Enhancement of the cleavage rates of DNA-armed hammerhead ribozymes by various divalent metal ions

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ABSTRACT

In order to characterize structure–function relationships, the kinetic behavior of chimeric RNA/DNA ribozyme was compared with that of all RNA ribozyme. Determined \(k_{\text{cat}}\) values were proven to represent the chemical-cleavage step and not the product-dissociation step. In agreement with the finding by Dahm and Uhlenbeck [Biochemistry 30, 9464–9469 (1991)], various metal ions, including \(\text{Co}^{2+}\) and \(\text{Ca}^{2+}\) with the ionic radius of 0.65 and 1.0 Å, respectively, could support hammerhead cleavage for both types of ribozyme. Measurements of kinetic parameters in the presence of various divalent metal ions revealed that DNA arms always enhanced \(k_{\text{cat}}\) values. Chemical-probing data using dimethylsulfate indicated that the catalytic-loop structures of all-RNA and chimeric ribozymes were nearly identical with the exception of enhanced termination of primer extension reactions at C3 in the case of the chimeric ribozyme. These observations and others demonstrate that DNA substitution in non-catalytic-loop regions increases chemical-cleavage activity, possibly with an accompanying very subtle change in the structure.

INTRODUCTION

As part of our efforts to characterize structure–function relationships, we previously synthesized several chimeric DNA/RNA hammerhead ribozymes (1–3). Examination of kinetic parameters revealed that the introduction of DNA arms into binding stems I and III enhanced \(k_{\text{cat}}\) values (2,3). Similar observations were made by Rossi’s group (4) and Jennings’ group (5,6). Enhancement of \(k_{\text{cat}}\) values by DNA arms was the result of two distinctly different phenomena: (i) in case of the study by Rossi’s group (4), the enhancement originated from the acceleration of dissociation of the products from the reaction complex \(k_{\text{diss}}\) (Figure 1); and (ii) in case of the study by Hendry et al. (5), the rate-limiting step for the chimeric DNA/RNA ribozyme was the chemical-cleavage step \(k_{\text{cleav}}\) although, for their all-RNA ribozyme, the rate-limiting step appeared to be a step prior to cleavage (and not product-dissociation) (5). Since it is important to define what is represented by \(k_{\text{cat}}\), in order to avoid kinetic complexities, we have been using short substrate-binding arms (stems I and III) (1–3). Note that when a longer binding sequence is used, the product-dissociation step \(k_{\text{diss}}\) rather than the chemical-cleavage step \(k_{\text{cleav}}\) becomes the rate-limiting step (Figure 1) (7).

In the present study, since ribozymes appear to be metalloenzymes (8–10), we examined possible differential effects of various divalent metal ions on the chimeric DNA/RNA hammerhead ribozyme, under conditions where the chemical-cleavage step \(k_{\text{cleav}}\) is the rate-limiting step. We report here that, in the presence of various divalent metal ions, DNA arms always enhance \(k_{\text{cleav}}\) values. Furthermore, in order to investigate possible structural changes in the catalytic loop as a result of the presence of the DNA arms, chemical probing with dimethylsulfate (DMS) was performed.

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**Figure 1.** The reaction of the hammerhead ribozyme consists of at least three steps. The substrate (and magnesium ion) first binds to the ribozyme \(k_{\text{bind}}\). The phosphodiester bond of the bound substrate is cleaved, probably by the action of a magnesium ion \(k_{\text{cleav}}\). The cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events \(k_{\text{diss}}\).
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Figure 2. Structures of all-RNA ribozyme (R32) and chimeric DNA/RNA ribozyme (DRD32). RNA and DNA regions are indicated by bold and outlined letters, respectively. The substrate-binding regions (stems I and III) are short to avoid the rate-limiting product-dissociation step (Fig. 1; k_{obs}). Also, the binding sequence was designed so that self- and inter-substrate complementations could be avoided.

MATERIALS AND METHODS

Synthesis of ribozymes

All substrates and ribozymes shown in Figures 2 and 4(a) were chemically synthesized on a DNA synthesizer [38OB; Applied Biosystems, Inc. (ABI), Foster City, CA] as described previously (3). RNA reagents were purchased from American Bioinformatics, Inc. (ABN; Hayward, CA). Other reagents were purchased either from ABI or ABN. Purification of the synthesized oligonucleotides was performed as described in the ABI user bulletin (no. 53; 1989) with minor modifications.

