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Dependence on Mg^{2+} ions of the activities of dimeric hammerhead minizymes

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Abstract **A minizyme is a hammerhead ribozyme with short oligonucleotide linkers instead of stem/loop II. In a previous study we demonstrated that a minizyme with high-level activity forms a dimeric structure with a common stem II [Amontov and Taira (1996) J. Am. Chem. Soc. 118, 1624–1628]. We now demonstrate that the stability of the dimeric structure is influenced by Mg^{2+} ions. We found that the dependence on Mg^{2+} ions of the activity of homodimeric minizyme (a dimer with two identical binding sites) has composite biphasic characteristics. When the concentration of Mg^{2+} ions reached a specific critical level, the dependence on the concentration of Mg^{2+} ions lost its tendency to reach a plateau. In the case of the heterodimeric minizyme (a dimer with two different binding sites), we investigated the kinetic behavior of two different forms of the dimer, namely, free dimer and the complex of the dimer with an uncleavable substrate. The kinetic behavior of the free heterodimer was very similar to that of the homodimeric minizyme. In contrast, in the presence of the uncleavable substrate at a concentration as high as that of the minizyme, the curve for the dependence on Mg^{2+} ions showed normal saturation kinetics. While, at low concentrations of Mg^{2+} ions, the activity of the heterodimers was much higher when the dimeric structure was stabilized by the presence of the uncleavable substrate, at high concentrations of Mg^{2+} ions, this difference in activity became less and less significant. Thus, high concentrations of Mg^{2+} ions were able to stabilize the dimeric minizymes in the absence of the uncleavable substrate.**

Key words: Mg^{2+} dependence; Minizyme; Kinetics

1. Introduction

Recent publication of crystallographic data for hammerhead ribozymes has shed light on a number of very important details of the tertiary structure of this class of catalytic RNAs [1,2]. However, some problems remain as to the precise mechanism of hammerhead catalysis. A pivotal role in the cleavage of RNA by hammerhead ribozymes is played by Mg^{2+} ions, and it is now clear that Mg^{2+} ions have multiple functions in hammerhead catalysis. The first important function is the direct participation in the chemical cleavage of the phosphodiester bond. The mechanism whereby Mg^{2+} ions mediate such cleavage has been analyzed by numerous authors [3-12]. The second, and no less important function, is the critical role of Mg^{2+} ions in the folding processes that yield the correct active conformation [13,14]. The influence of some divalent metal ions on the tertiary structure has been demonstrated not only in the case of specifically hammerhead structures, but also, more generally, in the case of three-way junctions and Holiday junctions [15-18]. It remains unclear, however, whether only one principal active conformation is possible or whether some specific conditions can result in alternative active conformations.

We found recently that hammerhead minizymes with short oligonucleotide linkers instead of a stem-loop II region (Fig. 1) can form homodimers that are actually very active hammerhead ribozymes [19]. They seem to form divalent structures with two catalytic centers, two identical binding sites and one common stem II (Fig. 1c). The activity of the dimeric minizyme was comparable with that of the full-sized ribozyme [19]. As a next step, we designed analogous heterodimeric structures in which the sequences of binding sites were different and which could, thus, target two different substrates (Fig. 1d) [20]. All these dimeric structures are not only stabilized by Watson-Crick base pairs that yield the common stem II of the dimeric structure. A major contribution to the stabilization of such dimers is also made by additional non-Watson-Crick base pairing and coordination of Mg^{2+} ions within the catalytic core [14]. Interaction with substrates also has a significant stabilizing effect on the dimeric structure [19].

Here we describe our investigations of the influence of different concentrations of Mg^{2+} ions on the activity of homodimeric and heterodimeric minizymes. In the case of the heterodimeric minizyme, we found that two different kinds of Mg^{2+} dependence could be observed depending on the presence or absence of an uncleavable substrate.

2. Materials and methods

2.1. Synthesis of minizymes

Minizymes and their corresponding substrates were synthesized on an ABI DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified by HPLC and polyacrylamide gel electrophoresis as described previously [19-23]. Reagents for manipulations of RNA were purchased from ABI. The oligonucleotides were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications.

