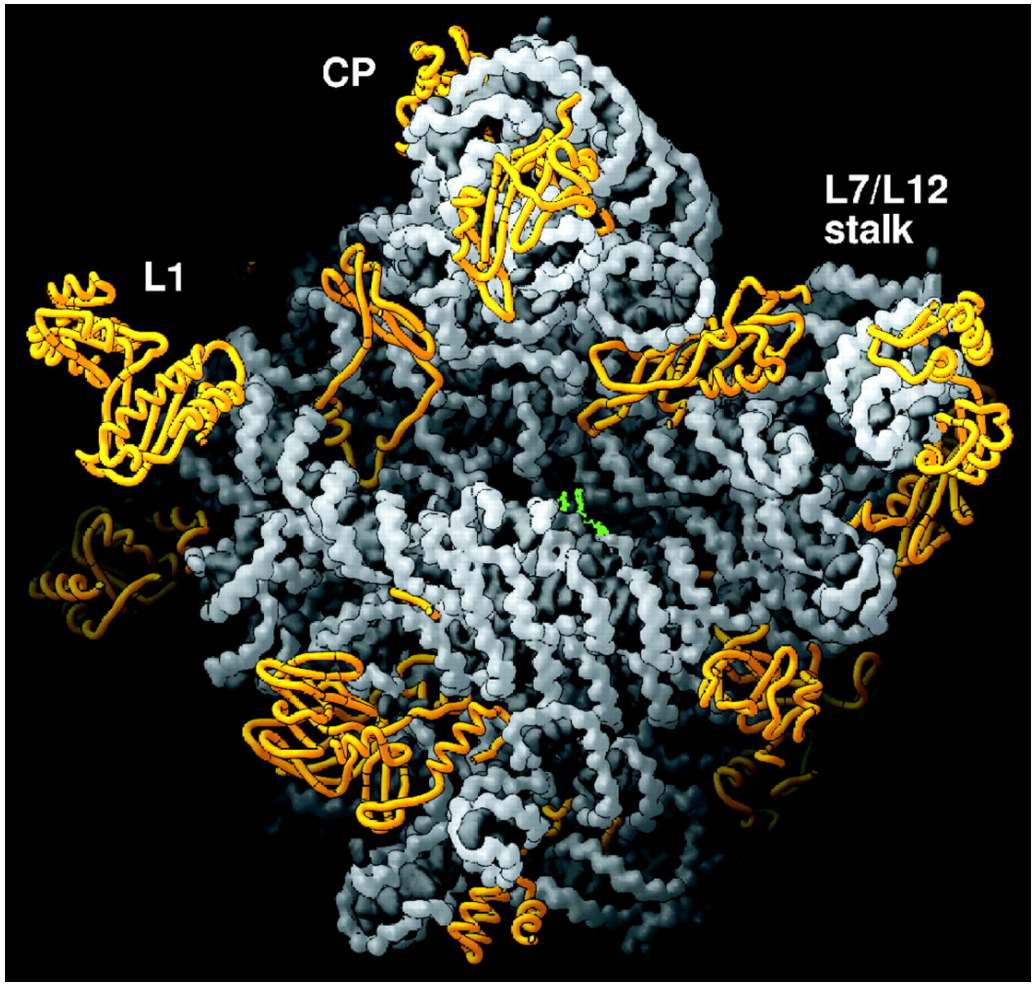
Lecture 6. 2023
RNA Does Chemistry

What is a ribozyme?
RNA + Enzyme

Group I introns (and Group II introns), the ribosome, the spliceosome, and RNase P are all ribozymes; and then there are small RNAs that cleave themselves that are also ribozymes.

This x-ray crystal structure of a bacterial large ribosomal subunit shows the side that sits on the small subunit. The RNA is white, proteins are gold. Green is a puromycin bound at the active site where catalysis occurs. The nearest protein is 18 Å away from the catalytic center.

Ribosomes are RNA enzymes – the RNA does the chemistry.



N. Ban et al., Science 289, 905 -920 (2000)

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Science
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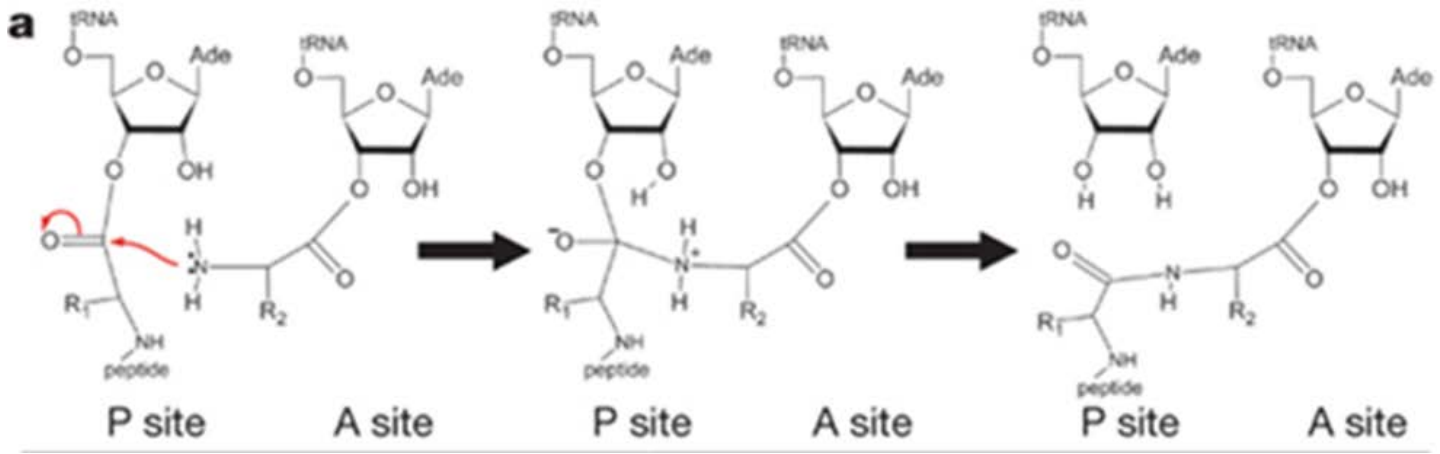
The ribosomal RNA catalyzes peptide bond formation



Five small nucleolytic ribozymes

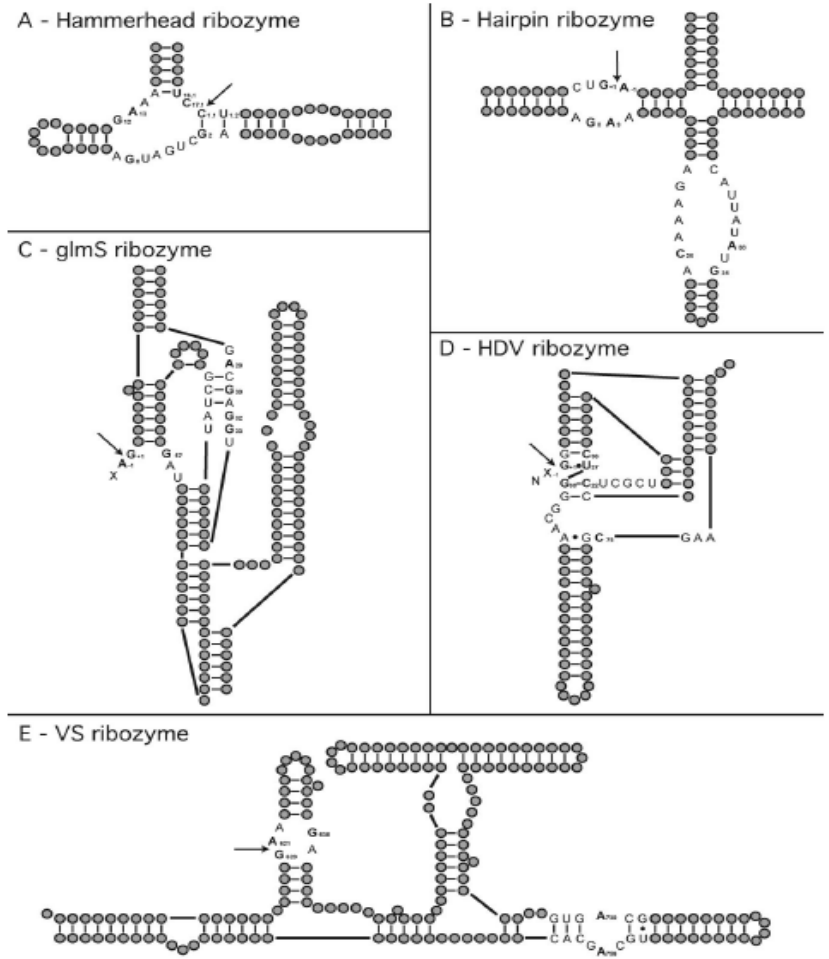
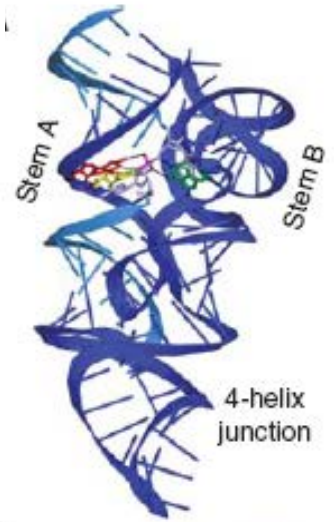