Kinetic measurements

Kinetic measurements were made at 37°C, in 50 mM Tris—HCl (pH 7.0) and 25 mM metal ions. Since K_{m} values for R32 and DRD32 in 25 mM MgCl_{2} were 0.02 μM and 1 μM, respectively, at pH 8.0 (3), in order to ensure the condition of k_{obs} = k_{cat}, concentrations of ribozyme and substrate were set at 0.5 μM and 100 μM, respectively. Reactions were stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution that 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 (BA100; Fuji Film, Tokyo).

The burst kinetics shown in Figure 3 were obtained similarly at 37°C, in 50 mM Tris—HCl (pH 7.0) and 25 mM MgCl_{2}. Final concentrations of ribozyme and substrate were 0.1 μM and 2.17 μM, respectively.

Chemical modifications by dimethylsulfate

For chemical modification, general procedures were essentially those described by Krol & Carbon (11). For each modification reaction, about 5 pmol of unlabelled R32 or DRD32 ribozyme, to which a primer-binding RNA sequence of 3'-CUA-GGGGUA-AAG-5' had been attached [Figure 4(a)], were used. Since it is known that changing or adding sequences of hammerhead ribozymes can have dramatic effects on cleavage, we checked the catalytic activity of these new ribozymes to which a primer-binding RNA sequence had been attached: the activity of these ribozymes was found to be nearly the same as that of the corresponding original ribozymes. Controls were treated in parallel with omission of the chemical reagents. For probing the corresponding original ribozymes. Controls were treated in parallel with omission of the chemical reagents. For probing the corresponding original ribozymes. Controls were treated in parallel with omission of the chemical reagents. For probing the corresponding original ribozymes. Controls were treated in parallel with omission of the chemical reagents. For probing the corresponding original ribozymes.
RESULTS AND DISCUSSION

Structures of the chemically synthesized hammerhead ribozymes are shown in Figures 2 and 4(a). R32 is an all-RNA 32-mer ribozyme. DRD32 is a chimeric ribozyme consisting of DNA (stem I), RNA (catalytic loop, stem II and its loop), and DNA (stem III) regions. Similarly, a cleavable all-RNA substrate (R11) and an uncleavable chimeric substrate (RdC11), which contains deoxy-C at the cleavage site [at C17 of Figure 4(a)], were synthesized. Kinetic parameters were measured at 37°C, in 25 mM solutions of divalent metal ions with 50 mM Tris-HCl (pH 7.0) under saturating conditions, and they are listed in Table I. Our kinetic analysis on DNA-armed chimeric ribozymes revealed that deoxyribozymes have an extra RNA annealing sequence at the 3'-end were treated with dimethylsulfate (DMS) under various conditions. Lanes 0 and 1, control (without DMS treatment, natural stops); lane 2, R32 + Mg²⁺; lane 3, R32; lane 4, DRD32 + Mg²⁺; lane 5, DRD32; lane 6, R32 + RdC11 + Mg²⁺; lane 7, R32 + RdC11; lane 8, DRD32 + RdC11 + Mg²⁺; lane 9, DRD32 + RdC11. The sequencing ladders (U, G, C, A) are shown.

Figure 4. Chemical probing of R32 and DRD32 ribozymes. (a) Ribozyme sequence used for the chemical probing. C2.5, G2.4, G2.3, G2.2, G2.1, G5.3, G15.4, and C15.5 were made of DNA for the chimeric ribozyme. (b) Two types of ribozyme with an extra RNA annealing sequence at the 3'-end were treated with dimethylsulfate (DMS) under various conditions. Lanes 0 and 1, control (without DMS treatment, natural stops); lane 2, R32 + Mg²⁺; lane 3, R32; lane 4, DRD32 + Mg²⁺; lane 5, DRD32; lane 6, R32 + RdC11 + Mg²⁺; lane 7, R32 + RdC11; lane 8, DRD32 + RdC11 + Mg²⁺; lane 9, DRD32 + RdC11. The sequencing ladders (U, G, C, A) are also shown.

