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Cleavage Activity of a Hammerhead Ribozyme Domain Containing 2',5'-Phosphodiester Linkages

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Abstract: The effects of the introduction of 2',5'-phosphodiester linkages within a hammerhead ribozyme domain have been examined in order to determine the regions which can be modified without impairing the cleavage activity and whether a substrate analogue, containing a 2',5'-linkage at the scissile position, could be cleaved.

Abolition of cellular gene expression by the antisense strategy requires the control of many critical factors. In particular, at the RNA level, the antisense oligonucleotides should 1) hybridize with an accessible RNA target and 2) mediate its cleavage. This crucial phosphodiester cleaving step depends on the recruitment of an RNase such as RNases H, L or P the expression of which might vary from one type of cell to another.[1] In contrast to antisense oligonucleotides, ribozymes are catalytic RNA molecules which can be designed both for target recognition and its cleavage. Accordingly, ribozymes exercise their activity in a RNase-independent manner. In this series, the now well studied hammerhead ribozymes offer the advantage of being constituted of short sequences which can be chemically assembled.[2] This is important in view of the future therapeutical applications of ribozymes by exogenous administration. However, unfortunately, as other oligonucleotide-based therapeutics, unmodified hammerhead ribozymes cannot be used *in vivo* in reason of their instability in a biological context. Thus, to increase their stability in biological fluids and in a cellular environment, a number of chemical modifications have been designed.[3] Since substitutions which are made within the domain of conserved residues strongly affect the hammerhead catalytic activity, most ribonucleoside analogues such as 2'-deoxyribonucleosides,[4] 2'-*O*-alkylribonucleosides[5] or phosphorothioate linkages,[4c] which appeared to be well tolerated, have been introduced in the non-conserved domain of the ribozyme, *i.e.* in the stem loop II and the hybridizing arms I and III (with the exception of the conserved position $A_{15.1}$).[6]

Herein, we have investigated whether the introduction of 2',5'-phosphodiester bonds in the non-conserved domain of the hammerhead ribozyme would maintain the catalytic activity. We reasoned that, in comparison with 3',5'-phosphodiester linkages, the corresponding 2',5'-oligonucleotides should exhibit an enhanced nuclease stability.[7] Moreover, 2',5'-oligonucleotides are known for their capacity to hybridize with their complementary RNA (and not with DNA).[7] It is also worthy of note that their slow degradation would lead to natural nucleosides only, which is not the case for chemically modified oligonucleotides containing unnatural residues which might induce unexpected severe toxic effects in the long run. With these observations in mind, we proposed to probe the nuclease stability and the effect on the cleavage activity of the introduction of 2',5'-phosphodiester linkages within a well-studied hammerhead ribozyme system which consists of the 35-mer ribozyme (**Rz**) and its 14-mer substrate (**S**) (fig. 1).[8]

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RNase resistance and catalytic activity of 2',5'-modified hammerhead ribozymes.

Using commercial 2'-nucleoside phosphoramidites, we synthesized the partially 2',5'-modified oligonucleotides **Rz-1**, **Rz-2** and **S**(2'-5') as analogues of **Rz** and **S**, respectively. Among these oligonucleotides, **S**(2'-5') is identical to **S** except for the cleavable 3',5'-phosphodiester bond (between C_{17} and $U_{1.1}$) which was replaced by a 2',5'-phosphodiester linkage in order to probe both the capacity of **Rz** to hybridize and cleave this substrate analogue. Thus, after RNase digestion following a previously described procedure.[9] While all 3',5'-phosphodiester bonds were sensitive to cleavage by RNase T_2 in **Rz** and **S**, their 2',5'-counterparts in **Rz-1**, **Rz-2** and **S**(2'-5') revealed fully resistant to this enzyme. In addition cytidine specific RNases (Cl_3, A) cleaved the $C_{17}pU_{1.1}$ bond of **S** but not the 2',5'-one of **S**(2'-5'). These data demonstrate the improved RNase resistance of these oligomers.

