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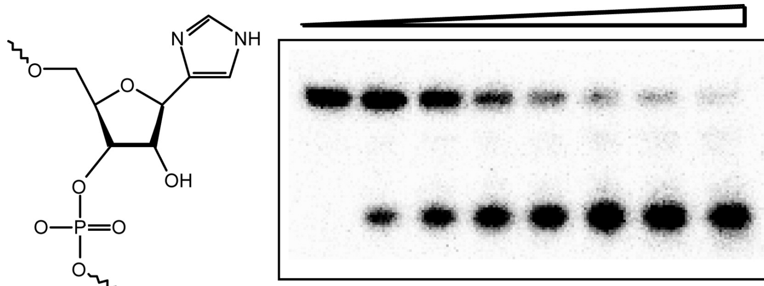

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Nucleobase Participation in Ribozyme Catalysis

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RNA catalysis occurs in all cells, yet the mechanisms of ribozymes generally remain poorly understood. The cleavage reaction of the nucleolytic ribozymes is mediated by nucleophilic attack on the scissile phosphate by the adjacent 2′ oxygen. One of the possible strategies for accelerating this reaction uses acid−base catalysis to improve the nucleophilicity of the 2′ oxygen by removal of the hydroxyl proton and protonation of the 5′ oxyanion leaving group. Another is transition state stabilization. A nucleobase might participate in one or more of these roles. In the HDV ribozyme, the nucleobase of cytosine 75/76 has been proposed to act as a general acid[1] or base[2] in the cleavage reaction. The reaction is pH-dependent, corresponding to the titration of a group with a pK_a = 6.1. Substitution of this nucleotide by U or G leads to loss of activity, but some activity could be restored by addition of exogenous imidazole base.[3]

The VS ribozyme is the largest of the nucleolytic ribozymes (Figure 1), and there is evidence suggesting that nucleobase catalysis could be important. We have suggested[4] that the substrate stem-loop binds into a cleft formed between helices II and VI, where it can make a close interaction with the A730 loop in helix VI. Evidence indicates that the A730 loop is an important component of the active site of the ribozyme.[5−7] Within the A730 loop, sequence variants at position 756 are especially impaired in cleavage[6,7] and ligation activity.[8,9] Cleavage activity is particularly sensitive to changes in the Watson−Crick edge of the nucleobase of A756.[10] A756 can be cross-linked to the cleavage site.[11]

It is possible that the nucleobase of A756 participates in acid−base catalysis. The rate of approach to equilibrium in the ligation reaction is sensitive to pH, corresponding to a pK_a = 5.7.[9] Strobel and co-workers[8] showed that the rate of ligation is dependent on the state of protonation of the base at position 756. However, unlike corresponding experiments with the HDV ribozyme,[3] addition of 200 mM exogenous imidazole failed to restore activity in an A756G or A756 abasic VS variant.[10] This might be due to a lack of accessibility to imidazole from the medium or a structural reorganization of the modified loop; no restoration of activity was observed using exogenous adenine base, suggesting that free adenine could not gain access to the required position in the correct conformation. Base rescue of abasic sites in the hammerhead ribozyme showed variability of activity with position.[12,13] By contrast, the active site region of the HDV ribozyme is a cleft that is quite open to the solvent.[2,14] We therefore adopted a new strategy in which imidazole base was placed into the A730 loop covalently as a pseudonucleoside.

We previously synthesized 4(5)-(β-D-ribofuranosyl) imidazole,[15] in which imidazole is connected via its C4(5) to the C1′ of ribose, a pseudonucleoside in which the nucleobase is replaced by imidazole. We have made an N^{im}-pivaloyloxymethyl-protected phosphoramidite of this compound[16] that is compatible with *t*BDMS synthesis of RNA (Figure 1D). In this study, we have introduced this into the VS ribozyme at position 756 (Figure 1A). This form of the ribozyme has an opened helix VI; such forms are active (k_{obs} = 0.28 min^{-1}).[6]

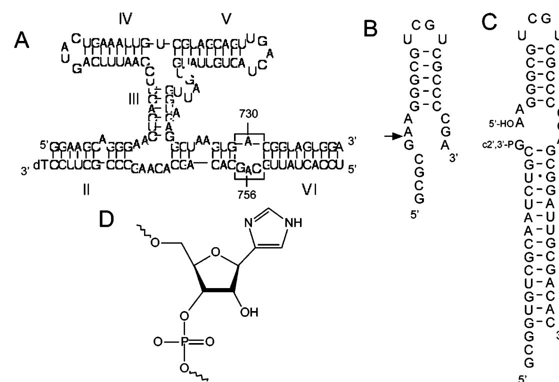

Figure 1. Sequence of VS ribozyme and substrates and the structure of the imidazole nucleotide. (A) The trans-acting VS ribozyme used in these studies. The A730 loop is boxed. Variants of the 34 nt oligonucleotide with imidazole or guanine replacing adenine at position 756 were synthesized. (B) Substrate for the cleavage reaction, with the cleavage position arrowed. (C) Substrate for the ligation reaction. This comprises two RNA oligonucleotides, generated by the action of the VS ribozyme in separate reactions[9] to give the required 2′3′-cyclic phosphate and 5′-hydroxyl termini. (D) Structure of the imidazole nucleotide when incorporated into an oligoribonucleotide.

