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IMPORTANCE OF PURINE-PYRIMIDINE HYDROXYL AND PURINE AMINO GROUPS FOR HAMMERHEAD RIBOZYME CLEAVAGE

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SUMMARY: The importance of the 2'-hydroxyl and 2-amino groups of guanosine residues for the catalytic efficiency of a hammerhead ribozyme has been investigated. The three guanosines in the central core of a hammerhead ribozyme were replaced by deoxyinosine, inosine, and deoxyguanosine, and ribozymes containing these analogues were chemically synthesized. Most of the modified ribozymes are drastically decreased in their cleavage efficiency. However, deletion of the 2-amino group at G_8 (replacement with inosine, deoxyguanosine, deoxyinosine) caused little alteration in the catalytic activity relative to that obtained with the unmodified ribozyme. Whereas, deletion of the 2'-amino group at G_{12} and G_5 (replacement with inosine, deoxyinosine, and deoxyguanosine) resulted in ribozymes with drastic decrease in the catalytic activity relative to that obtained with the unmodified ribozyme. In contrast, two uridine residues, U_7 and U_4, in the ribozyme sequence were replaced by deoxyuridine (dU). The dU_4 complex resulted in a decrease in the catalytic rate, with relative cleavage activity that was about half that observed for the native complex. By comparison, the dU_7 complex exhibited a relative cleavage activity within 3.3-fold of that observed with native ribozyme/substrate complex. This result suggests that the 2'-hydroxyl group at U_7 is not essential for activity.

Certain small RNA molecules pathogenic to flowering plants are able to undergo site specific cleavage of their phosphodiester backbones in the presence of a divalent metal ion and in the absence of proteins. This self-cleavage reaction generates 5-OH and 2',3'-cyclic phosphate termini and is believed to be an integral step in the proposed rolling-circle replication of a number of plant viroids and virsoids (1-3). Secondary structural homology exists around the site of self-cleavage for nine self-cleaving RNAs, and consists of three base-paired stems and 13 conserved nucleotides which form a "hammerhead" structure (4-7). The structural model consists of three RNA double helices that delimit the consensus nucleotides CUGAUGA and GAAAC in the ribozyme and GUC in the substrate, which have been the focus of nucleotide replacement studies in order to ascertain their role in the cleavage mechanism. Such studies include replacement of the 2'-hydroxyl groups by 2'-substitution derivatives (F, NH_2, OR, and H) or of purine bases (adenine and guanine) by

inosine, 7-deazaadenosine, and 2-aminopurine (8-20).

We now describe the effect on the catalytic efficiency resulting from the deletion of purine base amino or purine and pyrimidine 2'-hydroxyl groups in a hammerhead ribozyme (Fig. 1).

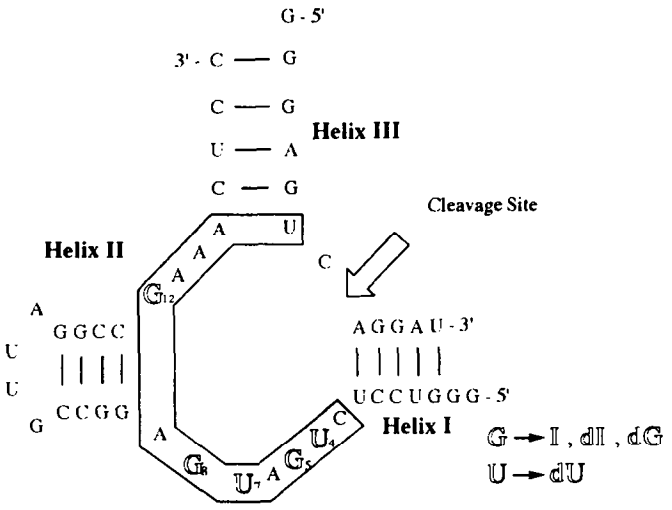

Figure 1. Structure of ribozyme-substrate complex: shaded area, central core region.

