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Extraordinary enhancement of the cleavage activity of a DNA-armed hammerhead ribozyme at elevated concentrations of Mg^{2+} ions

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Abstract As part of an ongoing effort to characterize structure–function relationships, activities of all-RNA and DNA-armed hammerhead ribozymes were examined. An analysis of the dependence on the concentration of Mg^{2+} ions of cleavage rates revealed that, whereas the k_{cat} of the reaction catalyzed by the all-RNA ribozyme reached a maximum value of about 18 min^{-1} at a concentration of about 200 mM Mg^{2+} ions, that of the DNA-armed ribozyme increased linearly as the concentration of Mg^{2+} ions was increased above 300 mM, finally reaching a value of more than 100 min^{-1} at 700 mM Mg^{2+} ions. These results suggest that the potential activity of a hammerhead ribozyme might be greater than is usually recognized.

Key words: Ribozyme; Cleavage activity; Dependence on Mg^{2+} ion; DNA substitution

1. Introduction

Hammerhead ribozymes are RNA enzymes that act in *cis* in nature [1]. They have also been engineered such that they act in *trans* [2,3] and their potential as therapeutic agents has been demonstrated [4]. In an attempt to enhance our understanding of structure–function relationships and also to stabilize the ribozyme against nucleases, the hybridizing arms of a hammerhead ribozyme (stems I and III; Fig. 1) were replaced by deoxyribonucleotides [5–7]. As a result, an increase in the cleavage activity, which was a consequence of the enhancement of either the product-release rate [5] or the chemical cleavage rate [6–9], was observed. In order to investigate the cleavage reaction catalyzed by the DNA-armed ribozyme in further detail, an analysis of the dependence on the concentration of Mg^{2+} ions of the value of k_{cat} was made. Here we report a surprising increase in the activity of the DNA-armed ribozyme at elevated concentrations of Mg^{2+} ions.

2. Materials and methods

2.1. Synthesis of all-RNA and chimeric DNA/RNA oligonucleotides

The substrate and the ribozymes shown in Fig. 1 were chemically synthesized on a DNA synthesizer (model 380B; Applied Biosystems Inc. (ABI), Foster City, CA). RNA-related reagents were purchased from American Bionetics Inc. (ABN; Hayward, CA). Other reagents were purchased either from ABI or ABN. Purification of chemically synthesized oligonucleotides has been described elsewhere [7].

2.2. Kinetic measurements of dependence on Mg^{2+} ions

Kinetic measurements of dependence on Mg^{2+} ions were performed in 50 mM Tris-HCl (pH 8.0) at 37°C. In the standard reaction, the concentrations of the substrate, R11, and the ribozyme, R32, were 43 μM and 0.090 μM, respectively. In the reaction with a chimeric ribozyme, the concentrations of the substrate, R11, and the chimeric ribozyme, DRD32, were 53 μM and 0.022 μM, respectively. Time courses of the reactions were individually adjusted to correspond to cleavage activities [10]. Concentrations of oligonucleotides in solution were determined by measuring UV absorbance at 260 nm. Solutions of both [^{32}P]5′-end-labeled substrate and non-radio-labeled ribozyme were preincubated separately at 37°C. The reaction was initiated by addition of the substrate to the solution of ribozyme. The reaction was stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution of 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromophenol blue, with subsequent snap-cooling on ice. Intact substrate and 5′-cleaved product 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 measurement of radioactivity in the bands of the substrate and the 5′-product with a Bio-Image Analyzer (model BA100 or BA2000; Fuji Photo Film Co. Ltd., Tokyo, Japan).

