Catalytic activities of hammerhead ribozymes with a triterpenoid linker instead of stem/loop II

Hiroshi Sugiyama[a], Ken Hatano[a], Isao Saito[a], Sergei Amontov[b,*], Kazunari Taira[b,c]

[a]*Department of Synthetic Chemistry and Biological Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606-01, Japan*
[b]*National Institute of Bioscience and Human Technology, AIST, MITI, 1-1 Higashi, Tsukuba Science City 305, Japan*
[c]*Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba Science City 305, Japan*

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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, *J. Am. Chem. Soc.*, 118 (1996) 1624–1628). As a continuation of this study, we recently examined whether a short oligonucleotide linker at stem/loop II could be replaced by a triterpenoid linker and whether the resulting minizymes with bulky hydrophobic groups would form dimeric structures. In contrast to the minizyme with high-level activity that we characterized in the previous study, minizymes that contained a triterpenoid group had low cleavage activities. The nature of the dependence of the activity on the concentration of ribozyme revealed that these minizymes with a triterpenoid group do not form dimeric structures. Thus, the low activities of these structures can be attributed to their failure to form dimers.

Key words: Ribozyme; Hammerhead; Dimer; Triterpenoid; Kinetics

1. Introduction

Hammerhead ribozymes are a class of catalytic RNAs. They were first recognized as RNAs with a sequence motif that consisted of three duplex stems and a conserved 'core' of two non-helical segments that were responsible for the self-cleavage of the satellite RNAs of certain viruses [1]. Thus, natural hammerhead ribozymes are *cis*-acting ribozymes. Later Uhlenbeck [2] and Haseloff and Gerlach [3] designed *trans*-acting hammerhead ribozymes with an antisense section (stem I and stem III) and a catalytic domain closed by a hairpin loop [3]. Such structures can cleave oligoribonucleotides at specific sites (most effectively at GUC) [4–8]. This specific endoribonucleolytic activity of *trans*-acting hammerhead ribozymes makes them attractive as potential therapeutic agents.

Hammerhead ribozymes are among the most extensively studied ribozymes in terms of both reaction mechanism and structure. Mg^{2+} ions play a pivotal role in the cleavage of RNA by hammerhead ribozymes. The mechanism whereby Mg^{2+} ions mediate such cleavage in ribozyme-mediated reactions in general has been examined by numerous authors [9–18]. Another important function of Mg^{2+} ions is their pivotal role in the folding that yields the correct active conformation of the ribozyme [19,20]. Recent crystallographic studies have also shed light on a number of very important details of the tertiary structure of hammerhead ribozymes [21,22].

We showed recently that hammerhead minizymes with short oligonucleotide linkers instead of a stem-loop II region can form homo- or heterodimers that are very active (Fig. 1b) [23]. These minizymes seem to form divalent structures with two catalytic centers, two binding sites and a single, common stem II (Fig. 1b). The activity of a homodimeric minizyme was found to be similar to that of the full-sized ribozyme [23,24]. Such dimeric hammerhead structures have a number of additional advantages, in particular they are very compact divalent structures that (in the case of heterodimers) can be targeted individually to two different cleavage sites. We designed analogous heterodimeric structures in which the sequences of binding sites were different and which could, thus, simultaneously cleave a single substrate at two independent sites [25].

One problem associated with dimeric minizymes is that the stability of the dimeric structures (which can be characterized by the K_d of the dimerization process) depends on a number of factors, such as the concentration of Mg^{2+} ions, interactions with substrates and within both catalytic cores, and the stability of the stem II (formed intermolecularly) of the hammerhead dimers [23–25]. Stem II is not a region that is conserved in hammerhead dimers and, thus, it should be possible to introduce considerable modifications into this region that might enhance the stability of the entire dimeric structure. We are using several different approaches in attempt to stabilize the dimeric structures [25]. One possibility is to introduce hydrophobic groups that would stabilize stem II via strong hydrophobic interactions. In the present study we introduced a triterpenoid group as such a hydrophobic moiety. Such kind of steroid-like group has an important potential advantage: such a group could play the role of messenger to facilitate the delivery of such compounds to cells via the system that normally delivers steroid hormones in the blood stream to the specific steroid receptors of cells.