Table I. Dependence of kcat on divalent metal ions for the cleavage of R11*

<table>
<thead>
<tr>
<th>Divalent ion</th>
<th>Ionic radius</th>
<th>R32 kcat</th>
<th>DRD32 kcat</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂</td>
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</tr>
<tr>
<td>BaCl₂</td>
<td>1.35</td>
<td>~0</td>
<td>~0</td>
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</tbody>
</table>

*aKinetic measurements were made at 37°C, in 50 mM Tris-HCl (pH 7.0) and 25 mM metal ions. Since K_M values for R32 and DRD32 in MgCl₂ were 0.02 μM and 1 μM, respectively (ref. 3), in order to maintain saturation conditions, concentrations of substrate and ribozyme were set at 100 μM and 0.5 μM, respectively.

*bIonic radius was calculated on the basis of presumed hexacoordination (ref. 22).


tutions in stems I and III increase values of both kcat and K_M (2,3). The next question is as follows: does the enhanced kcat really represent an enhancement of the rate of the cleavage step? One classical way to examine this question is to check for the possible existence of ‘burst kinetics’ (16,17). In the case of chymotrypsin, Ser-195 acts as the nucleophile in formation of a covalently linked enzyme—substrate intermediate. Since, in the case of chymotrypsin, the formation of the enzyme–substrate intermediate occurs more rapidly than the decomposition (hydrolysis) of the intermediate, the rapid liberation of the leaving group (up to an equimolar amount of substrate with respect to the amount of enzyme, similar to a single-turnover condition) is followed by the slower decomposition of the intermediate (decomposition of the intermediate to regenerate the active enzymes and a second liberation of leaving groups catalyzed by the regenerated enzyme under steady-state conditions), with a resultant biphasic fast reaction followed by a slower reaction (burst kinetics). Similarly, if the product-dissociation step (k_diss) in Figure 1 was the rate-limiting step, then biphasic burst kinetics would be anticipated (4,5,17). Figure 3 illustrates the results of studies designed to examine this possibility. The dashed line denotes the concentrations at which the concentrations of substrate and enzyme are equal. The absence of burst kinetics (the identical slopes on either side of the ‘equimolar line’) at the early stage of the reaction suggests that the rate-limiting step in these ribozyme-catalyzed reactions is the chemical-cleavage step rather than the product-release step (k_diss).

Having demonstrated the rate-limiting chemical-cleavage step (k_cat) for this sequence by mono-phasic reactions (Figure 3), we examined the dependence of k_cat on divalent metals under substrate-saturating conditions (Table I). When the effect of Mg²⁺ ions at various concentrations was examined for the all-RNA ribozyme (R32) at pH 8.0, the dependence was found to be very similar to that observed by Dahm and Uhlenbeck for a similar all-RNA ribozyme with a different binding sequence (18): k_cat reached a plateau value at higher concentrations of Mg²⁺ ions, with a limiting value of 18 min⁻¹ (>100 mM). By contrast, in the case of chimeric DRD32 ribozyme, it was of special interest to us that Mg²⁺ ions enhanced the catalytic activity to give a k_cat value with the surprisingly high value of >100 min⁻¹ at concentrations of Mg²⁺ ions above 1 M: even though at such a high Mg²⁺ ion concentrations, no saturation
point had been reached and thus it was not possible to calculate $K_{Mg}$ for the chimeric ribozyme. Since the dependence on Mg$^{2+}$ ions was not identical between the all-RNA (saturation curve) and the chimeric (no saturation) ribozymes, we arbitrarily chose a concentration of metal ions of 25 mM for our subsequent comparative studies. It is to be pointed out, based on the differential dependence on Mg$^{2+}$ ions, that the observed activity difference between all-RNA and chimeric ribozymes in Table I would increase at higher metal ion concentrations. A pH of 7.0 was employed in order to maintain the solubility of some ions: at pH 8.0, for example, some precipitation was recognized in a solution containing Mn$^{2+}$ ions.