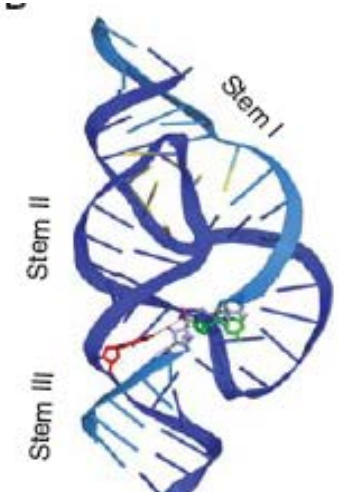
FIGURE 1. Secondary structures of the five nucleolytic ribozymes: (A) hammerhead; (B) hairpin; (C) *glmS*; (D) HDV; (E) VS. Key nucleotides are

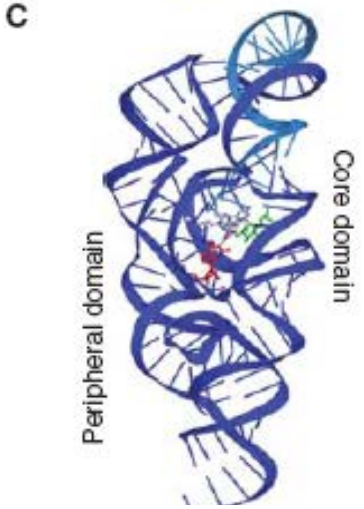
Hairpin Ribozyme

Residues thought to be part of general acid/base catalysis are green/red. Scissile phosphate and 2'OH are magenta. Substrates in light blue.

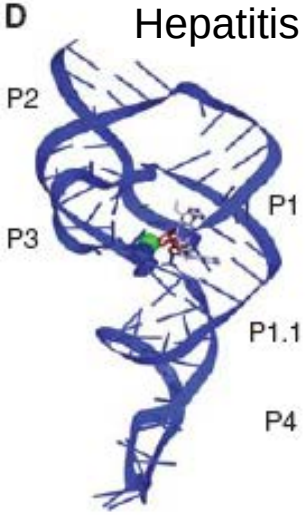


Hammerhead Ribozyme





glmS Ribozyme-riboswitch



Hepatitis Delta Virus (HDV) Ribozyme

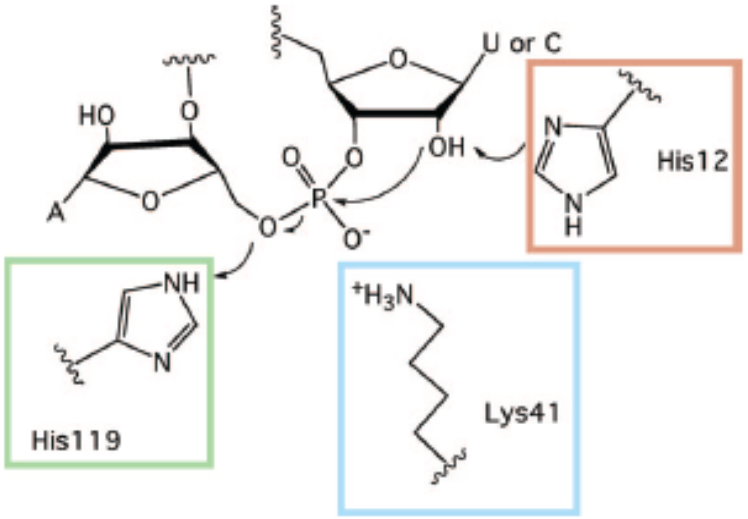


FIGURE 2. The catalytic reaction of RNase A. The general base, His12, is boxed in red. The general acid, His119, is boxed in green. The amino acid responsible for charge stabilization at the transition state, Lys41, is boxed in blue.

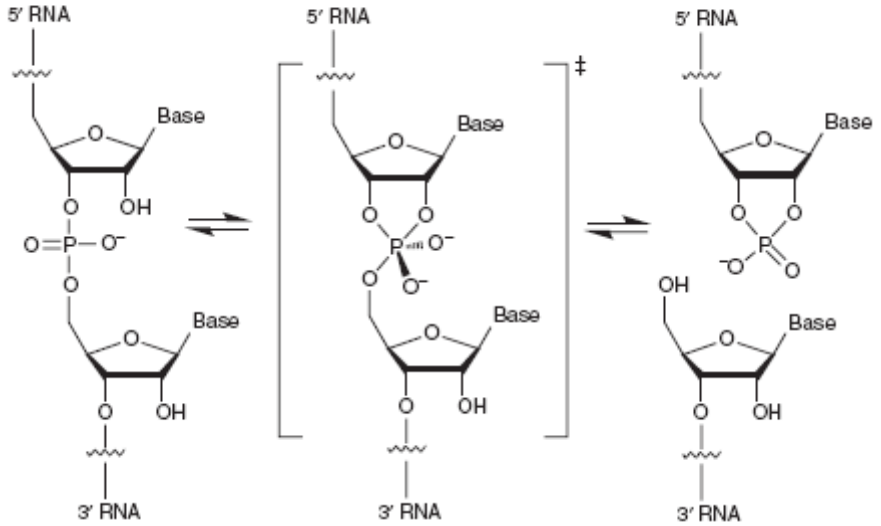
In **RNase A**, the unprotonated Histidine 12 acts as a general base catalyst, extracting the H from the 2′ OH, creating a reactive Oxygen that attacks the phosphate PO_4^- and produces a 2′,3′ cyclic phosphate.

The protonated Histidine 119 acts as a general acid and donates its H to the 5′ OCH_2 leaving group.

The ammonium group of Lys41 stabilizes the excess negative charge of the trigonal bipyramidal oxyphosphorane transition-state.

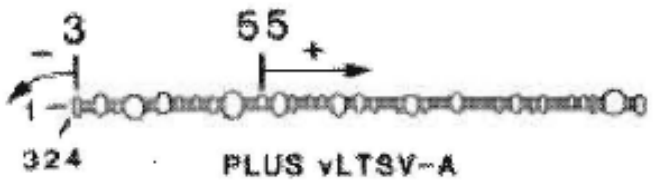
Hairpin, hammerhead, and *glmS* ribozymes use the same acid/base chemistry.

We will ask:
Where are the acid and the base?