We report here that, in contrast to the minizyme with high-level activity investigated in previous studies [23,24], hammerheads that contain a triterpenoid group have low cleavage activities. The nature of the dependence of catalytic activity on the concentration of ribozyme revealed that minizymes with a triterpenoid group do not form dimeric structures. It appears, therefore, that dimeric structures are mandatory for the establishment of minizymes with high-level activity.

2. Materials and methods

2.1. Chemicals

Pyridine and acetonitrile (HPLC grade) were dried over calcium hydride. Nucleoside β-cyanoethylphosphoramidite reagents (A, B, C, U) were obtained from Applied Biosystems (Foster City, CA). Column chromatography on silica gel was carried out using Wakogel C-200 (Wako, Ohsaka). Thin-layer chromatography was carried out on

*Corresponding authors. Sergei Amontov, Fax: (81) 298-54-6533, or Professor Kazunari Taira, Tel./Fax: (81) 298-53-4623

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a silica gel 60 PF254 plate (Merck, Gibbstown, NJ). ^{1}H-NMR spectra were recorded on a JEOL-JNM-GX400 spectrometer and a Varian-Gemini 200 spectrometer. FAB mass spectra were obtained with a JEOL-JMS-SX102A system.

2.2. Synthesis of 2-cyanoethylphosphoramidite

2-Cyanoethylphosphoramidite was synthesized by the scheme shown in Fig. 2A, as described in detail below.

2.3. Synthesis of compound 2 (reduction by $LiAlH_4$ of glycyrrhetinic acid)

Three grams of $LiAlH_4$ (LAH) were suspended in 100 ml of anhydrous tetrahydrofuran (THF). To this solution, 100 ml of a solution of 2.0 g of 18β-glycyrrhetinic acid in anhydrous THF were added and the solution was stirred overnight at room temperature. The excess LAH and the metallic complexes were decomposed by the careful addition of 3 ml of water, 3 ml of 15% sodium hydroxide, and then 9 ml of water. The solution was evaporated to dryness. White crystals of compound 2 were obtained by recrystallization from methanol (1.1 g, 2.41 mmol, 57%). Results of analysis were as follows. ^{1}H-NMR δ 0.81 (s, 3 H), 0.84 (s, 3 H), 0.88 (s, 3 H), 0.98 (s, 3 H), 1.09 (s, 3 H), 1.21 (s, 3 H), 1.37 (s, 3 H), 0.80–2.05 (m, 21 H), 3.22 (dd, 1 H, J = 9.6, 5.7 Hz), 3.47 (d, 1 H, J = 10.8 Hz), 3.54 (d, 1 H, J = 10.8 Hz), 4.28 (brs, 1 H), 5.29 (d, 1 H, J = 4.2 Hz). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 15.71, 18.10, 18.70, 19.37, 25.26, 26.38, 27.04, 27.12, 27.37, 28.35, 28.45, 29.55, 32.27, 33.21, 35.55, 36.34, 38.29, 38.68, 38.75, 39.50, 41.86, 42.52, 46.49, 52.48, 55.75, 66.63, 66.74, 79.06, 126.04, 147.97. HR-FABMS (positive ion) m/z 457.3681 (M+H, 459.3838; calculated for $C_{30}H_{51}O_3$).