3. Results and discussion

We previously synthesized *trans*-acting hammerhead ribozymes, as shown in Fig. 1 [11], in which the sequence of the substrate was carefully designed so that self- and inter-substrate aggregation would be avoided and the k_{cat} value was proved to represent the rate of chemical cleavage under the conditions of our kinetic measurements ($k_{cat} = k_{cleav}$ in Fig. 2) [8]. Kinetic measurements showed an increase in k_{cat} upon introduction of DNA in stems I and III, in either hybridizing arms of the ribozyme [7,8] or in the substrate [9]. In this study, we compared the dependence of k_{cat} of the all-RNA (R32) and the DNA-armed (DRD32) ribozymes on the concentration of Mg^{2+} ions using an all-RNA (R11) substrate (Fig. 1). The results are shown in Fig. 3. The concentrations of the substrate in the reactions were kept high with respect to the K_m values of 20 nM for the R32 ribozyme and 1.3 μM for the DRD32 ribozyme [7,9] in order to ensure k_{cat} measurements. In the case of the R32 ribozyme, a typical saturation curve was obtained as previously reported [2,12–14]; the k_{cat} reached a plateau value of about 18 min^{-1} at about 200 mM Mg^{2+} ions in our case although the concentration at which the saturation by Mg^{2+} ions is achieved is different among the reports [2,12–14]. However, by contrast to the results for R32, the results for DRD32 showed that the k_{cat} value tended initially to reach a plat-

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Abbreviation: HDV, human hepatitis delta virus.

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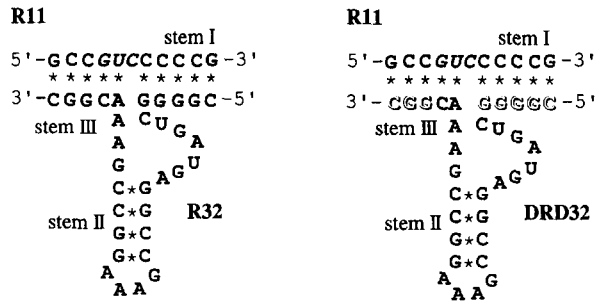

Fig. 1. The *trans*-reaction system of a hammerhead ribozyme. An all-RNA substrate (R11), an all-RNA ribozyme (R32) and a DNA-armed ribozyme (DRD32) are shown. Regions of RNA and DNA are indicated by bold and outlined letters, respectively.

eau but then, increased abruptly from about 300 mM Mg^{2+} ions, reaching more than 100 min^{-1} at about 700 mM Mg^{2+} ions.

The great increase in activity obtained with the DRD32 ribozyme appeared not to be due to factors other than a change in the chemical cleavage rate, such as non-specific cleavage of the substrate at higher concentrations of Mg^{2+} ions, which would result in an observed decrease in the level of the substrate, or to the effect of mere ionic strength. In such cases, the k_{cat} values for both the R32 and the DRD32 ribozymes would show the same profile of dependence on the concentration of Mg^{2+} ions. Fig. 4 shows the results of cleavage reactions catalyzed by the DRD32 ribozyme at higher concentrations of Mg^{2+} ions. DRD32 cleaved a large amount of the R11 substrate in a short time and no products of non-specific cleavage were observed. Furthermore, no enhancement was observed of the rate of the cleavage reaction catalyzed by the DRD32 ribozyme at the standard concentration of 25 mM $MgCl_2$ after addition of 2.5 M NaCl to the assay mixture (data not shown). The dependence on the concentration of Mg^{2+} ions of the cleavage rate in a wild-type reaction with or without NaCl has been examined by Dr. P. Hendry (personal communication). The cleavage rates with 1.0 M NaCl are lower than those without NaCl at the concentrations of Mg^{2+} ions below 40 mM. However, at 50 and 100 mM Mg^{2+} ions, the rates in the presence of 1.0 M NaCl are greater than those in the absence of NaCl.