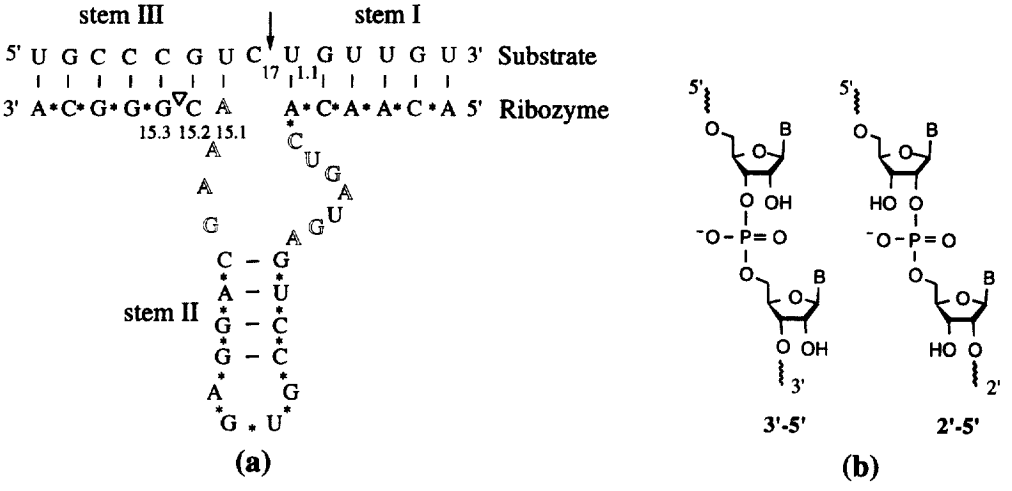

Figure 1: a) Structures and sequences of the standard (**Rz**) and modified (**Rz-1**, **Rz-2**) hammerhead ribozymes. The internucleotidic linkages that were replaced by 2',5'-phosphodiester bonds are indicated by stars. The triangle corresponds for ribozyme **Rz-1** to a 3',5'-linkage and for **Rz-2** to a 2',5'-linkage. In the ribozyme strand, the conserved residues are indicated by outline letters. The ribozyme substrate analogue **S**(2'-5') differs from **S** by the presence of a 2',5'-phosphodiester bond at the cleavage site between C_{17} and $U_{1.1}$. The cleavage site is shown by the arrow. b) Structure of 3',5'-RNA and 2',5'-RNA (B = nucleic base).

Under cleavage conditions (pH 8, 20 mM Mg^{2+}, 37° C) all three ribozymes revealed active yielding the same 5' ^{32}P-labelled octamer as judged by comparative gel migration. Under multiple-turnover conditions, both **Rz-1** and **Rz-2** were able to cleave more than 100 molecules of substrate **S**/molecule of ribozyme and follow a Michaelis-Menten kinetics (fig. 2). However, the k_{cat} values of **Rz-1** and **Rz-2** were reduced 8 and 38 fold compared to **Rz** (k_{cat} =15 min^{-1}). Since hybrids between a 2',5'-RNA with a complementary 3',5'-RNA are less stable than their corresponding full 3',5'-RNA duplexes,[7] it is excluded that the rates of 3'- and 5'-product departure account for the decreased activity of the modified ribozymes. However, it could result from discrete changes in the geometry of their helices I, II and III, due to the preferred C-2'-*endo*-puckering of 2',5'-linked ribose units in contrast to the C-3'-*endo*-pucker of their 3',5'-linked analogues.[10] Most likely this conformational preference disfavours the conversion of the conformation which characterizes the ribozyme-substrate

complex in its ground state, into the one which it needs to adopt in its transition state leading to the cleavage reaction. Accordingly, the presence of 2',5'-linkages in close proximity of the catalytic core are expected to be more deleterious to the activity. Indeed, **Rz-2** which, compared to **Rz-1**, is further modified at the $C_{15.2}$-$G_{15.3}$ linkage was found five time less effective than **Rz-1** (fig. 2). The catalytic efficiency of **Rz** and **Rz-1** were found closely similar ($k_{cat}/K_M \approx 2.10^7$ $M^{-1}.min^{-1}$), a value close to the rate constant for the double helix formation.[11] This indicates that the rate constant for the ribozyme-substrate complex dissociation k_{-1} remains much lower than k_{cat} in agreement with the value k_{-1} ($2.10^{-3}.min^{1}$) determined for a RNA substrate identical to **S** except for a carbocyclic cytidine at the cleavage site.[9] Due to uncertainties in K_M determination for **Rz-2**, the k_{cat}/K_M values ranged from 0.8 to 2.10^7 $M^{-1}.min^{-1}$.