2.4. Synthesis of compound 3 (oxidation of compound 2 by MnO_2)

An aliquot of 1.1 g of compound 2 was dissolved in 200 ml of CH_2Cl_2. Seven grams of MnO_2 were added to this solution and the mixture was stirred overnight at room temperature. MnO_2 was removed by filtration and the filtrate was evaporated to dryness. White crystals of compound 3 were obtained by recrystallization from methanol (553 mg, 122 mmol, 51%). Results of analysis were as follows. ^{1}H-NMR δ 0.78 (s, 3 H), 0.83 (s, 3 H), 0.89 (s, 3 H), 0.97 (s, 3 H), 1.09 (s, 3 H), 1.10 (s, 3 H), 1.35 (s, 3 H), 0.62–2.10 (m, 19 H), 2.30 (s, 1 H), 2.75 (m, 1 H), 3.20 (dd, 1 H, J = 9.8, 5.7 Hz), 3.44 (d, 1 H, J = 11.2 Hz), 3.59 (d, 1 H, J = 11.2 Hz), 5.56 (s, 1 H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 15.56, 16.35, 17.46, 18.69, 23.39, 26.37, 26.70, 27.27, 27.32, 28.07, 28.56, 29.38, 32.28, 32.74, 35.41, 35.92, 37.07, 39.11, 40.29, 43.39, 45.37, 46.98, 50.80, 54.92, 61.76, 66.21, 79.04, 128.31, 169.87, 200.25. HR-FABMS (positive ion) m/z 457.3681 (M+H, 457.3681; calculated for $C_{30}H_{49}O_3$).

2.5. Synthesis of compound 4

Compound 3 (295 mg, 0.67 mmol) was dried 3 times by co-evaporation with dry pyridine. The residue was dissolved in 10 ml of dry pyridine, and then 480 mg (1.42 mmol) of 4,4′-dimethoxytrityl chloride were added to the mixture. The solution was stirred for 6 h at room temperature. The reaction mixture was evaporated to dryness, and then the residue was extracted with CH_2Cl_2 and water. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was subjected to column chromatography on silica gel (in hexane and acetone, 2 : 1, v/v). Compound 4 was a colorless syrup and the yield was 294 mg (0.39 mmol, 58%). Results of analysis were as follows. ^{1}H-NMR (CD_3OD, 200 MHz) δ 0.50 (s, 3 H), 0.80 (s, 3 H), 1.00 (s, 3 H), 1.05 (s, 3 H), 1.07 (s, 3 H), 1.10 (s, 3 H), 1.30 (s, 3 H), 0.6–2.1 (m, 19 H), 2.3 (s, 1 H), 2.6–2.8 (m, 1 H), 2.90 (brd, 2 H), 3.20 (brt, 1 H), 3.76 (s, 6 H), 5.39 (s, 1 H), 6.7–6.9 (d, 4 H, J = 8.8 Hz), 7.2–7.5 (m, 9 H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 15.23, 16.03, 17.27, 18.40, 23.08 26.98, 27.08, 27.72, 28.06, 28.23, 28.25, 29.18, 31.61, 32.49, 34.84, 35.17, 36.84 38.86, 40.07, 43.15, 45.06, 46.45, 50.37, 54.98, 55.01, 61.53, 78.42, 112.78, 127.50, 127.87, 128.85, 129.82, 129.91, 136.20, 145.13, 169.81, 199.81. HR-FABMS (positive ion) m/z 759.4971 (M+H, 759.4988; calculated for $C_{51}H_{67}O_5$).

2.6. Synthesis of 2-cyanoethylphosphorodiamidite, compound 5

The dimethoxytrityl triterpenoid derivative 4 was dried by co-evaporation with pyridine (3 times) and redissolved in acetonitrile. This solution was suplemented with 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (1.1 eq.) and 0.5 M tetrazole (1.1 eq.) in acetonitrile, and the mixture was stirred overnight at room temperature under argon. The reaction mixture was poured into ice-cooled water and extracted with ethyl acetate. The organic layer was washed with saturated $NaHCO_3$, dried over anhydrous Na_2SO_4 and concentrated. Crude compound 5 was used in an automated DNA synthesizer without further purification as described in Section 2.7. FABMS (positive ion) analysis of compound 5 yielded an m/z of 960 (M+H, 960; calculated for $C_{60}H_{84}O_6N_3P$).