2.2. Kinetic measurements

Reaction rates were measured under minizyme-saturating (single-turnover) conditions at 37°C. In all cases, kinetic measurements were made under conditions where all the available substrate was expected to form a Michaelis-Menten complex, with excess and sufficiently high concentrations of minizymes.

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 (BA2000; Fuji Film, Tokyo).

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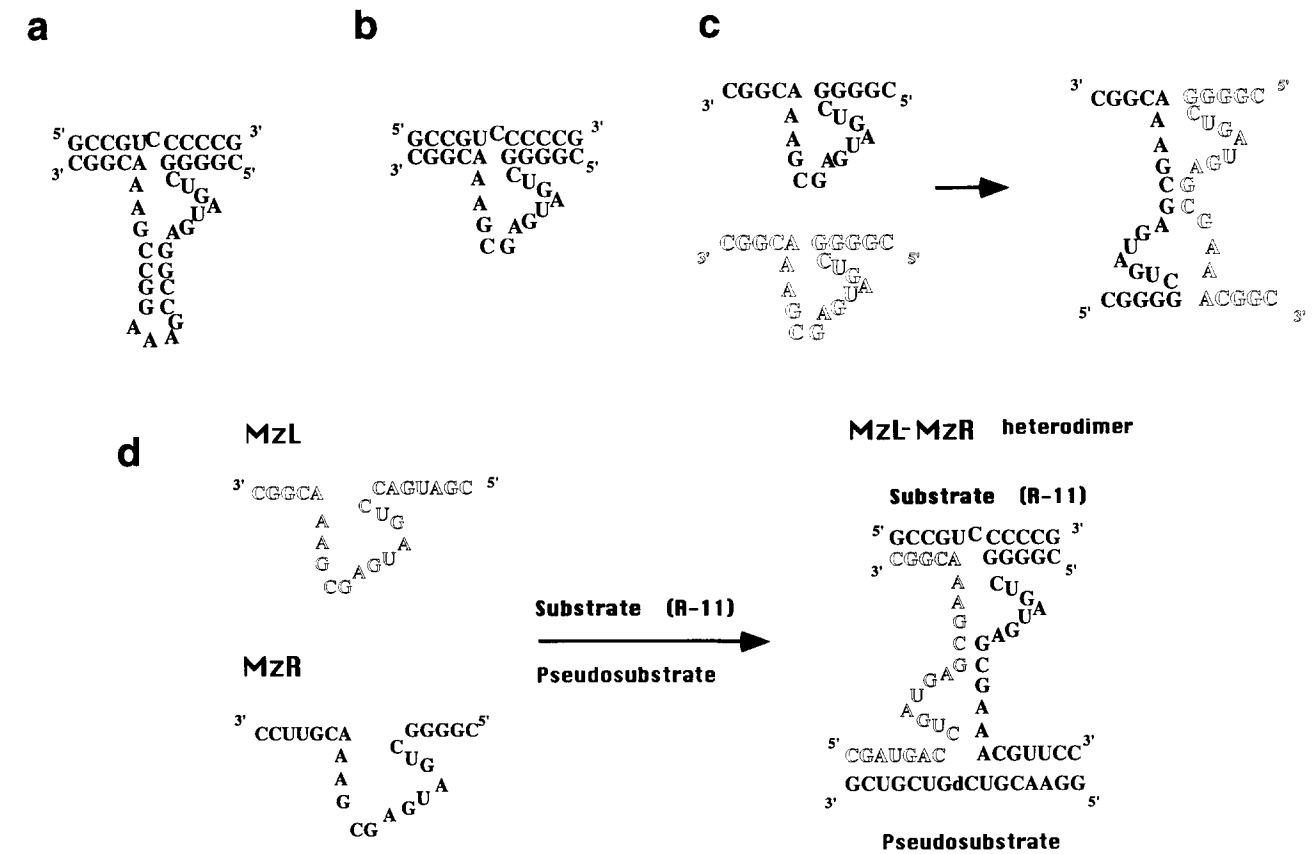

Fig. 1. The secondary structures of (a) the hammerhead ribozyme (R32) and its substrate (R11); (b) a complex of the minizyme MzGC and its substrate; (c) the dimerized minizyme MzGC (formation of the homodimer). (d) Schematic representation of the MzL-MzR heterodimer. The MzL-MzR heterodimer can generate two different binding sites: one is complementary to the sequence of R11 (top binding site) and the other (bottom binding site) is complementary to an uncleavable pseudosubstrate. The presence of the pseudosubstrate stabilizes the dimer [19].