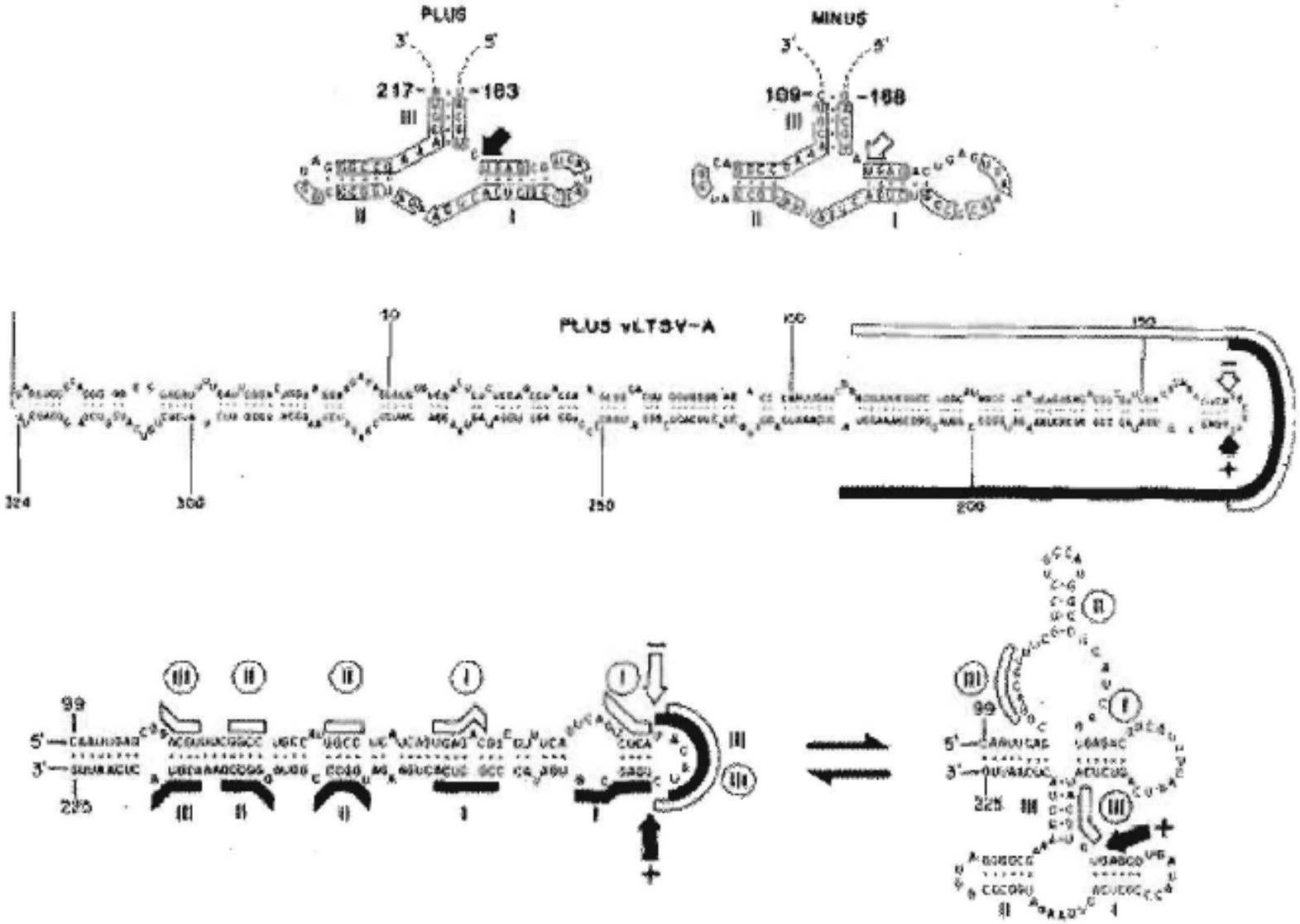


History of the Hammerhead Ribozyme.

It was first identified in the genome of a virusoid. A virusoid is a "circular single-stranded RNA dependent on plant viruses for replication and encapsulation" (Forster & Symons, 1987, *Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites*, Cell 49(2) 211-220). The plus strand of lucern transient streak virus (LTSV) is shown here:



The RNA structure must be rearranged during replication to allow formation of the structure that leads to auto-catalysis.


PLUS
3'
5'
217~
~183
III
II
I
MINUS
3'
5'
189~
~168
III
II
I
PLUS vLTSV-A
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300
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225
III
II
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+

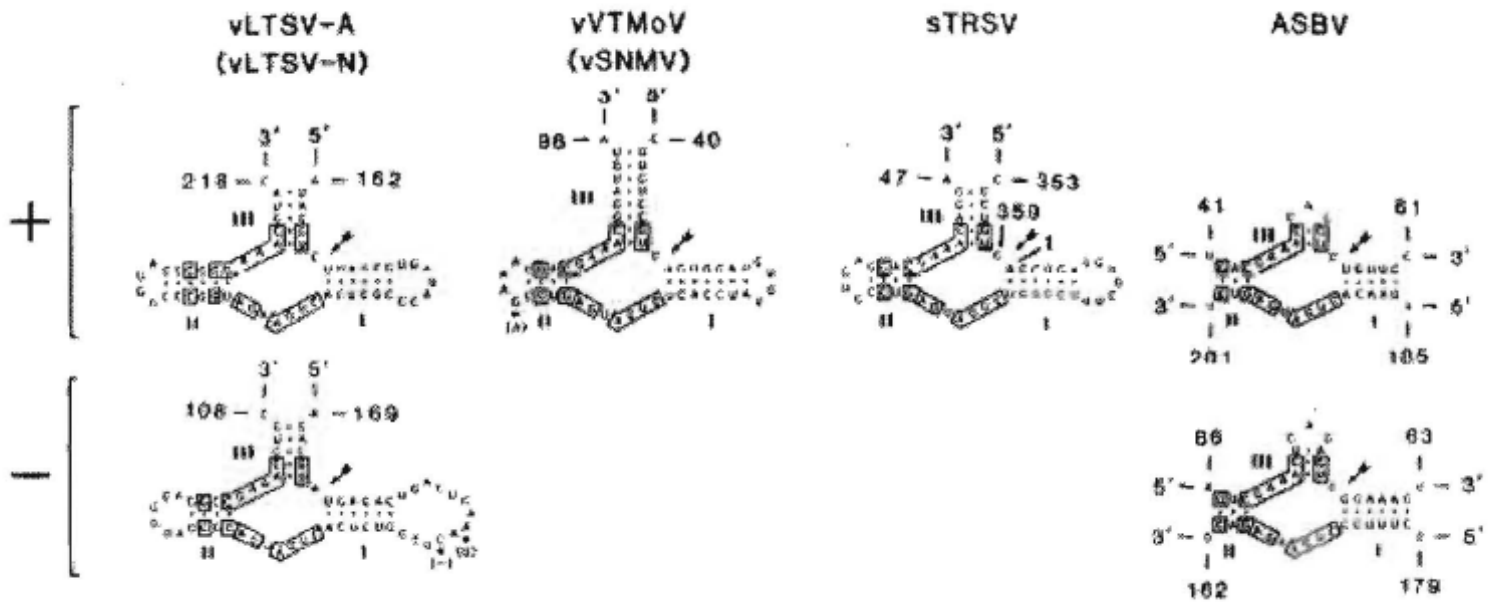
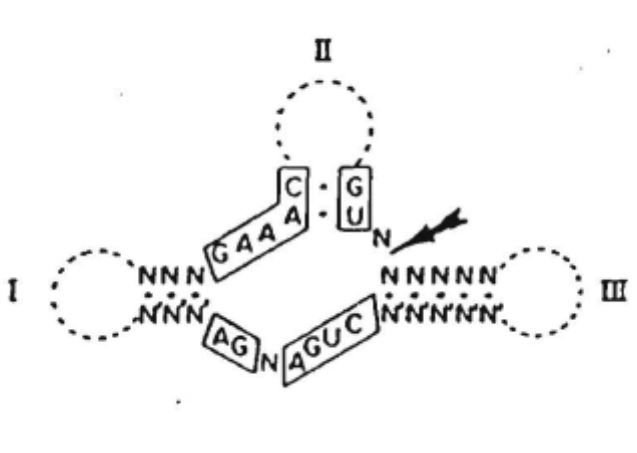


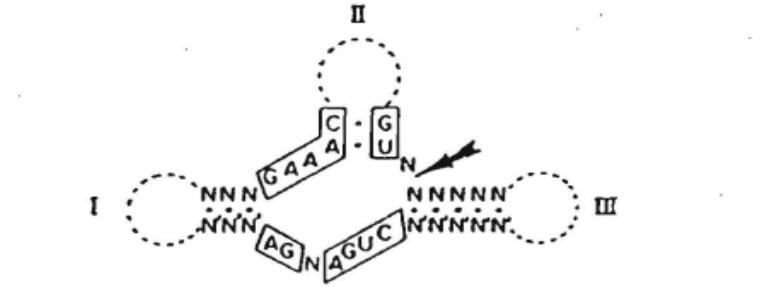
Figure 4. Secondary Structural Models for the Active Sites of Satellite and Viroid RNAs Known or Predicted to Self-Cleave

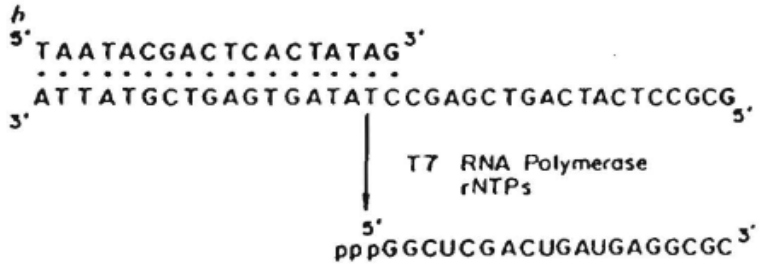
Many other virusoids share this structure, and it has become known as the hammerhead ribozyme, or the littlest enzyme.



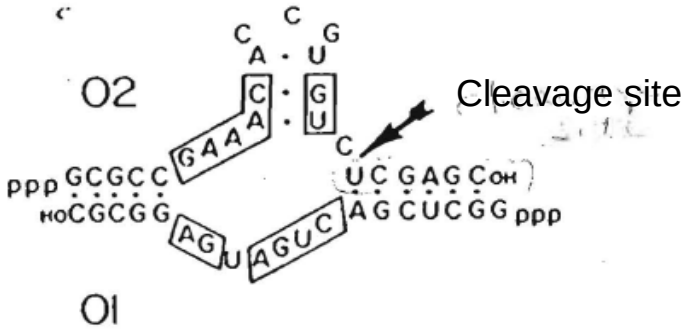
The Hammerhead can be removed from the viral genome and studied on its own. Uhlenbeck [*Nature 1987 328:596*] showed that it could be engineered to become a true enzyme.