2.7. Synthesis of ribozymes and substrates

Ribozymes and their corresponding substrates were synthesized with a DNA/RNA synthesizer (model 392; Applied Biosystems, Foster City, CA) and purified by HPLC and polyacrylamide gel electrophoresis as described previously [7,16,26,27]. For the introduction of a triterpenoid group at a specific position, 2-cyanoethylphosphorodiamidite, compound 5, was used in place of the regular phosphorodiamidite. Reagents for manipulations of RNA were purchased from American Bionetics, Inc. (ABN; Foster City, CA). Other reagents were purchased from either ABI or ABN. The synthesized oligonucleotides were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications.

2.8. Kinetic measurements

Reaction rates were measured, in 25 mM $MgCl_2$ and 50 mM Tris·HCl (pH 8.0), under ribozyme-saturating (single-turnover) conditions with trace levels of 5′-^{32}P-labeled substrate at 37°C. Thus, in all cases, kinetic measurements were made under conditions where all the available substrate was expected to form a Michaelis-Menten complex, with high concentrations of ribozyme (0.1–10 μM).

Reactions were 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. 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 the substrate and product with a Bio-Image Analyzer (BA2000; Fuji Film, Tokyo).

3. Results and discussion

Hammerhead minizymes with short oligonucleotide linkers instead of a stem-loop II region were found previously to form homo- or heterodimers, some of which were as active as the parental wild-type hammerhead ribozyme (Fig. 1b) [23]. They formed divalent structures with two catalytic centers, two binding sites and one common stem II in which the first base pair ($G_{10.1}$:$C_{11.1}$) was required for retention of maximum activity (Fig. 1b) [23–25,28–32]. The first $G_{10.1}$:$C_{11.1}$ pair in stem II appears to provide a binding site for a structurally important Mg^{2+} ion [21,22]. Other parts of the stem II region could be replaced by non-nucleotide linkers, such as polyethyleneglycols [30–32]. In some cases, the possibility was not excluded that ribozymes with polyethyleneglycol linkers instead of a stem-loop II region [31] might form dimeric structures [23]. Therefore, we synthesized another type of ribozyme, with a triterpenoid linker instead of the stem-loop II region, and we examined the activities of and the possibility of the formation of dimers by such modified ribozymes. Four such ribozymes, designated Mz-0-Tp-0, Mz-0-Tp-C, Mz-G-Tp-0 and Mz-G-Tp-C (Fig. 2B), were characterized in this study. Only the last variant was expected to retain the first $G_{10.1}$:$C_{11.1}$ pair of the common stem II for each catalytic site if the monomers were to form a dimeric structure. We considered Mz-G-Tp-C to be a derivative of the highly active minizyme Mz-GC that we characterized in a previous study (Fig. 1) [23].

As in our previous study, we used single-turnover conditions to examine the activities and kinetic behaviors of ribo-