Figure 2A shows the cleavage of a substrate stem-loop by 1 μM imidazole−756 VS ribozyme (A756Imz) *in trans*, under single turnover conditions in the presence of 10 mM Mg^{2+}. The modified ribozyme catalyzed the almost-complete conversion of substrate into product, compared to ∼5% cleavage by A756G ribozyme under the same conditions (Figure S1). The rate of the A756Imz reaction was approximately 3 orders of magnitude slower than that of the corresponding natural sequence ribozyme, but was comparable to that of HDV C76U ribozyme in the presence of exogenous imidazole.[3] The electrophoretic mobility of the 5 nt product was identical to that generated by the natural VS ribozyme. However, at the longest times of incubation, a second product of faster mobility was observed. RNA of identical mobility was also generated from the product of the natural ribozyme when incubated under the same conditions (Figure 2B), and the same product could also be generated by acid treatment of the slower product generated using natural VS ribozyme (Figure 2C). We therefore assign the slower product as the 2′3′-cyclic phosphate, and the faster species as the result of hydrolysis of the cyclic phosphate. The fraction of substrate and the two products fitted well to the integrated rate equations corresponding to the sequential formation of cyclic phosphate and open product (Figure S2), giving rates of k_{obs} = 1.6 × 10^{-3} and 8.7 × 10^{-4} min^{-1} for the formation of cyclic and open

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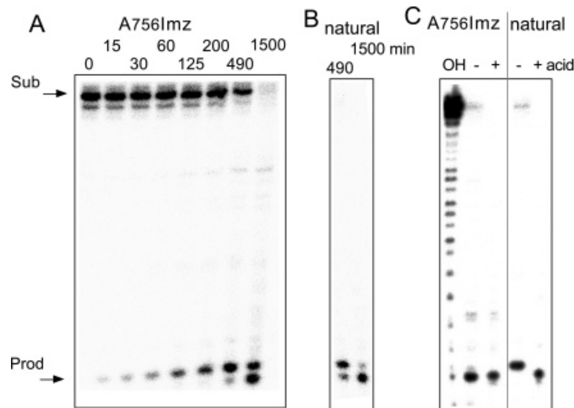

Figure 2. Cleavage activity of the imidazole-containing VS ribozyme. (A) Time course of a cleavage reaction using 1 μM A756Imz ribozyme under single turnover conditions, in the presence of 50 mM Tris−HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM spermidine, 25 mM KCl at 37 °C. Samples were removed at the indicated times (min), and the products were resolved on a sequencing gel. (B) Products of cleavage using the natural ribozyme with extended times of incubation (min). (C) Effect of acid treatment. The products of extended incubation with the A756Imz ribozyme or 10 min incubation with the natural ribozyme were electrophoresed without or with 4 h incubation with 0.1 M HCl. Track 1 contains the result of partial hydroxide cleavage of the RNA to act as a size marker.

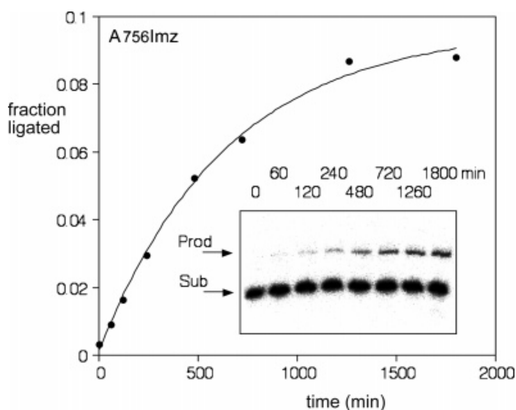

Figure 3. Imidazole-containing VS ribozyme catalyzes a ligation reaction. The reaction was performed using 1 μM A756Imz ribozyme and a substrate comprising a trace (<1 nM) of radioactively [5′-^{32}P]-labeled 5′ section terminated by a cyclic 2′3′-phosphate plus an excess (100 nM) of the 3′ section terminated by a 5′-OH (Figure 1C). The reaction was performed in the presence of 50 mM Tris−HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM spermidine, 25 mM KCl at 25 °C.

phosphate products. The good fit to the sequential model indicates that the initial product of the imidazole-substituted ribozyme is the cyclic 2′3′-phosphate (i.e., the same product that would be formed by the natural VS ribozyme).

The rate of the cleavage reaction mediated by the A756Imz VS ribozyme could be increased by elevating the concentrations of ribozyme and Mg^{2+} ions. An optimized reaction performed using 3 μM ribozyme in the presence of 30 mM Mg^{2+} gave a rate of k_{obs} = 0.01 min^{-1} (Figure S3). The reaction requires a hard divalent metal ion; it is not supported by Zn^{2+} ions, for example (data not shown).