The hammerhead RNA can be synthesized in two pieces



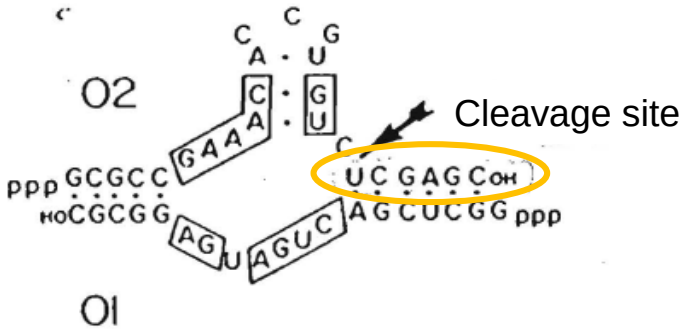


The top strand O2 is the “substrate”
The bottom strand O1 is the “enzyme”

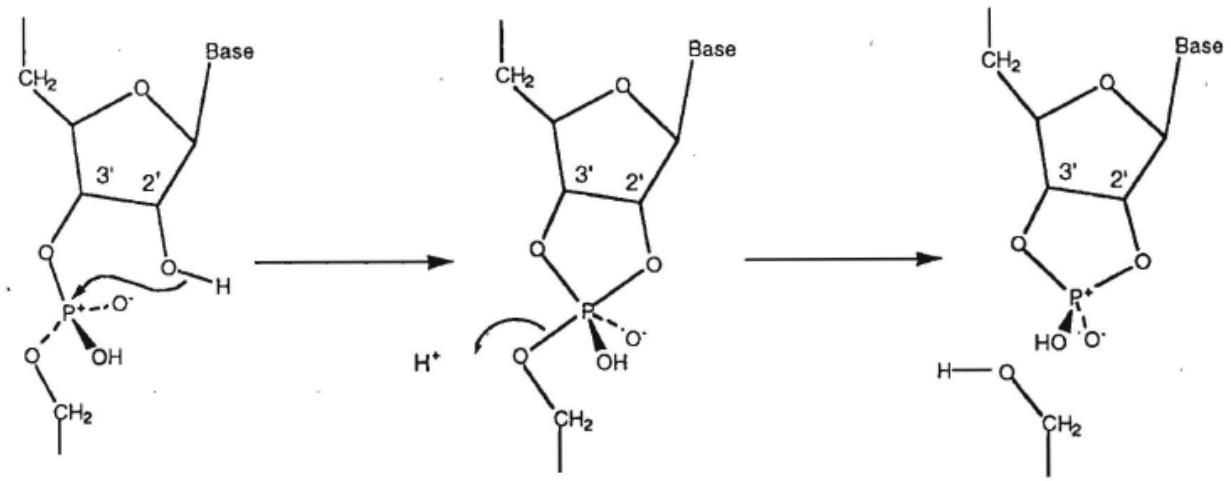


Add equimolar amounts of O1 and O2.
Incubate in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 37°C, 1 hr
85% of O2 “substrate” is cleaved to give two products.

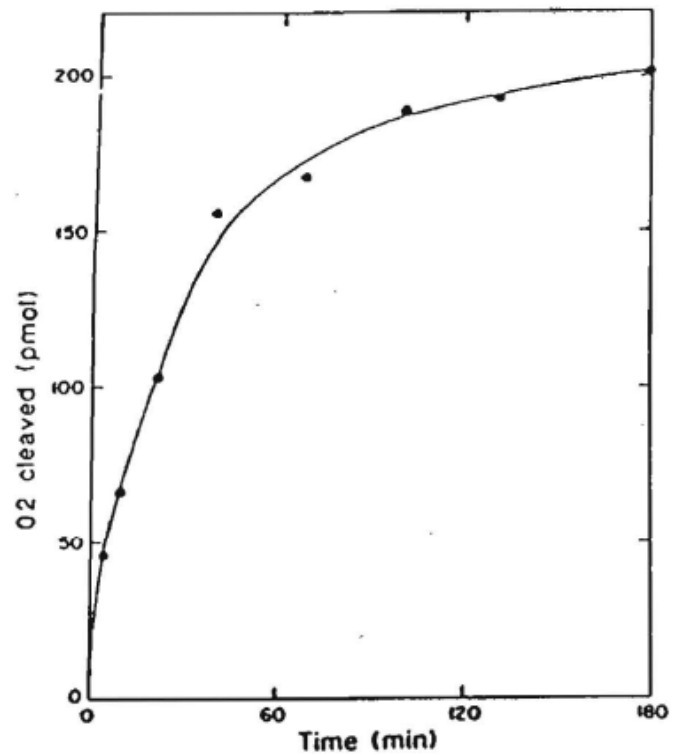
The O1 “enzyme” is unchanged.



The products were characterized and found to have a 5′ OH on the short product $_{OH}UCGAGC_{OH}$
and a 2′,3′ cyclic phosphate on the long product

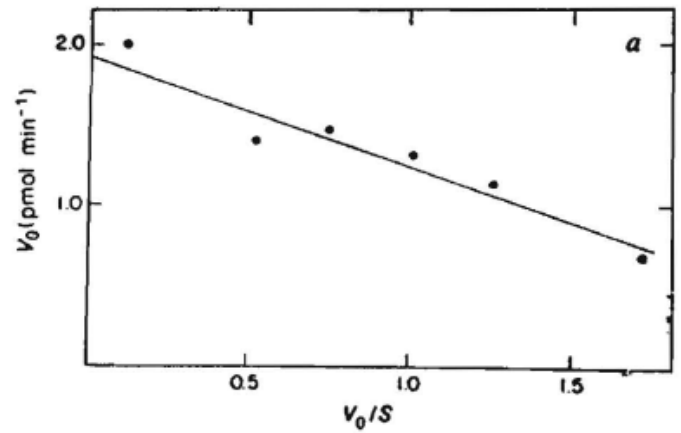


The next experiment was to look for multiple turn-over of substrates. These experiments used 208 pmol of substrate and 10 pmol of enzyme. The reaction was run at 55°C, where the rate is rapid and product release is favored.



Result: > 90% of substrate is cleaved in 3 hr, so the rate is about 4 pmol/min. This is the same rate seen at 55°C for equimolar E and S, and means that product dissociation is not rate limiting.

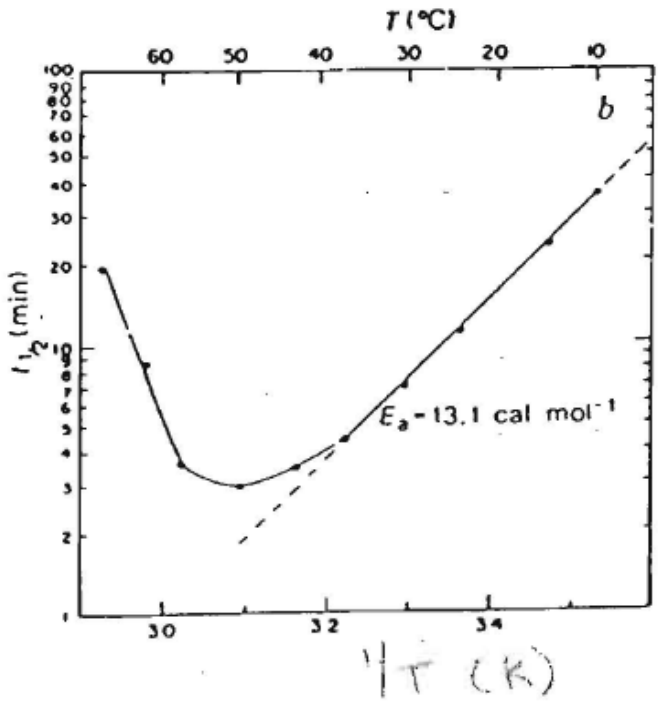
Analyze this reaction by standard enzyme kinetics, using an "Eadie-Hofstee" plot.



V_o: initial rate of reaction, measured with constant [E] and variable [S]. K_M= 0.63 μM; V_{max}=1.9 pmol/min; k_{cat} ~0.5 min^{-1}

The cleavage reaction is temperature-dependent.

The slope of the Arrhenius plot of the reaction is the activation energy. ($\ln k = -E_a/RT + C$; slope $= -E_a/R$)

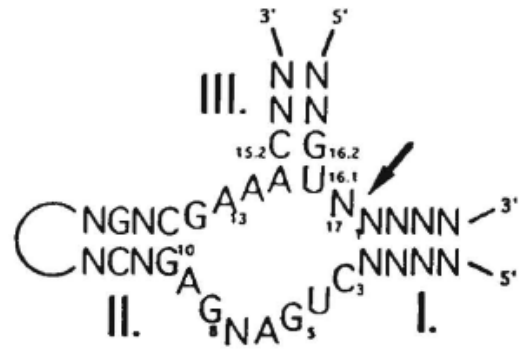


Cleavage rate increases until 50 °C, then decreases. Why?