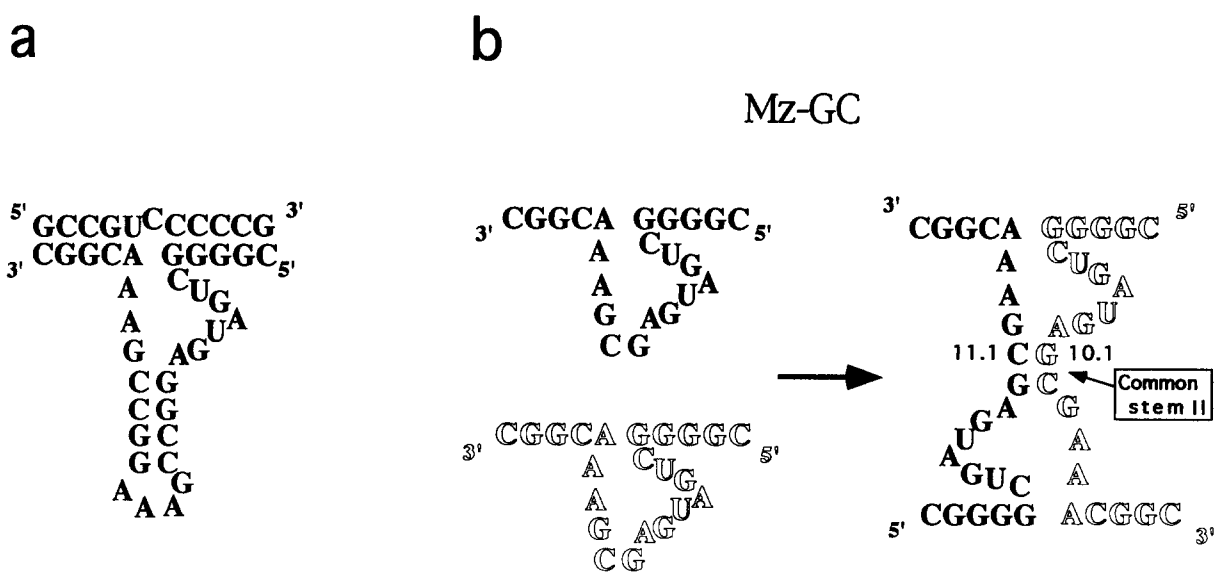

Fig. 1. Structures of a wild-type ribozyme (a) and minizymes with high-level activity (Mz-GC; b). Dimerization of the minizymes, Mz-GC, is also shown. (For clarity, two different kinds of letters are used to denote the same Mz-GC.)

zymes that contained a triterpenoid groups. The rate constants that can be calculated from results of kinetic experiments under single-turnover conditions provide the most direct characterization of the catalytic competence of an enzyme–substrate complex. In particular, in the case of multimolecular systems, such as the dimeric hammerhead system, with its four independent components (the two catalytic molecules that form the divalent hammerhead structure itself and the two molecules of substrate that interact with this dimeric hammerhead structure), single-turnover conditions not only allow us to draw reliable conclusions about the chemical step of the catalytic cleavage reaction, but, sometimes, they also simplify the complicate kinetic picture that is a consequence of the multimolecular character of the enzyme [23–25]. Conversely, if the active unit of the enzyme is a single molecule, single-turnover conditions can allow us to characterize the monomolecular nature of the enzymatically active unit.