3. Results and discussion

We demonstrated previously that Mg^{2+} ions can convert an inactive form of the ribozyme shown in Fig. 1a to an active ribozyme complex with its substrate [14]. A previous study [19] also demonstrated that minizyme with high-level activity (Fig. 1b) forms a dimeric structure with a common stem II (Fig. 1c). The dimeric minizyme (MzGC, Fig. 1c) retained 65% cleavage activity of the parental ribozyme (Fig. 1a) when the same substrate was used under minizyme-saturating conditions [19]. Since there was a possibility that MzGC might exist in an inactive form at low concentrations of Mg^{2+} ions, we initiated a study of the Mg^{2+}-dependent changes in activity of the homodimeric minizyme.

We found initially that, in contrast to conventional hammerhead ribozymes, the enzymatic reaction catalyzed by the homodimeric minizyme (MzGC) demonstrated unusual dependence on Mg^{2+} ions under single-turnover conditions (with an excess of minizyme; Fig. 2). Under these conditions, minizyme exists as (i) free monomers, (ii) free dimers, and (iii) complexes of the dimer with one molecule of substrate (the second binding site is free). We found, moreover, that under these conditions, the Mg^{2+}-dependence curve had two phases (Fig. 2). The first phase resembled a standard saturation curve (with increasing concentrations of Mg^{2+} ions, the rate of the reaction catalyzed by the minizyme had a tendency to reach a plateau). This result is in accordance with the proposed mechanism, which includes, as a first step, the interaction of a Mg^{2+} ion with a specific Mg^{2+}-binding site in the ribozyme-substrate complex. However, when the concentration of Mg^{2+} ions reached a critical level, the character of the curve changed. Further increases in the concentration of Mg^{2+} ions

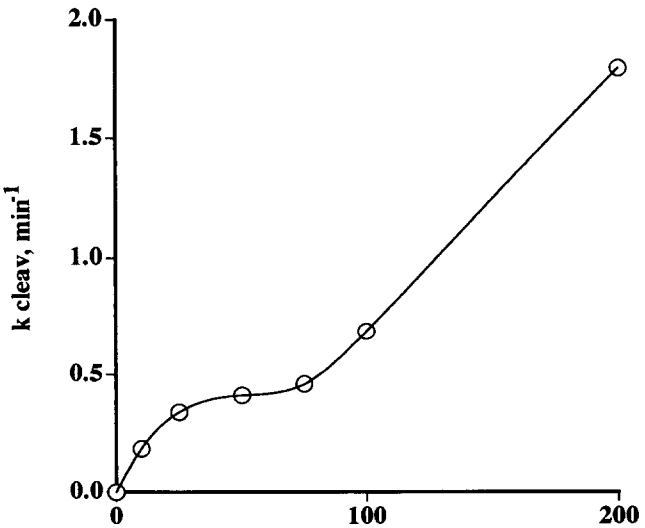

Fig. 2. Dependence on Mg^{2+} ions of the cleavage activity of homodimeric MzGC under single-turnover conditions (Tris-HCl (pH 7.0), 50 mM; ribozyme, 10 μM; 5′-^{32}P-substrate, 2–5 nM; $MgCl_2$, 0–200 mM; 37°C). The cleavage reaction was started by addition of the substrate.