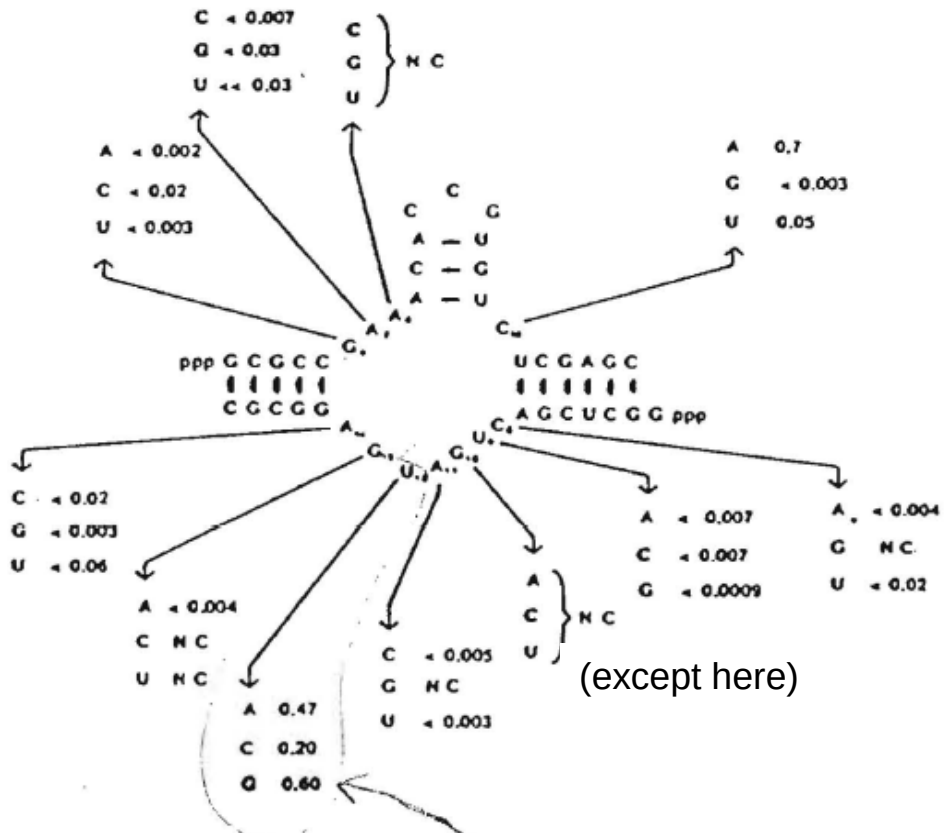
What features of the ribozyme are critical for its activity?

Look for mutants. First, identify conservation and variation, using phylogenetic comparison.

The Hammerhead consensus sequence.
There is a conserved core
with variable arms.
N17 can be C or A in
this context.



Each nucleotide in the central core was replaced. The mutant enzyme or substrate was combined with their respective wild type substrate/enzyme, and assayed for cleavage. The numbers are the relative cleavage rates with respect to wild-type enzyme/substrate.
NC = no cleavage after 2 hr



Conclusion: No mutations in the core are tolerated (Ruffner et al., (1990) Biochem 29:10695-10702).

The original hammerhead enzyme had very short stems.
It was later understood that it lacked stabilizing tertiary interactions.

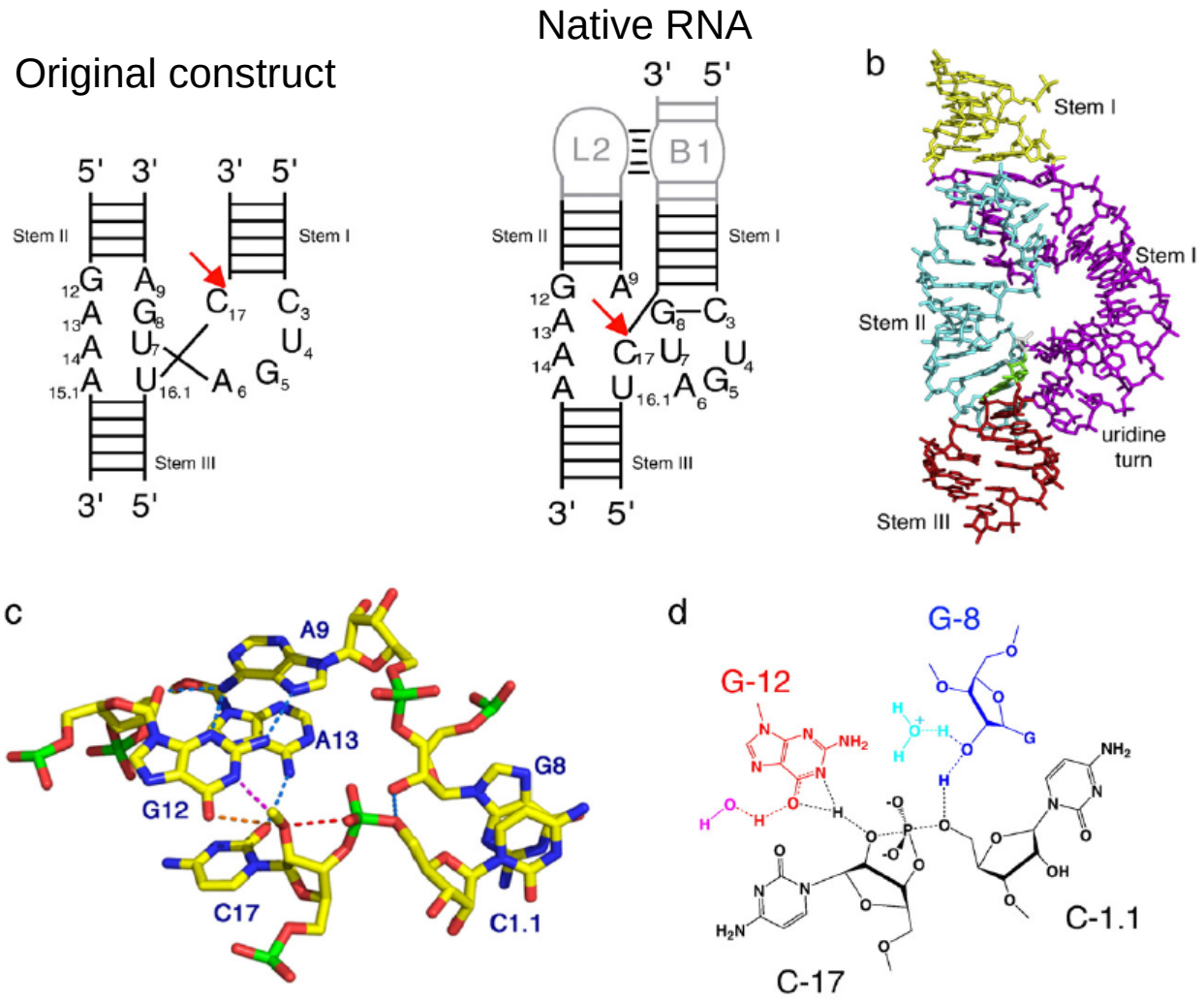
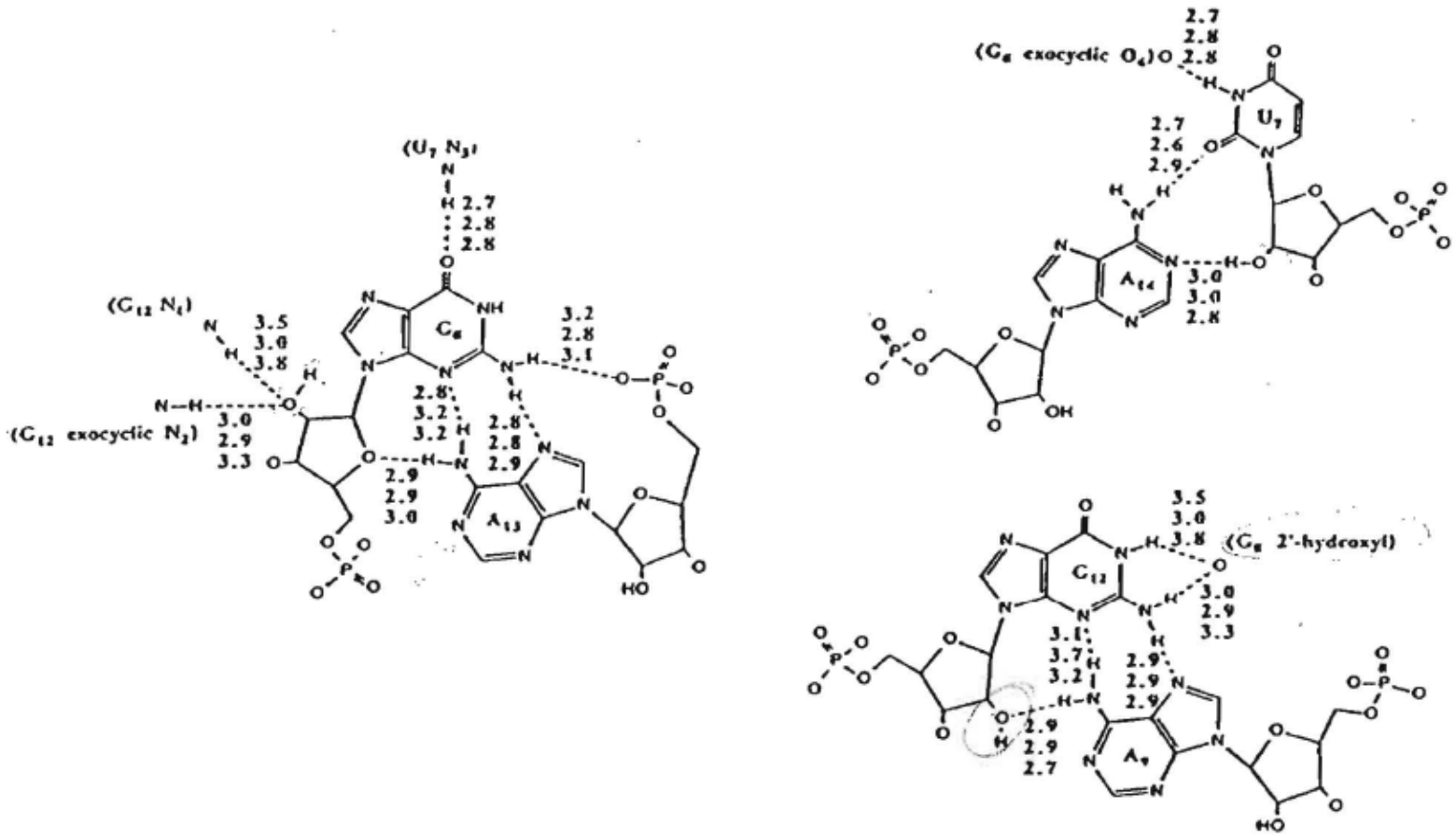
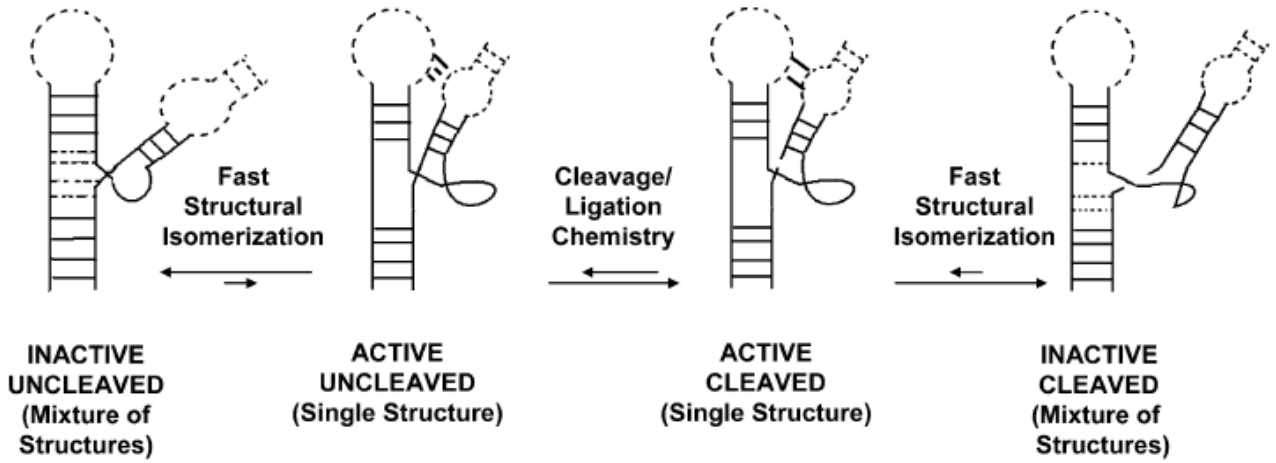