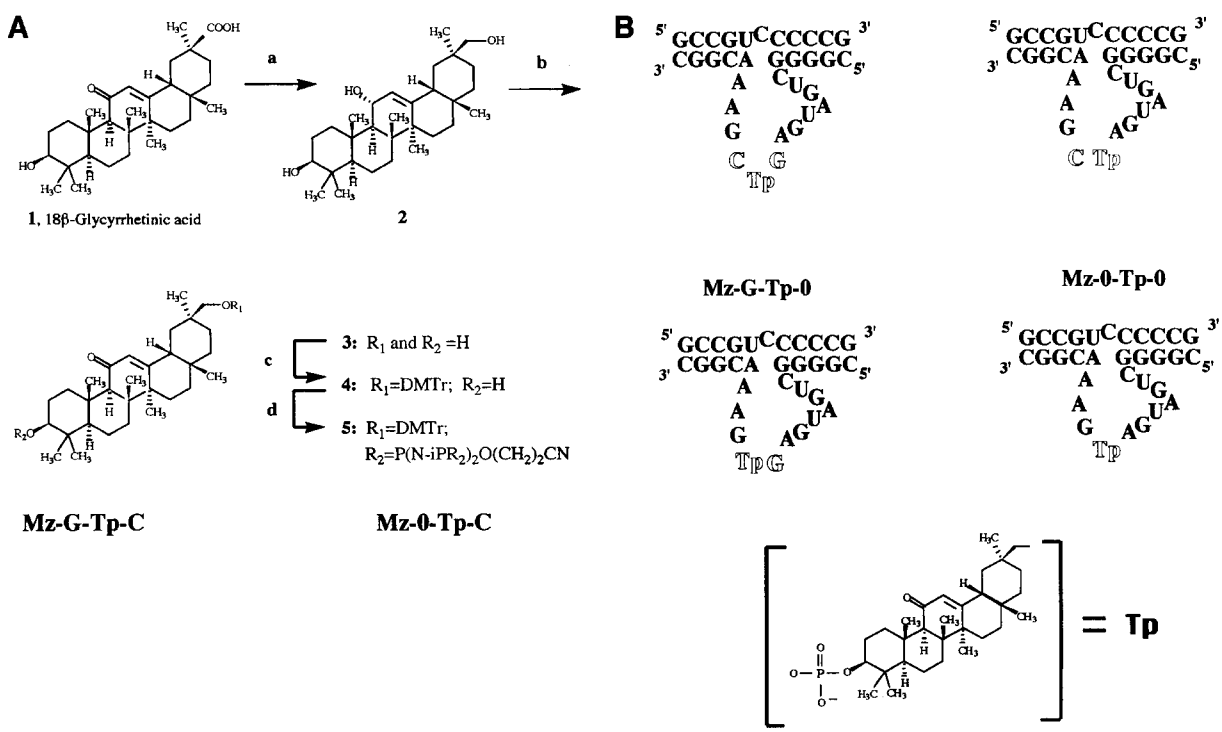

Fig. 2. (A) Scheme for the synthesis of the triterpenoid phosphoramidite. Reagents used in each step were: a, $LiAlH_4$/THF, 57%; b, MnO_2/CH_2Cl_2, 51%; c, DMTrCl/pyridine, 58%; d, $P(N\text{-}iPr_2)_2O(CH_2)_2CN$, tetrazole. See text for full details. (B) Monomeric structures of four different chimeric ribozymes that contained a triterpenoid group (Tp). The chemical structure of the triterpenoid is shown at the bottom.

We found that, in contrast to the dimeric hammerhead structures investigated in our previous study (Mz-GC and Mz-GCGC) [23], the new variants of hammerheads with a triterpenoid linker did not have high cleavage activity (Fig. 3). We expected that Mz-0-Tp-0 would be inactive because of the absence of the $G_{10.1}$:$C_{11.1}$ base pair, which is an important component of the hammerhead catalytic center [28–31]. The low activity of the other two hammerheads (Mz-G-Tp-0 and Mz-0-Tp-C) was also expected for the same reason: a point deletion in the $G_{10.1}$, $C_{11.1}$ region. We originally designed these compounds as negative controls. However, we found that Mz-G-Tp-C also had low activity (Fig. 3).

There are several different explanations for the low activity of the Mz-G-Tp-C ribozyme. (1) The monomers might form a dimeric structure analogous to that of Mz-GC (dimers in which each of the two oligonucleotides is involved in formation of half the catalytic core at each site; antiparallel mode of interaction) (Fig. 4a), but the efficiency of the cleavage reaction catalyzed by the corresponding hammerhead dimer is low as a result of the imperfect conformation of the hammerhead catalytic core that is caused by steric hindrance by the bulky hydrophobic linker. (2) The Mz-G-Tp-C ribozyme might not form a dimer. In this case, the low activity of all three structures (Mz-0-Tp-C, Mz-G-Tp-0 and Mz-G-Tp-C) could be attributed to the monomeric ribozyme structure, since we found in our previous study that a number of minizymes with low-level activities were incapable of forming a common stem II because of mismatched base pairs [23]. (3) Bulky triterpenoid linkers have a very strong tendency to develop mutual hydrophobic interaction, and it is quite probable that monomers might form another type of dimer (dimers in which each of the two oligonucleotides is involved in formation of one unit of the catalytic core of the dimer; parallel mode of interaction) (Fig. 4b). In such cases, the hammerhead catalytic cores of the monomers do not participate in the dimerization process and, thus, the conformation of the catalytic core of each dimer most likely remains identical to that of the corresponding monomers. The activity of such dimers is likely to be the same as that of the monomers.