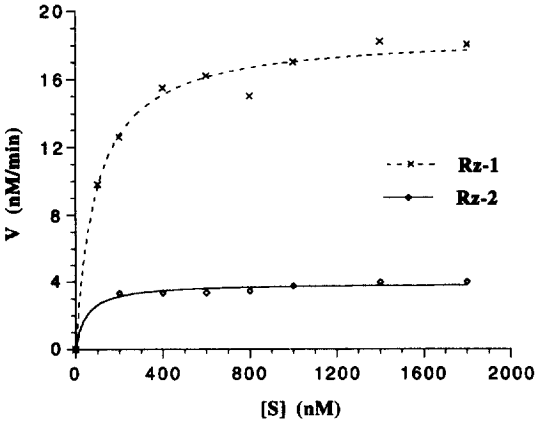

Figure 2: Comparison of catalytic activity of modified ribozymes **Rz-1** and **Rz-2**. The plots of initial velocities of cleavage as a function of substrate concentration are represented. Cleavage reactions were carried out under multiple-turnover conditions in the presence of 20mM $MgCl_2$ in 50 mM Tris-HCl buffer (pH 8) at 37° C with 10 nM ribozyme.

Introduction of a 2',5'-phosphodiester bond in the hammerhead ribozyme substrate.

In order to determine whether the hammerhead ribozyme is able to cleave a 2',5'-phosphodiester bond by promoting the attack of the free 3'-OH on the vicinal phosphorus, we synthesized the substrate analogue **S**(2'-5') containing a unique 2',5'-phosphodiester bond at the cleavage site. It should be noted here that a hairpin ribozyme was shown to be inhibited by the presence of such a 2',5'-phosphodiester bond.[12]

Under low ionic strength conditions, Mg^{2+} or analogous divalent cations are absolutely required to promote the hammerhead ribozyme cleavage reaction. Analysis of the cleavage mechanism has shown that it involves an in-line attack of the 2'-OH, proximal to the scissile phosphodiester bond, on the adjacent phosphorous leading to the formation of a pentacoordinated phosphorane intermediate which is followed by the cleavage of the P-O-5' bond.[13] In addition to their role, in adequately folding the ribozyme-substrate complex, the Mg^{2+} ions are widely considered to be the primary catalytic species triggering the reaction. A variety of mechanisms have been proposed which involve either a single magnesium hydroxide or two catalytic Mg^{2+} ions.[13] However, these considerations have been recently questioned after it could be observed that, in the absence of Mg^{2+}, high concentrations (4M) of monovalent cations (Li^+, Na^+, NH_4^+) do promote the efficient cleavage.[14] This might

indicate that folding of the ribozyme-substrate complex in the presence of a dense positive charge suffices to carry out the catalytic function. Hence, one should conclude that either the initial steps of the cleavage mechanism are distinct in the presence of Mg^{2+} (20 mM range) or monovalent ions in high concentration, or that the role of Mg^{2+} ions is only structural. In this respect, it was of interest to check whether S(2'-5') containing a 2',5'-phosphodiester bond at the cleavage site could be cleaved under these ionic conditions. We observed that S(2'-5') was not cleaved (37° C, pH 8) in the presence of either 20 mM Mg^{2+} or 4M monovalent salts (Li^+, Na^+, NH_4^+) and 25 mM EDTA while under the same conditions the parent substrate S was efficiently cleaved. Hence, whatever the ionic conditions, the presence of a 2',5'-linkage at the cleavage site either prevents formation of the necessary in-line arrangement of the 3'-OH-phosphorous-5'-O leaving group, or deprotonation of the 3'-OH group, or both. This failure cannot be ascribed to the absence of complex formation between **Rz** and S(2'-5'). Indeed, this substrate analogue was shown to bind efficiently to the ribozyme with an estimated K_D of 0.04 nM as determined by electrophoresis mobility shift assays at pH 7.5, 10 mM $MgCl_2$, 25° C. This demonstrates that, like classical enzyme reactions, ribozyme reactivity is placed under a severe stereospecific control.

In conclusion, we have demonstrated that the introduction of 2',5'-phosphodiester linkages within the three stems I, II and III of a ribozyme domain, as in **Rz-1** and **Rz-2**, does not impair the ribozyme catalytic activity. In term of nuclease resistance, our preliminary observations indicate the potential advantages of introducing in therapeutic hammerhead ribozymes such easy to perform modifications which utility might compare well with other ones recently proposed.[3] Moreover, although S(2'-5') display a high affinity for ribozyme S the latter proved unable to cleave this oligomer indicating that the ribozyme cleavage reaction is under high stereoselective control and, at least in the present case, specific for 3',5'-phosphodiester linkages.

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