By the principle of microscopic reversibility, A756Imz should also function in ligation. We used the same two-piece VS ribozyme construct, with a substrate comprising two strands as described previously.[9] The two chains of the substrate are held together by the 10 bp lower helix, allowing the internal cleavage/ligation reaction to come to equilibrium. The A756Imz ribozyme catalyzes the ligation of the substrate *in trans* (Figure 3), at a rate comparable with the cleavage reaction. The extent of the ligation reaction is lower than that observed using natural ribozyme,[9] probably due to the loss of cyclic phosphate substrate by hydrolysis during the extended incubation.

In summary, the A756Imz VS ribozyme catalyzes the cleavage and ligation of its substrate at the correct position. Virtually complete cleavage has been obtained, with rates of 0.01 min^{-1}. The low rate is not surprising. Neither ring nitrogen could be superimposed with the N1 of an adenine base at position 756, and thus the rate is likely to be limited by the fraction of time that the best reaction geometry is brought about by thermal fluctuation. Nevertheless, the functionality of imidazole at position 756 is further evidence that the nucleotide at this position is in close proximity to the substrate. Imidazole is very efficient in acid−base catalysis at neutral pH, and thus our results would be consistent with such a role for A756 in the natural VS ribozyme. However, the data would also support stabilization of the dianionic transition state by a protonated base at this position, and these two possibilities cannot be distinguished at this time.

The approach that we have described could be applied to other situations in which nucleobase participation is suspected. The imidazole nucleoside can be incorporated at any site in an RNA species that can be generated by chemical synthesis. While other modified nucleosides with imidazole attached to the nucleobase have been described,[17,18] our compound places the base at a more natural position in the RNA. For the VS ribozyme it is clearly functional, lending powerful support to an important role for nucleobase participation in the chemistry of the ribozyme.

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Supporting Information Available: Experimental details and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Nakano, S.; Chadalavada, D. M.; Bevilacqua, P. C. *Science* **2000**, *287*, 1493−1497.
(2) Ke, A.; Zhou, K.; Ding, F.; Cate, J. H.; Doudna, J. A. *Nature* **2004**, *429*, 201−205.
(3) Perrotta, A. T.; Shih, I.; Been, M. D. *Science* **1999**, *286*, 123−126.
(4) Lafontaine, D. A.; Norman, D. G.; Lilley, D. M. J. *EMBO J.* **2002**, *21*, 2461−2471.
(5) Beattie, T. L.; Collins, R. A. *J. Mol. Biol.* **1997**, *267*, 830−840.
(6) Lafontaine, D. A.; Wilson, T. J.; Norman, D. G.; Lilley, D. M. J. *J. Mol. Biol.* **2001**, *312*, 663−674.
(7) Sood, V. D.; Collins, R. A. *J. Mol. Biol.* **2002**, *320*, 443−454.
(8) Jones, F. D.; Strobel, S. A. *Biochemistry* **2003**, *42*, 4265−4276.
(9) McLeod, A. C.; Lilley, D. M. J. *Biochemistry* **2004**, *43*, 1118−1125.
(10) Lafontaine, D. A.; Wilson, T. J.; Zhao, Z.-Y.; Lilley, D. M. J. *J. Mol. Biol.* **2002**, *323*, 23−34.
(11) Hiley, S. L.; Sood, V. D.; Fan, J.; Collins, R. A. *EMBO J.* **2002**, *21*, 4691−4698.
(12) Peracchi, A.; Beigelman, L.; Usman, N.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11522−11527.
(13) Peracchi, A.; Matulic-Adamic, J.; Wang, S.; Beigelman, L.; Herschlag, D. *RNA* **1998**, *4*, 1332−1346.
(14) Ferré-d'Amaré, A. R.; Zhou, K.; Doudna, J. A. *Nature* **1998**, *395*, 567−574.
(15) Harusawa, S.; Murai, Y.; Moriyama, H.; Imazu, T.; Ohishi, H.; Yoneda, R.; Kurihara, T. *J. Org. Chem.* **1996**, *61*, 4405−4411.
(16) Araki, L.; Harusawa, S.; Yamaguchi, M.; Yonezawa, S.; Taniguchi, N.; Lilley, D. M. J.; Zhao, Z.; Kurihara, T. *Tetrahedron Lett.* **2004**, *45*, 2657−2661.
(17) Santoro, S. W.; Joyce, G. F.; Sakthivel, K.; Gramatikova, S.; Barbas, C. F., III. *J. Am. Chem. Soc.* **2000**, *122*, 2433−2439.
(18) Gourlain, T.; Sidorov, A.; Mignet, N.; Thorpe, S. J.; Lee, S. E.; Grasby, J. A.; Williams, D. M. *Nucleic Acids Res.* **2001**, *29*, 1898−1905.

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