To clarify the situation, we investigated the dependence of activity on the concentration of Mz-G-Tp-C, to examine whether Mz-G-Tp-C could form a dimeric structure (Fig. 4a). If the dimer were the catalytically active unit, the activity under single-turnover conditions should increase with increasing concentrations of Mz-G-Tp-C if we examine the kinetics of the reaction before the activity reaches a plateau value at concentrations that are much higher than the K_d of the dimerization process. We observed such a phenomenon in our previous study with the highly active minizymes Mz-GC and Mz-GCGC and it provided one of the major preliminary clues that led to our proposal of dimeric hammerhead enzymes [23]. If the monomer were the only catalytically active unit, the activity under single-turnover conditions should be basically constant over a range of concentrations that are significantly higher than the K_m for the substrate-hammerhead ribozyme complex and, thus, the activity should be concentration-independent.

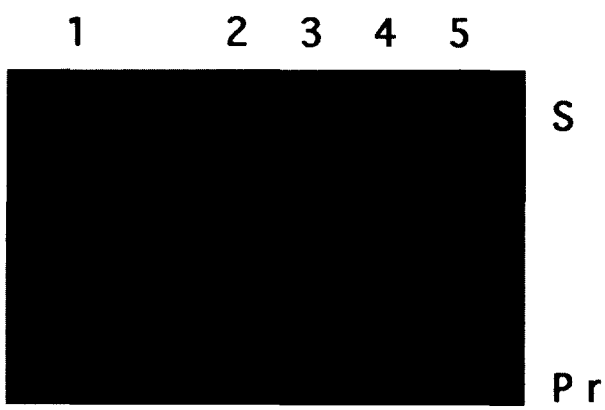

Fig. 3. Activities of oligonucleotides that contained a triterpenoid group. Lane 1, control (substrate alone, S); lanes 2, 3, 4 and 5, respectively, show the activities of Mz-0-Tp-0, Mz-0-Tp-C, Mz-G-Tp-0 and Mz-G-Tp-C. Pr denotes the cleavage product.

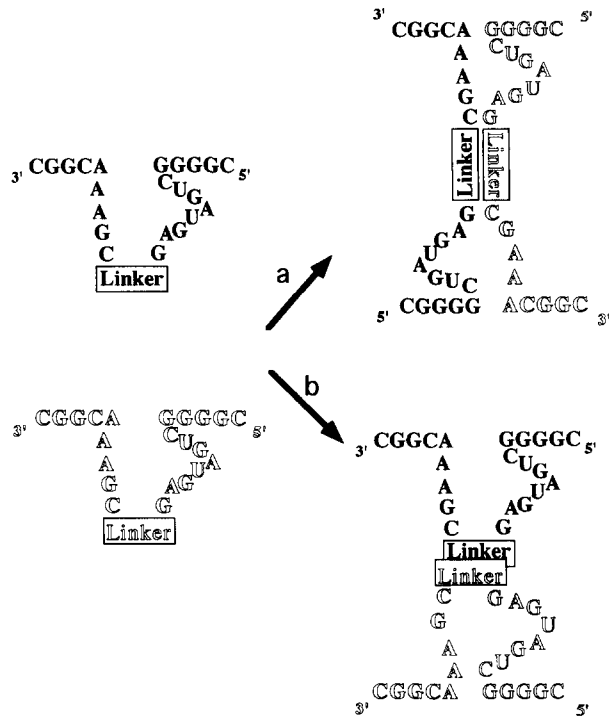

Fig. 4. Two possible dimerization processes. (a) The dimerization is driven by the interaction between the two linkers in an antiparallel mode and elements of the catalytic core of both monomers are involved in formation of the dimer. (b) The dimerization is driven only by the interaction between the two linkers in a parallel mode.

