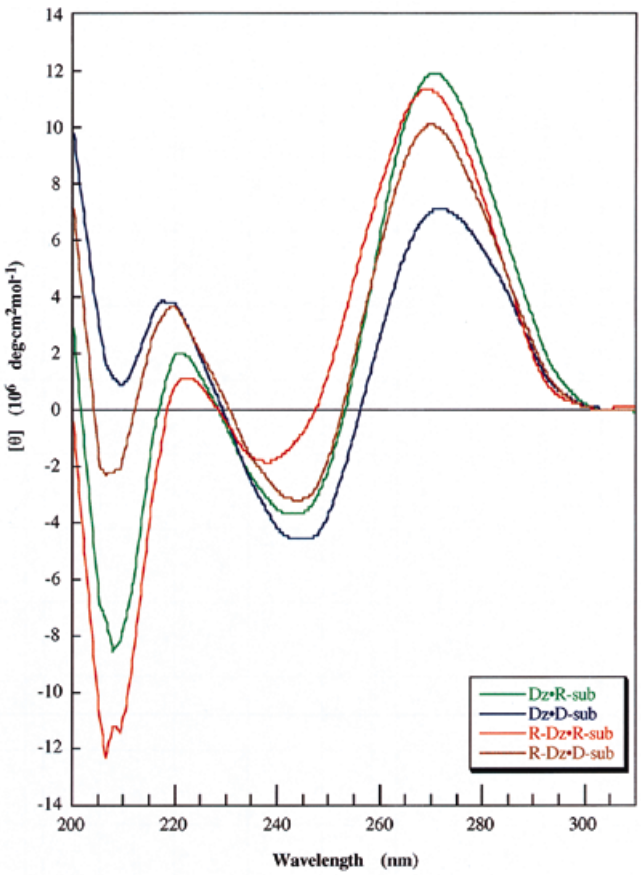

Figure 6. CD spectra of the four kinds of complex. It is known that the wavelength of the peak of the spectrum is correlated with the extent of B-form helix in a duplex. The peak wavelength of the spectrum for Dz·D-sub, which had the maximum k_{cat}, was 272 nm, higher than those for the other complexes, whereas that for R-Dz·R-sub, which had the minimum k_{cat}, was lowest, 269 nm. Thus, the wavelength of the peak of the spectrum shifted from 269 to 272 nm with increases in k_{cat}. The k_{cat} value was, therefore, correlated with the extent of B-form helix in each complex.

Since we observed a similar phenomenon with the DNAzymes, namely DNA arms within the binding helix increased the rate of catalysis, we investigated the helical structures of chimeric DNAzymes. We first examined whether the measured K_m values really reflected the stability of the complexes. According to our kinetic measurements (Fig. 3), the order of K_m values was R-Dz and D-sub > Dz and R-sub = Dz and D-sub > R-Dz and R-sub. The order of K_m values was consistent with the order of free energies of the corresponding complexes, as estimated by the nearest neighbor method (data not shown; 51–54). To obtain further support for our conclusion, we measured the T_m of each complex. Figure 5 shows the derivative curves of the plots of absorbance at 260 nm versus temperature. The T_m values of complexes were obtained by subtraction of the curves for the DNAzyme and the substrate from that for the corresponding complex (Fig. 5E). The T_m values obtained for R-Dz·R-sub, Dz·R-sub and Dz·D-sub were 42, 26 and 21°C respectively. The T_m value of the least stable complex, R-Dz·D-sub, with the largest K_m value, was too low to be determined under the conditions of our measurements. This is probably because a double helix consisting of a pyrimidine-rich RNA strand and a purine-rich DNA strand is very unstable (55) and so it might be expected that the double helix formed between the 3′-arm of the DNAzyme and the 5′-end of the substrate would be very unstable in the case of the R-Dz·D-sub complex. It is to be noted that in the measurements of T_m values, since Mg^{2+} ions were omitted from the solution, the difference in the stability of the complex might be more discernible. A comparison of the rank orders of K_m and T_m values confirmed that the K_m values reflected the stability of the various complexes.

We then analyzed the structure of the binding helix of each complex by CD spectroscopy (Fig. 6). The spectra of Dz·D-sub, Dz·R-sub, R-Dz·D-sub and R-Dz·R-sub had peaks at 272, 270.5, 270 and 269 nm respectively. This rank order was the same as the rank order of the cleavage rates. It is generally known that a DNA·DNA duplex favors formation of a B-form helix, whereas an RNA·RNA duplex favors formation of an A-form helix (55–59). Moreover, nucleic acids with a B-form helix have CD peaks at higher wavelengths than those with an A-form helix (56–58). It is clear from the spectra shown in Figure 6 that the extent of formation of a B-form helix by each complex of a DNAzyme and its substrate was correlated with the cleavage rate, as we anticipated in the case of DNA-armed hammerhead ribozymes (47), as well as for DNAzymes (Fig. 3). Thus it appears that the explanation for the increase in the reaction rate is the same for hammerhead ribozymes and the DNAzyme. Differences in the structure of duplexes among the various complexes of a DNAzyme and its substrate probably caused differences in the position and orientation of bases and other functional moieties within the catalytic core. A resultant change in the positioning of catalytic Mg^{2+} ions would then affect the efficiency of catalysis. Almost the same conclusion can be reached for reactions catalyzed by hammerhead ribozymes, since a similar correlation was observed between the extent of B-form-like structures in the binding helix and the cleavage rate (47).

Conclusion

In this study, we synthesized chimeric DNAzymes and substrates composed of DNA and RNA and determined the kinetic parameters of the corresponding cleavage reactions. The values of k_{cat} showed that as the amount of DNA in the binding helix increased, the rate of cleavage of phosphodiester bonds also increased. According to CD measurements, the cleavage rate was, in turn, correlated with the extent of B-form-like helix in the DNAzyme·substrate complex. The mechanism of action of DNAzymes appears to be very similar to that of hammerhead ribozymes. The reactions have identical dependence on pH (Fig. 4). Moreover, both demonstrate an inverse correlation between the pK_a of metal hydrates and activity and solvent isotope effects and thio effects on the reactions are identical (Q.-C.He and K.Taira, unpublished result). All these observations support involvement of the ‘double-metal-ion mechanism’ of catalysis in both cases. It is likely that a slight change in the structure of the binding helix, caused by the introduction of DNA, changes the positioning of catalytic divalent metal ions, such as Mg^{2+} and Mn^{2+} ions, and can alter the rate of the cleavage reaction by as much as 50-fold. Hammerhead ribozymes exist in the natural world, whereas DNAzymes have been generated artificially. Despite the significant difference in the origin of these nucleic acid enzymes, their properties and the mechanisms by which they cleave RNA appear to be very similar. These results indicate that nature has adopted

a rather narrowly defined common mechanism for the cleavage of RNA and that the mechanisms of various nucleic acid enzymes might converge into one unique and universal mechanism. Such a mechanism is exploited not only by various kinds of ribozyme, but also by the artificially generated metal ion-dependent DNAzymes. We can anticipate that it will also be exploited by other RNA-cleaving agents that will be identified in the future.

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