Fig. 2. The structure of the hammerhead ribozyme. (a) shows schematic diagrams of the secondary structures of the minimal and full-length hammerheads, respectively, and (b) shows an all-atom representation of the tertiary structure of the full-length hammerhead. A distal contact between Stems I and II in the full-length hammerhead, shown schematically in (a), stabilizes the active site structure, the details of which are shown in (c). (d) shows a proposed transition state structure extrapolated from (c).

There are two G:A interactions and one C:C interaction, in addition to an abnormal A:U pair.

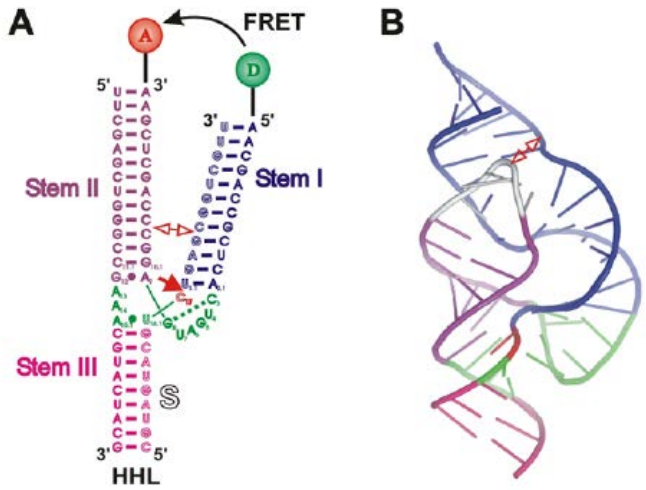


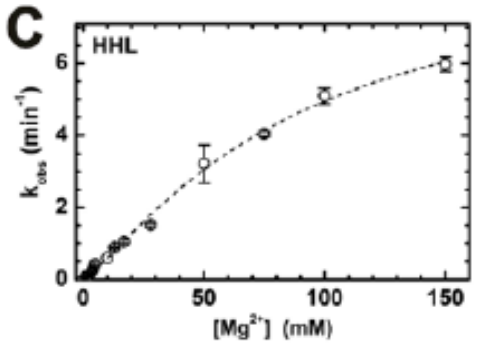


A schematic pathway of the hammerhead reaction illustrates both how a conformational transition positions the active site and how it might allow for the reverse ligation reaction. In the inactive, uncleaved state, a mixture of structures could be present; an even more heterogeneous mixture could be present in the inactive cleaved state.

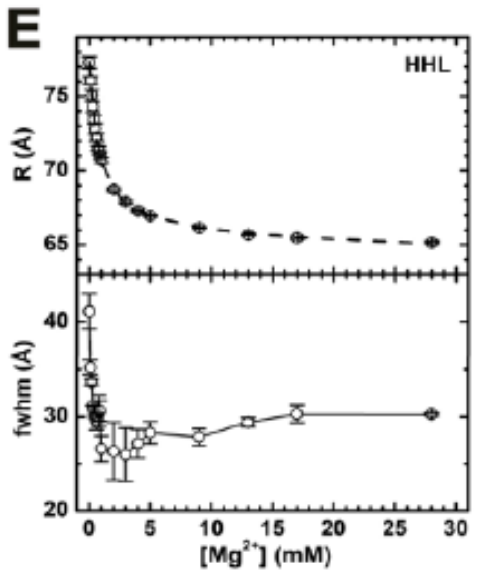
If the RNA persists for some time (how long?) in its active cleaved state, it could re-ligate the product since this is a reversible transesterification reaction.
If the tertiary interaction is too strong, then ligation would also be favored.
If the product:enzyme helix is too long, product release would be dis-favored and so lead to ligation.
This is a dynamic process!

Use ensemble measurements to look at the relative orientation of the arms of the small hammerhead (by lengthening the duplexes)