What factors determine the cleavage activity of a hammerhead ribozyme? One factor reflects intrinsic properties of the enzyme: the concentration of the Michaelis complex. A large

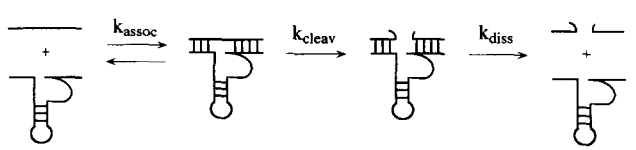

Fig. 2. Schematic representation of the kinetics of the ribozyme-catalyzed reaction. The reaction catalyzed by the hammerhead ribozyme consists of at least three steps. The substrate (and Mg^{2+} ions) first binds to the ribozyme (k_{assoc}). The phosphodiester bond of the bound substrate is cleaved by the action of Mg^{2+} ions (k_{cleav}). The cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}). Under the conditions of our kinetic measurements in this study, the obtained k_{cat} represents k_{cleav}.

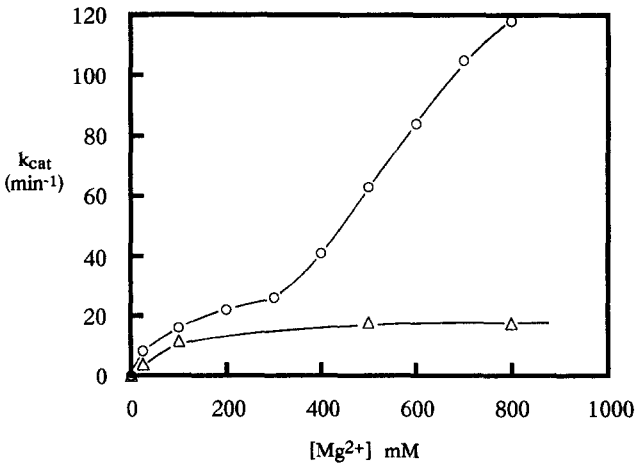

Fig. 3. Dependence on the concentration of Mg^{2+} ions of k_{cat} for R32 (△) and DRD32 (○). The concentrations of R11, R32, and DRD32 used in this study were, respectively, 53 μM, 0.038 μM, and 0.022 μM. The k_{cat} only for the DNA-armed ribozyme DRD32 could be increased to > 100 min^{-1} by increasing the concentration of Mg^{2+} ions (○).

excess of substrate can constantly maintain the complex at a saturated level in our measurements. The other factor is the concentration of Mg^{2+} ions. Cleavage reactions of hammerhead ribozymes require Mg^{2+} ions [12–18]. The suggested mechanism, based on solvent isotope effects and ab initio molecular orbital calculations [17,18], indicates that the active complex of substrate and ribozyme includes Mg^{2+} ions (Fig. 5). Therefore, cleavage activity should increase with an increase in the concentration of Mg^{2+} ions until saturation by Mg^{2+} ions is achieved. Fig. 3 clearly indicates that the profile of the dependence on the concentration of Mg^{2+} ions of the DRD32 ribozyme has two phases. The curve that levels off at lower concentrations is similar to that of the R32 ribozyme. However, there follows a linear increase at higher concentrations during which Mg^{2+} ions seem not saturated. It may be that an alternate structure, with higher activity and a higher dissociation constant for Mg^{2+} ions than those of the usual structure, is formed with the DNA-armed hammerhead at higher concentrations of Mg^{2+} ions. However, the DNA arms seem not to be a crucial requirement for such a dramatic increase in k_{cat} because a derivative of the R32 ribozyme that lacks stem II, which inevitably has much lower activity than the R32 ribozyme, showed a similar dependence on the concentration of Mg^{2+} ions to that of the DRD32 ribozyme (Amontov and Taira, unpublished results). A similar abrupt increase in cleavage activity with increases in the concentration of Mg^{2+} ions was also reported in the case of the HDV ribozyme [19] although its rate-determining step does not seem to be the chemical cleavage step, at least at moderate concentrations of Mg^{2+} ions (Kawakami et al., unpublished results).