We wondered whether the DNA arms of DRD32 might lead to a differential effects of divalent metal ions. However, as can be seen from Table I, no such differential effects were recognizable. In all cases examined, the relative reactivities of the chimeric DNA/RNA ribozyme (DRD32) were nearly identical to those of the all-RNA ribozyme (R32). This result indicates that the interaction with the metal ions in DRD32 remains the same as that in R32. With this sequence the catalytic activity by Mn$^{2+}$ is greater than that for other sequences which were measured at slightly higher pH (18,19): the enhancement originates, at least in part, from a unit lower pKa of Mn(OH)$_2$$^{2+}$ compared to that of Mg(OH)$_2$$^{2+}$. As mentioned above, in no cases, precipitation was recognized at pH 7.0. Clearly, various metal ions, including Co$^{2+}$ and Ca$^{2+}$ with the ionic radius of 0.65 and 1.0 Å, respectively, could support hammerhead cleavage for both types of ribozyme. It is of particular interest that, in all cases examined, DNA arms always enhanced the chemical-cleavage activity.

How do the DNA arms enhance the chemical-cleavage step? In order to probe the structure of the catalytic loop, both all-RNA (R32) and chimeric DRD32 ribozymes were treated with various ribonucleases. Digestion patterns, in bovine serum, were examined for three kinds of chimeric ribozymes that were DRD32, DDRD32 (in addition to stems I and III, stem II and its loop are also DNA), and thio-DDRD32 (all DNA domains of DDRDRD32 contained phosphorothioate linkages) (3). Since (i) the digestion patterns of all three chimeric ribozymes were nearly identical and that of the all-RNA ribozyme (R32) was different from the rest (those of chimeric ribozymes), and (ii) the digestion patterns for R32 and thio-DDRD32 were shown in Figure 3 of ref. 3, they were not repeated in this article. In short, when ribozymes were treated with fetal bovine serum, it became clear that the all-RNA R32 ribozyme was mainly degraded at the four phosphodiester linkages on the 3' sides of C3, U4, A6, and U7 [for the numbering, see Figure 4(a) and ref. 20]. By contrast, the chimeric DRD32 ribozyme was mainly degraded at U4 and U7 with a low rate of cleavage at C3. Thus, it is possible that, upon introduction of DNA arms with repetitive G's, the arms and the catalytic loop undergo a slight alteration in structure such that digestion at A6 is no longer possible.

However, one may not be able to draw structural conclusions by using serum as a source of nuclease activity since the serum may contain nucleic acid binding proteins which bind differentially to RNA and DNA sequences and will therefore differentially affect the structure of RNA and chimeric ribozymes. We therefore re-checked the structure by chemical probing analysis using dimethylsulfate (DMS). The reason why we chose DMS as a probing reagent was that DMS can modify more bases [it reacts with adenine (at N1), cytosine (at N3) and guanine (at N7)] than other reagents (DEPC, for example, is a N7 probe for adenines). Results of such analysis are shown in Figure 4(b). Chemical-probing data using dimethylsulfate (DMS) indicate that the catalytic-loop structures of all-RNA and chimeric ribozymes are nearly identical with the exception of enhanced termination of primer extension reactions at C3 in the case of the chimeric DRD32 ribozyme. The difference is recognizable only for the ribozyme–substrate complexes (lanes 6 and 7 vs. lanes 8 and 9). This difference in reactivity with DMS at C3 coincides with the difference in sensitivity of C3 to attack by RNases in fetal bovine serum. This kind of very small alteration in the structure may provide a clue to why the DNA arms enhance the chemical-cleavage step. However, the presence of Mg$^{2+}$ ions did not affect the degree of methylation; this is consistent with the conclusion based on NMR studies that there is no conformational changes mediated by Mg$^{2+}$ ions (21).

In conclusion, in the case of our sequence, we have unambiguously demonstrated that (i) DNA arms always enhance the chemical-cleavage reactions in the presence of various divalent metal ions, (ii) the interaction with various metal ions is very similar in all-RNA and chimeric ribozymes, and (iii) the structure of the ion pocket is nearly the same but may not be completely identical in both cases. These subtle differences may allow a more favorable positioning of the critical groups that are involved in catalysis. Since ribozymes are metalloenzymes and since divalent metal ions play a key role in catalysis (8–10), it is possible that the DNA arms fix the position of the catalytically indispensable metal ion in such a way that the transition state is more effectively stabilized than it is in the wild-type ribozyme.

REFERENCES