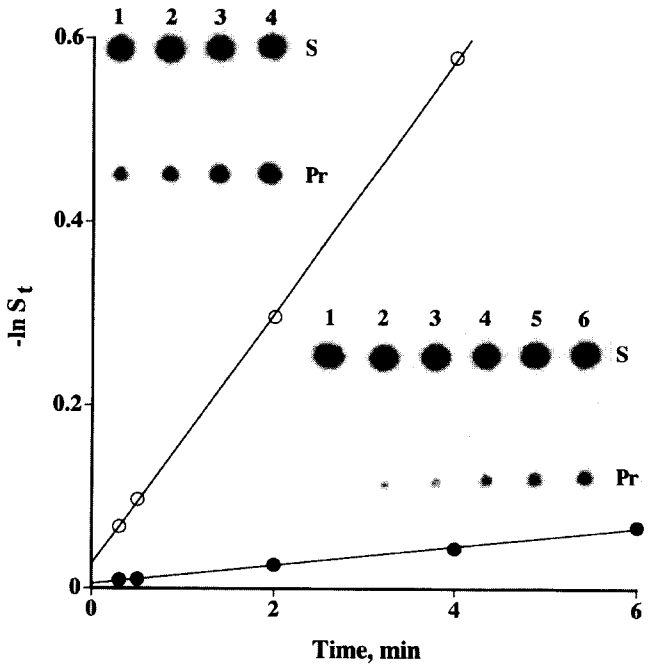

Fig. 3. Time course of the cleavage activity of the heterodimer under single-turnover conditions (Tris-HCl (pH 8.0), 50 mM; MzL, 2 μM; MzR, 1 μM; 5′-^{32}P-substrate, 1 μM; $MgCl_2$, 50 mM; 37°C). The cleavage reaction was started by addition of the substrate. (○) Uncleavable substrate at 1 μM; (●) in the absence of the uncleavable substrate. S_t designates the fraction of the undegraded substrate at time t, ($S_0 = 1$). The upper autoradiogram (lanes 1–4) represents the heterodimer-mediated cleavage in the presence of the uncleavable substrate and the lower one (lanes 1–6) represents that in the absence of the uncleavable substrate. S, substrate R11; Pr, product of the cleavage reaction.

caused a proportional increase in the reaction rate without noticeable tendency towards saturation.

A similar unusual dependence on Mg^{2+} ions was found in our laboratory in the case of a DNA-RNA chimeric hammerhead ribozyme [23], as well as by Hendry et al. in the case of some other hammerhead structures [24,25]. Thus, such a phenomenon is not unique but is, more likely, a manifestation of an alternative, but relatively common state of acting hammerhead ribozymes. As mentioned above, all rates of reactions catalyzed by dimeric minizymes were determined under single-turnover conditions and, consequently, the major population of catalytically active forms in the case of MzGC consisted of free dimers. It should be noted that the concentration of minizymes used in this study is above the corresponding K_d of the dimerization process [19]. Thus, the major population of minizymes is in their dimeric forms.

A heterodimeric minizyme provides the opportunity to investigate the activity of one member only of the dimeric hammerhead minizyme, if the dimer is allowed to form a complex with an uncleavable substrate (in previous studies we have already proven that this form is indeed active [19]), and we can compare this form directly with the free dimeric form of the same minizyme. We found that at relatively low concentrations of Mg^{2+} ions (10–50 mM), the activity of the complex of the dimer with the uncleavable substrate significantly exceeded that of the free dimer at the same concentrations of minizymes. Fig. 3 shows typical time courses of the cleavage activity of heterodimers in the presence (open circles) and absence (closed circles) of the uncleavable substrate at 50 mM Mg^{2+} ions, when an almost maximum difference in activity was observed (Fig. 4).

As shown in Fig. 4, whereas the dependence on Mg^{2+} ions of the complex of the dimer with the uncleavable substrate has monotonous saturation-like characteristics (open circles), the dependence on Mg^{2+} ions of the free heterodimer under a variety of conditions (closed circles) gave a biphasic curve that was qualitatively analogous to that obtained with the MzGC homodimer (Fig. 2). Therefore, the biphasic curve was observed only in the case of the complex of the homodimer or the heterodimer with one molecule of substrate bound while the second binding site was free.