The hammerhead ribozyme complex, identical to that described by Fedor and Uhlenbeck (21) was formed by the two RNA fragments of 12 and 34 nucleotides (Fig. 1). It possesses a high turnover number, reflecting the rate of chemical cleavage and not of product release. This complex is identical to that used by Tuschl et al. (20) for a corresponding study of a series of sequence mutations. The ribozymes with a base and sugar substitution at each of the guanosines (G5, G8, and G12) by inosine, deoxyinosine, and deoxyguanosine, or uridines (U4 and U7) with deoxyuridin were synthesized with an Applied Biosystems DNA synthesizer using the modified coupling program (22). The presence of the modified nucleosides in the oligoribonucleotides was confirmed by combined nuclease and alkaline phosphatase digestions and subsequent HPLC nucleoside analysis.

The ribozyme-substrate complexes were formed by substitution of the three conserved purine and two pyrimidine nucleoside residues present within the ribozyme sequences. The guanosine residues at positions 5, 8, and 12 were each replaced by deoxyguanosine (dG5, dG8, and dG12). inosine (I5, I8, and I12), and deoxyinosine (dI5, dI8, and dI12). In a similar fashion, the uridine residues at positions 4 and 7 were replaced by deoxyuridine (dU4 and dU7).

The effects of the replacement of the guanosines at positions 5, 8, and 12 by inosine or deoxyinosine in

the invariant region of the ribozyme are summarized in Table 1 (23). Guanosine modifications at positions 5, 8, and 12 resulted in a 7-300 fold reduced catalytic efficiency for I5, I12 , dI5, and dI12. due to a decrease in K_{cat}. In contrast to G5 and G12, the replacement of G8 by inosine resulted in a very slight loss of activity. Furthermore, the substitution of G8 by dI8 was tolerated, with cleavage efficiencies decreased by a factor of 30.

The effects of the amino group of the guanosines at positions 5, 8, and 12 of the invariant region are summarized Table 1. At positions 5 and 12, a significant effect on the cleavage activity was observed with the inosine. However, a significant difference was seen for the amino group of G8, which was a same factor to that of the corresponding unmodified ribozyme. Furthermore, the substitution of U4 by dU4 did not significantly alter the rate of reaction. However, a similar substitution at position 7 displayed a 3.3 fold increase in the catalytic activity. On the other hand, the deletion of the purine amino and 2'-hydroxyl groups from the guanosine at positions 5, 8, and 12 led to reductions in catalytic efficiencies as compared to the unmodified ribozyme (Table 1). Again, these decreased activities resulted principally from changes in the respective K_m values.

Table 1. Kinetic parameters for unmodified and modified hammerhead ribozymes

ribozyme	k_{cat}(min^{-1})	K_m (nM)	k_{cat}/K_m (μM^{-1} min^{-1})	relative (k_{cat}/K_m (K_{rel})
native	1.11	379	2.90	1.00
I5	0.01	177	0.66	0.02
I8	1.07	460	2.30	0.79
I12	0.006	83	0.07	0.02
dG5	0.10	1413	0.07	0.02
dG8	0.09	455	0.20	0.07
dG12	1.52	369	4.10	1.41
dI5	0.02	2359	0.008	0.003
dI8	0.05	591	0.08	0.03
dI12	0.02	55	0.40	0.14
dU4	0.38	248	1.50	0.52
dU7	2.59	269	9.60	3.31

Reactions were conducted 25°C in 40 mM Tris-HCl, pH 7.5/10 mM MgCl2 with 10 nM (dI5,40 nM) ribozymes at various substrateconcentrations.

Sequence mutations of the conserved nucleotides present in the central core of the hammerhead structure indicate that the conserved single strand nucleotide residues are critical for cleavage activity (24,25). These mutated ribozymes did not alter the ability of the hammerhead complex to form but cleavage activity was eliminated or the rates were differed by >1 order of magnitude. In particular, changes in three conserved guanosine residues, G5, G8, and G12, in the single strand nucleotides resulted in ribozyme with a drastic decreases in cleavage efficiency. The results can be explained in terms of a structure in which the conserved

residues orient the positions of specific functional groups in the active site such that base-base interactions or the access of the complex to Mg^{++}-nucleosides are optimized.