The k_{cat} values of more than 100 min^{-1} in our case are the highest ever reported for a hammerhead ribozyme, indicating that the potential activity of a hammerhead ribozyme might be greater than is usually recognized. Determination of pH–rate profile for a hammerhead ribozyme [14,18] suggests that the proportion of deprotonated 2′-alkoxide at the cleavage site, which can actually attack the adjacent phosphorus, seems low at usual conditions (near neutral pH). After further experi-

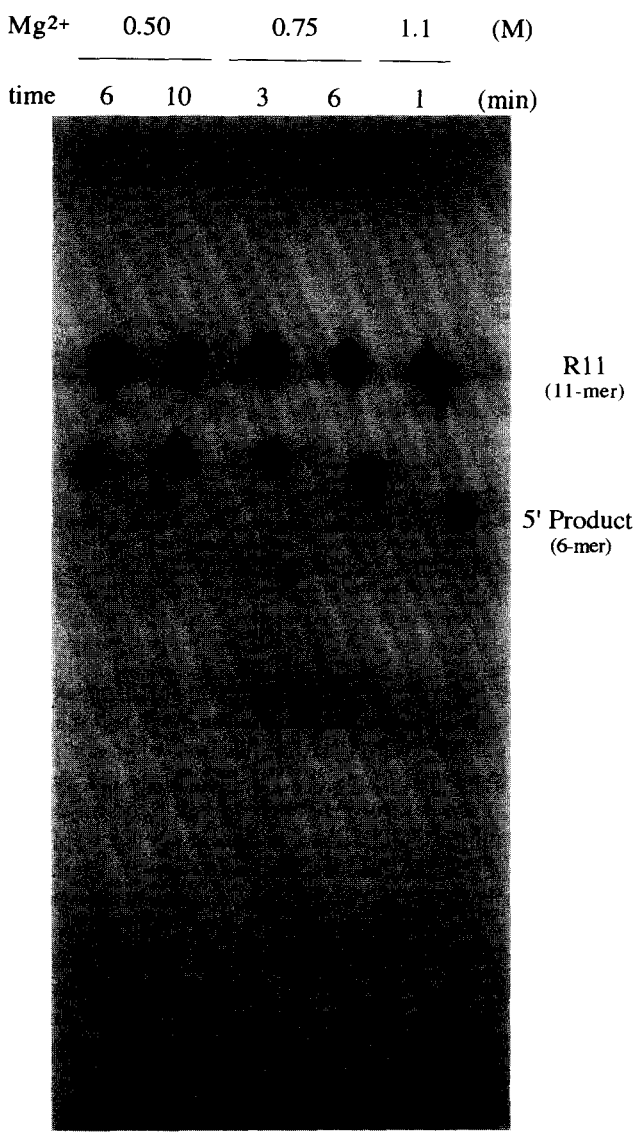

Fig. 4. Results of the cleavage of the R11 substrate by the DNA-armed ribozyme DRD32 at concentrations of Mg^{2+} ions at 0.50, 0.75, or 1.1 M. The concentrations of R11 and DRD32 used in this study were 43 μM and 0.030 μM, respectively. In no cases, any non-specific cleavage reactions had occurred because non-specific background reactions were much slower than the specific Mg^{2+}-mediated ribozyme reactions.

ments to explain the enhanced cleavage rate at elevated concentrations of Mg^{2+} ions, it might be possible to engineer much more active ribozymes that can be used to cleave specific RNAs.

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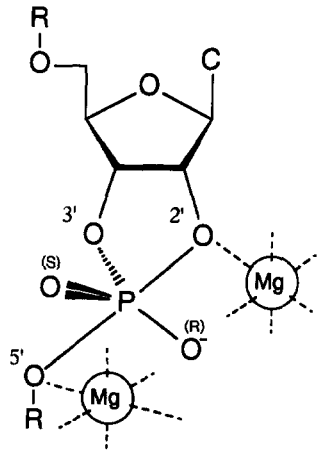

Fig. 5. Mechanism proposed for the cleavage of RNA by hammerhead ribozyme [17,18]. The *pro-R_p* and *pro-S_p* phosphoryl oxygens are labelled with (R) and (S), respectively. The *pro-R_p* oxygen is known to coordinate Mg^{2+} ion.

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