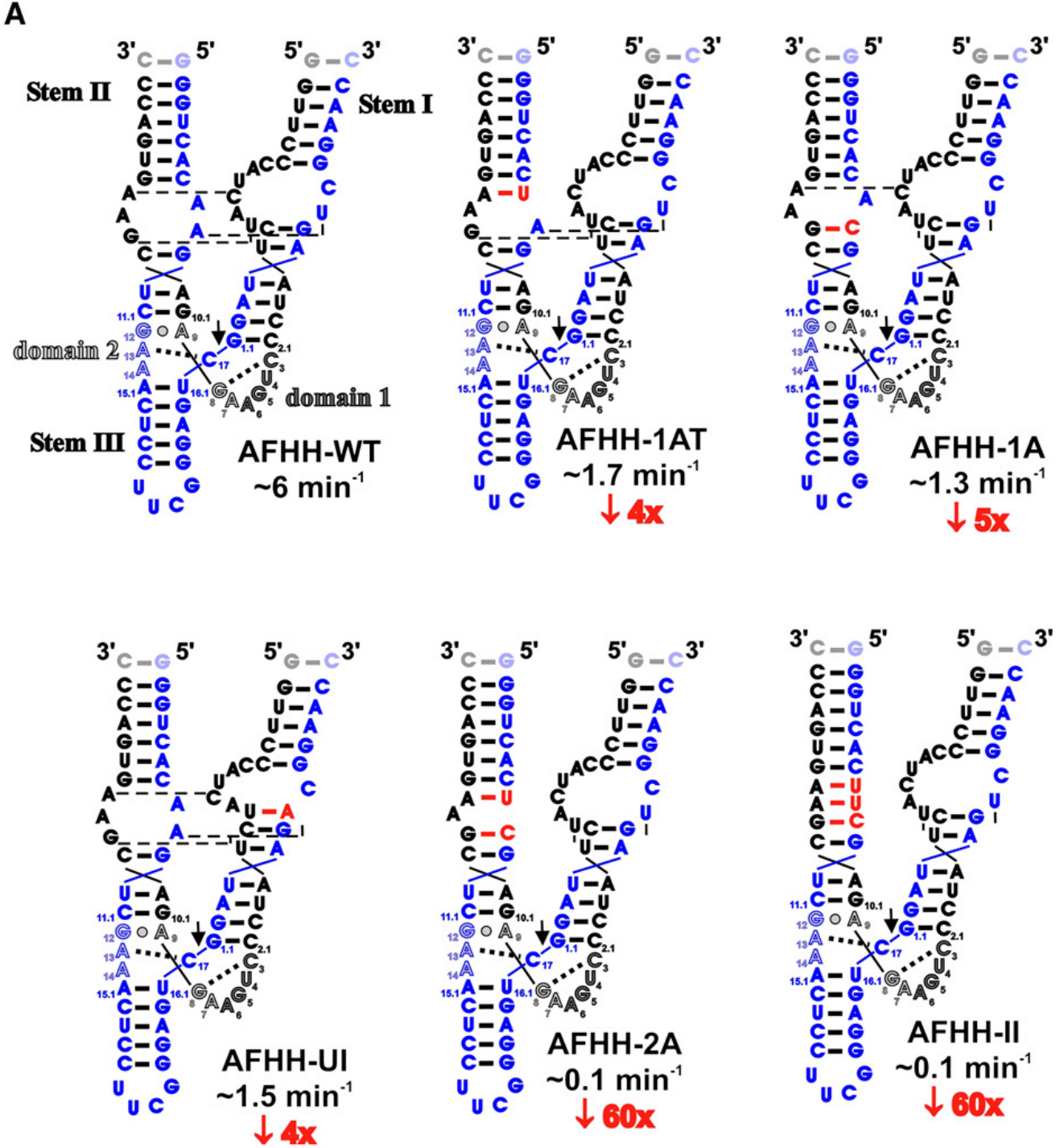




Mg2+ dependence of the cleavage rate



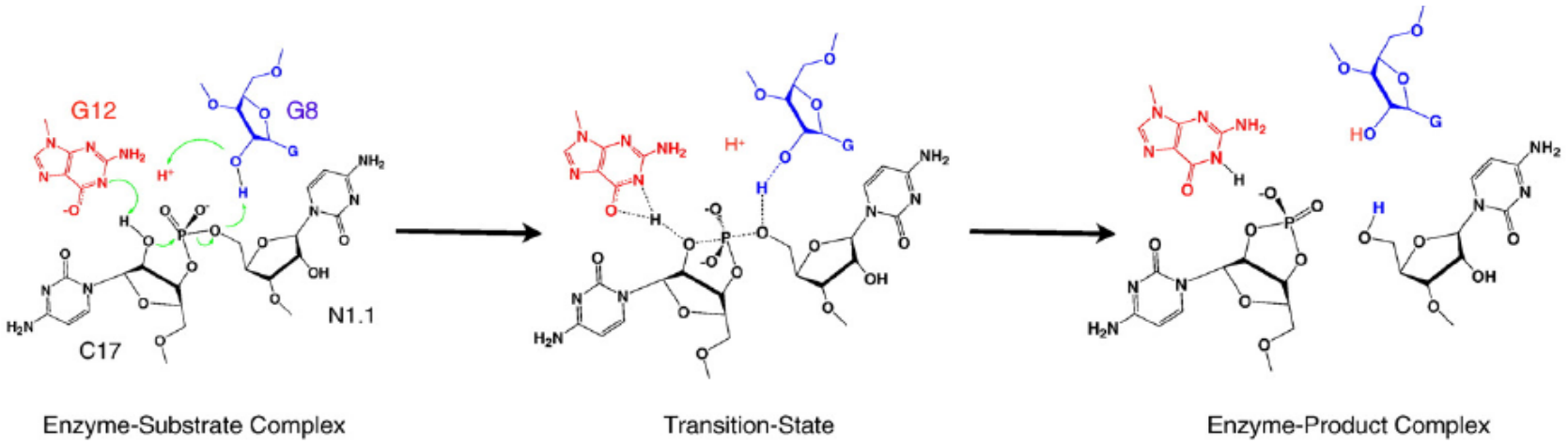
Mean distance and full width half maximum (fwhm) of the arms as a function of [Mg2+]



Long-range interactions control the rate of cleavage.

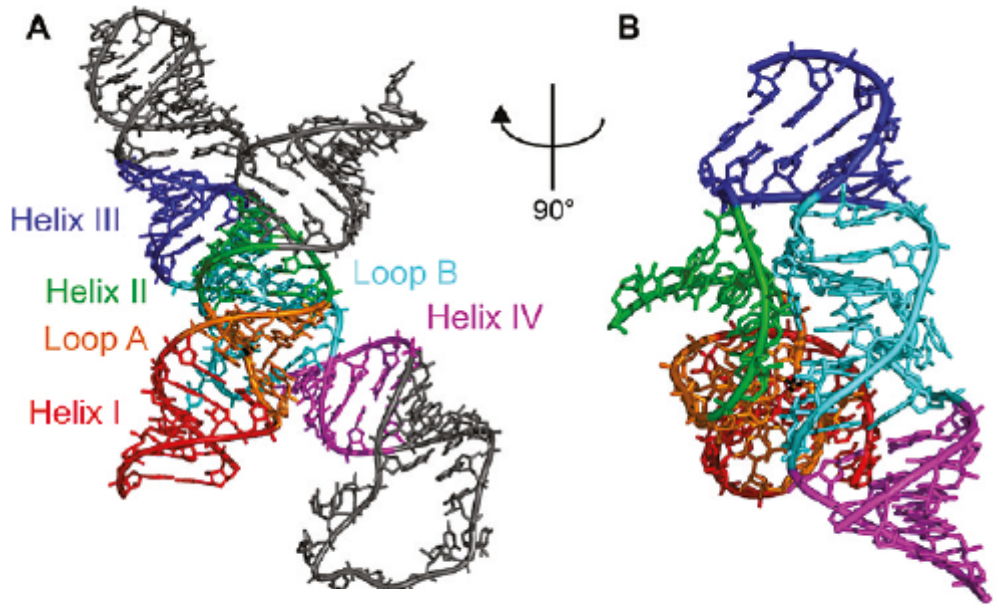
Mutations (red) disrupt tertiary contacts that reduce the cleavage rate via destabilizing the structure around the scissile CpG.

Proposed mechanism for acid/base catalysis in the hammerhead self-cleavage reaction.



G12 aromatic N hydrogen bonds to the nucleophilic 2′-OH. G12 is invariant in HH ribozymes, where it serves as the general base in the cleavage reaction. The ribose 2′ OH of G8 makes a hydrogen bond to the 5′-oxo leaving group (it acts as the general acid).

The **Hairpin Ribozyme** was discovered in a viroid RNA where it undergoes self-cleavage to produce a single copy of the RNA strand.