Furthermore, two uridine residues, U_7 and U_4, in the ribozyme sequence were replaced by deoxyuridine (dU). The dU_4 complex resulted in a decrease in the rate, with relative cleavage activity that was some 0.5-fold lower than that observed for the native complex. By comparison, the dU_7 complex exhibited a relative cleavage activity within 3.3-fold of that observed with the native ribozyme/substrate complex. This result suggests that the 2'-hydroxyl group at U_7 is not required. This observation is somewhat different from those in previous reports, which indicated that five critical functional groups are located within the tetrameric sequence $G_5A_6U_7G_8$ (17). The 2'-hydroxyl group of U_7 may be involved in specific interbase hydrogen-bonding interactions, direct chelation to the Mg^{++}, or interligand interactions involving the water molecules of the inner hydration shell of the metal cofactor (17). However, the results reported here do not support the effect of the 2'-hydroxyl group of U_7.

In conclusion, the exocyclic amino group at G_5 and G_{12} and the hydroxyls at G_5 and G_8 are important both for the ribozyme-substrate binding and for the Mg^{++} -catalyzed cleavage reaction. Furthermore, the dU_7 complex exhibited a relative cleavage activity within 3.3-fold of that observed with the native ribozyme/substrate complex. This result suggests that the 2'-hydroxyl group at U_7 is not required.

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22. Phosphoramidite. The nucleosides inosine and 2-aminopurine (26) were converted to the corresponding 5'-O-(dimethoxytrityl)-2'-O-(2-chloroethoxy)ethyl-3'-O-N,N-diisopropylamino-ß-cyanoethylphosphoramidite derivatives essentially by the procedures of Yamakage et al.(27). The 2'-deoxynucleoside phosphoramidites were obtained from MilliGen/Biosearch (Woburn, MA). The oligoribonucleotides were synthesized on controlled-pore glass supports with an Applied Biosystems 392-05 DNA/RNA synthesizer and were deprotected as previously described (28). The oligoribonucleotides were purified using anion exchange HPLC or on 15% denaturing polyacrylamide gels. The nucleoside composition was determined after snake venom phosphodiesterase/ bacterial alkaline phosphatase hydrolysis.

    A 50 μL reaction mixture containing 1 A_{260} unit of oligoribonucleotides, 10 mM $MgCl_2$, 5 mM dithiothrieitol (DTT), 0.2 mM Na_2EDTA, 20 units of T4 polynucleotide kinase, 0.1 mM ATP, and 300-600 mCi of [γ-^{32}P]ATP (1mCi = 37kBq) was incubated for 60 min at 37°C. The labeled RNA was separated by electrophoresis on 20% polyacrylamide/7 M urea gels. The RNA band was located by autoradiography was excised and eluted by crushing and soaking in 0.5 M ammonium acetate at 37°C for several hours, and was extracted with an equal volume of water-saturated phenol:chloroform (1:1) and precipitated with ethanol (2-3 vol) at -20°C.
23. Kinetic constants, K_{cat} and K_m, were determined from Eadie-Hofstee plots obtained from the initial velocities under multiple turnover conditions with a 5'-^{32}P-labeled substrate. Stock solutions of 100 nM ribozyme and 2 μM substrate RNA were prepared in 40 mM Tris-HCl (pH7.5), preheated separately at 90°C for 1 min, and cooled to 25°C for 15 min. After $MgCl_2$ was added to a final concentration of 10 mM, the stock solutions were incubated for another 15 min at 25°C. Ribozyme concentrations of 10 nM and substrate concentrations of 100-700 nM were used for the reactions in the presence of 10 mM $MgCl_2$ in 40 mM Tris-HCl (pH 7.5) at 25°C. For the catalytically less-efficient ribozymes, possessing a dI_{10}, the concentrations were 40 nM. The reactions were initiated by the addition of 5 μL of ribozyme stock

solution to 45 μL of the substrate solution.Initial rates were determined by transferring 8 μL aliquots into 16 μL of urea stop mixture (7 M urea, 50 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) at appropriate time intervals between 0 and 180 min of reaction time. The product was separated by a denaturing 20% PAGE (8 M urea). The reactions were quantified by radioanalytic imaging with a Bioimage analyzer BSA 2000 (Fujifilm).

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