The results of our analysis are shown in Fig. 5. In the case of the potentially active Mz-G-Tp-C and Mz-G-Tp-0 hammerheads, their cleavage activities under single-turnover conditions remained almost constant over a range of ribozyme concentrations from 100 nM, which is approximately 4–5 times higher than the K_m for the ribozyme-substrate Michaelis complex of the ribozyme with the same binding site (the K_m was 20 nM [7,26]), to 10 μM, which is a concentration that is higher than K_d for the dimerization process for Mz-GC (5 μM [23]). Thus, we can conclude that, in contrast to the highly active minizyme Mz-GC, Mz-G-Tp-C does not form an active dimeric hammerhead structure (or at least a type of dimer that we observed in the case of Mz-GC; Fig. 4a) and that the low residual activity of this hammerhead, which was similar to that of Mz-G-Tp-0 and Mz-0-Tp-C, was attributable to the monomeric form of the ribozyme (Fig. 2B). Although we

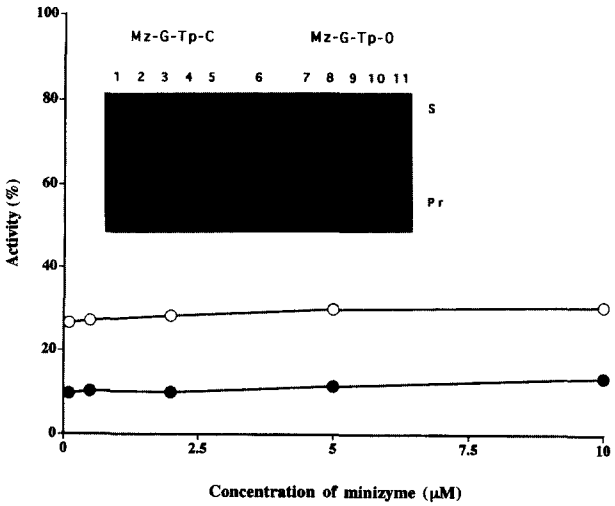

Fig. 5. Dependence of the cleavage activities on the concentrations of minizymes. ○, Mz-G-Tp-0; ●, Mz-G-Tp-C. Inset: Lanes 1 and 7, 10 μM minizyme; lanes 2 and 8, 5 μM; lanes 3 and 9, 2 μM; lanes 4 and 10, 0.5 μM; lanes 5 and 11, 0.1 μM; lane 6, control (no minizyme). The activity was determined after 4 h. 100% activity represents complete cleavage of the substrate.

cannot exclude, from our kinetic data (Fig. 5), the 'parallel' type of the dimeric structure (Fig. 4b), there is no reason to believe that 'parallel' type of dimers (Fig. 4b) are significantly more stable than 'antiparallel' type of dimers (Fig. 4a). The approximate evaluation of k_{cat} gave a value of about 10^{-3} min^{-1}. This value is in the same range as that for other minizymes with low activity [23]. Nonetheless, it was surprising to us, that despite the overall low activity of Mz-G-Tp-0, Mz-0-Tp-C and Mz-G-Tp-C, the Mz-0-Tp-C construct was still several-fold more active then the others (Fig. 3).

We failed to design a highly active dimeric hammerhead structure that was stabilized by the interaction of two hydrophobic linkers. It is likely that the hydrophobic triterpenoid group was not able to stabilize the dimeric form of the hammerhead structure. Nevertheless, we cannot exclude the possibility that other hydrophobic linkers, introduced into the stem II region of the dimer with minimal disturbance of the active conformation of the dimeric structure, might yield highly active and stable dimeric hammerhead constructs.

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