Although the exact origin of this kind of biphasic kinetic behavior remains obscure [23-25], several possibilities can be suggested. At low concentrations of Mg^{2+} ions, the free heterodimer, in contrast to the complex of the dimer with the uncleavable substrate, does not have the exactly correct, active conformation but it can, instead, assume a variety of different inactive (or, at least, imperfect) conformations. As a consequence, the free dimer has lower integral activity than the more stable complex of the dimer with the uncleavable substrate. However, beyond the critical concentration of Mg^{2+} ions, the free dimer (again as a result of its flexibility) changes from the less active conformation to the active one as the concentration of Mg^{2+} ions increases. We cannot exclude the possibility that the formation of the latter active conformer results in exposure of the cleavable bond directly to the solution and allows direct attack by Mg^{2+} moieties from the solution without their preliminary binding in the metal-binding pocket. The non-saturating increase in activity of the free dimer beyond the critical concentration of Mg^{2+} ions might be attributable to this metamorphosis. Although we attempted, in an effort to examine the effect of the uncleavable substrate, to follow kinetics at even higher concentrations of Mg^{2+} ions (up to 1 M), we failed to obtain reproducible data.

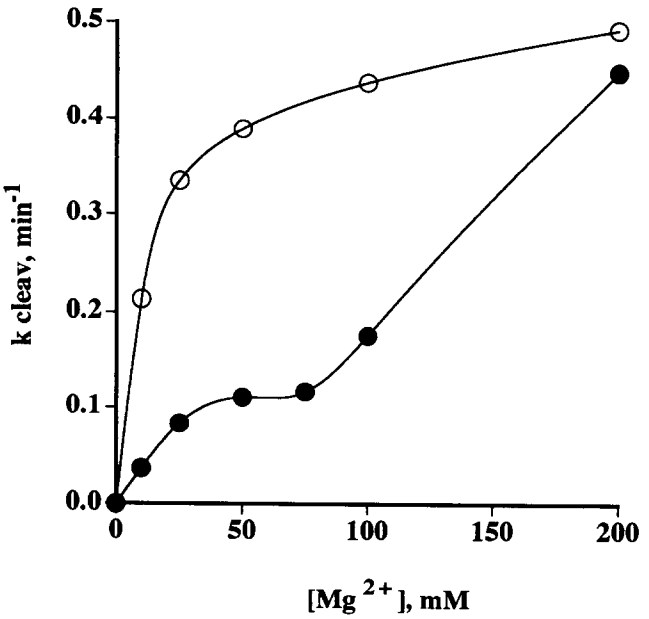

Fig. 4. Dependence on Mg^{2+} ions of the cleavage activity of the heterodimer under single-turnover conditions (Tris-HCl (pH 8.0), 50 mM; MzL, 2 μM; MzR, 1 μM; 5′-^{32}P-substrate, 0.5 μM; $MgCl_2$, 0–200 mM; 37°C). The cleavage reaction was started by addition of $MgCl_2$. (○) Uncleavable substrate at 2.5 μM; (●) in the absence of the uncleavable substrate.

Nonetheless, if the region of linear dependence on the concentration of Mg^{2+} ions were a reflection of the binding of another Mg^{2+} ion to the metal-binding pocket of the dimeric minizyme, such an association would be very weak.

Previous studies demonstrated that the dependence of ribozyme activity on the concentration of Mg^{2+} ions can be either monotonic with a plateau or biphasic, depending on the types of ribozyme used [23-25]. In this report, we have demonstrated that, in the case of the heterodimeric system, kinetic behavior of the heterodimer can be switched from one kind of Mg^{2+} dependence to the other. We could also identify which form of the heterodimeric complex was responsible for the respective Mg^{2+} dependence.

We have also demonstrated here that the stability (activity) of dimeric minizymes can be enhanced not only in the presence of a second, uncleavable substrate but also in the presence of high concentrations of Mg^{2+} ions. Since Mg^{2+} ions can induce conformational changes [13,14], the active conformer of dimeric minizymes could be generated at high concentrations of Mg^{2+} ions, even in the absence of a stabilizer of the dimers, namely, the uncleavable substrate.

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