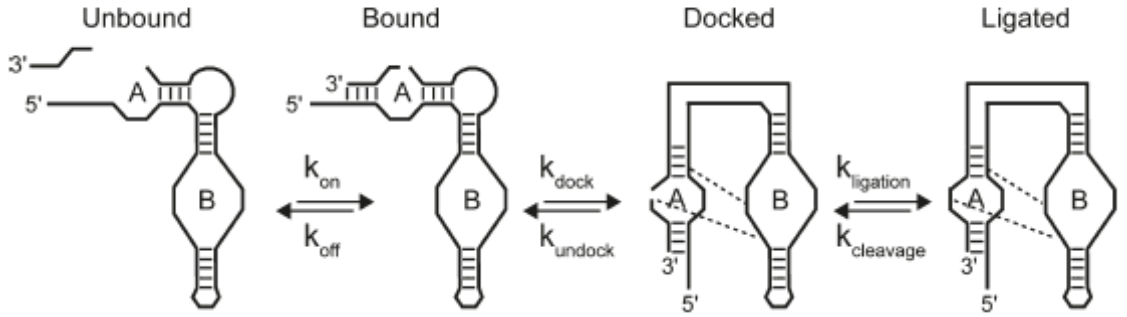
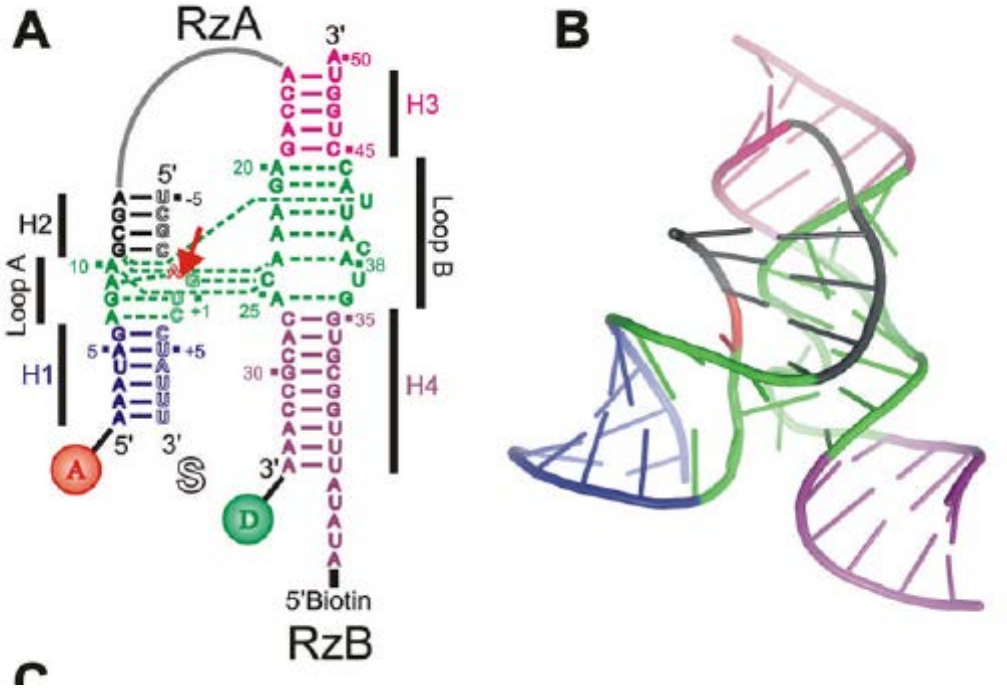


FIGURE 7: Minimum reaction sequence for hairpin ligation, consisting of substrate binding, the formation of a docked state requiring specific interactions between residues in loops A and B (dashed lines), and ligation between G(+1) and N(−1). All steps are reversible, with the equilibrium dependent on the energetics of substrate binding, the stability of the docked state, and the ribozyme-catalyzed equilibrium between ligation and cleavage.

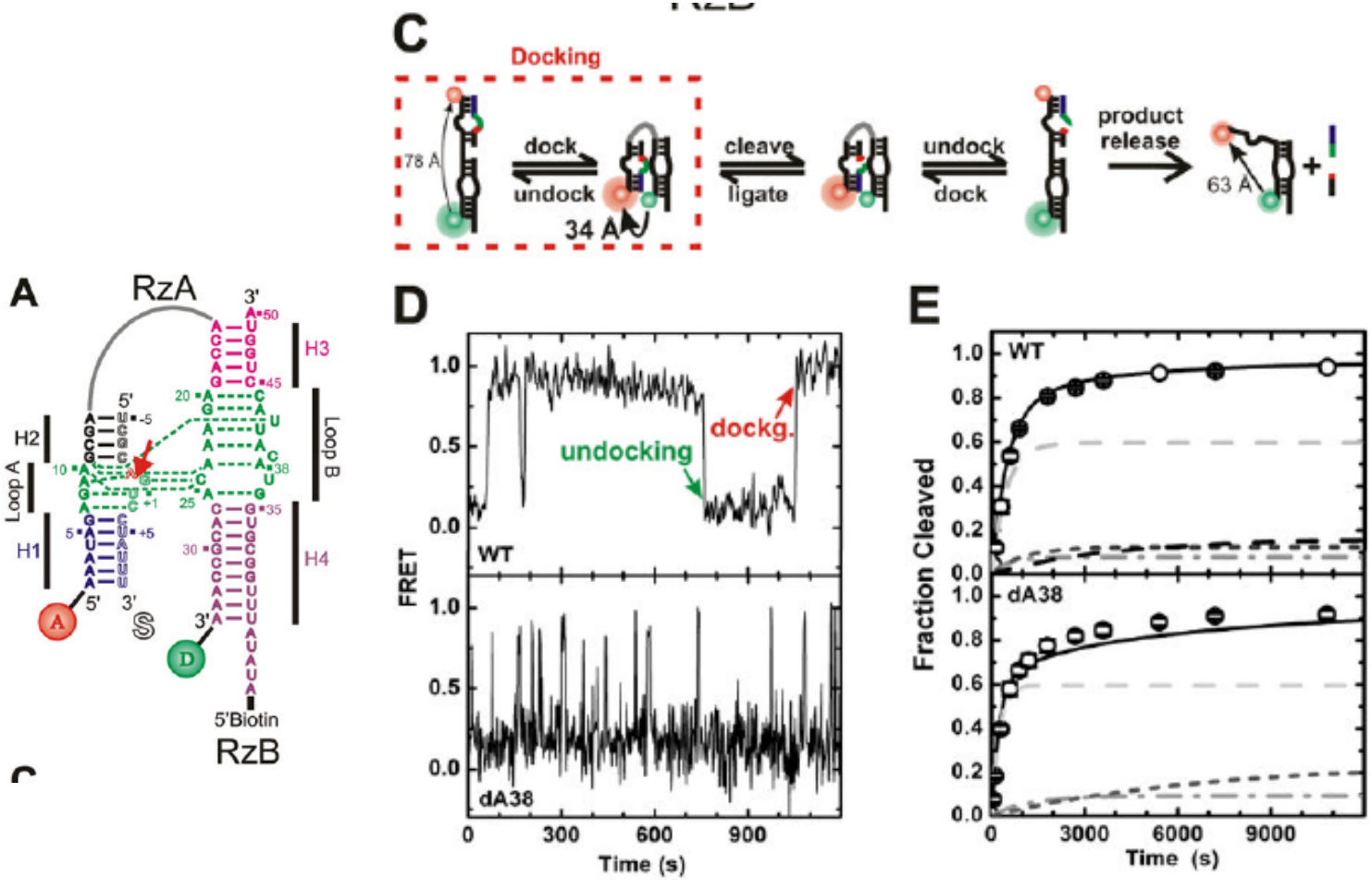
Dynamics of the hairpin ribozyme



Engineer a three-piece RNA. Tether one strand to glass via biotin/streptavidin.

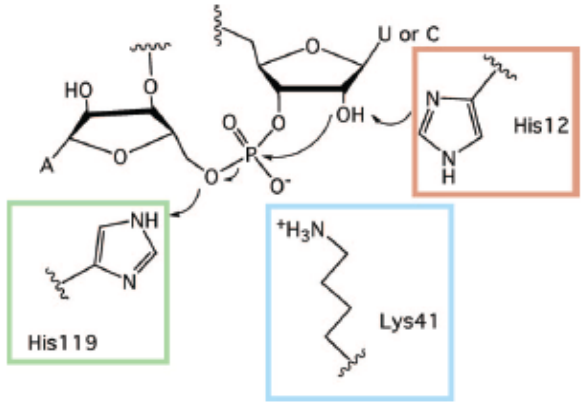
On one end of the enzyme strand, add a donor and on the other end an acceptor FRET pair.

Single Molecule study of the Hairpin Ribozyme.



dA38 accelerates undocking, since there is a loss of a hydrogen bond.
But the rate and extent of cleavage was preserved.

Cleavage chemistry: acid and base contributions from the nucleobase



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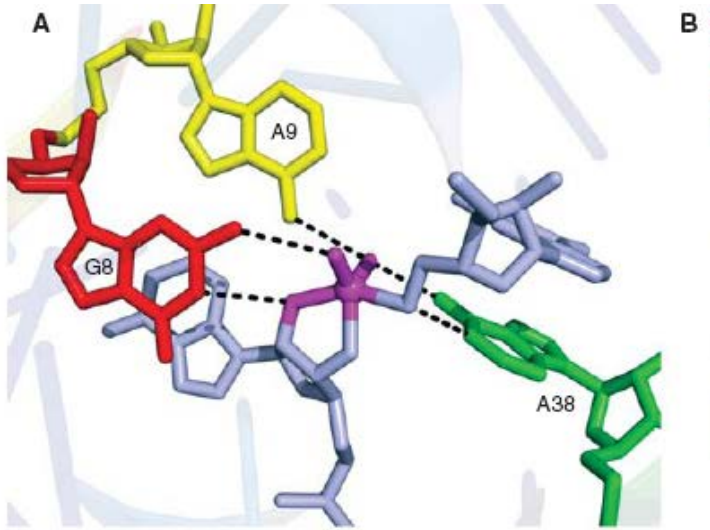
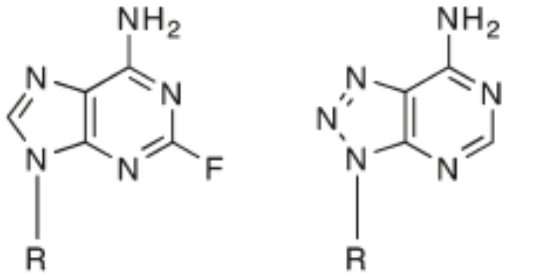


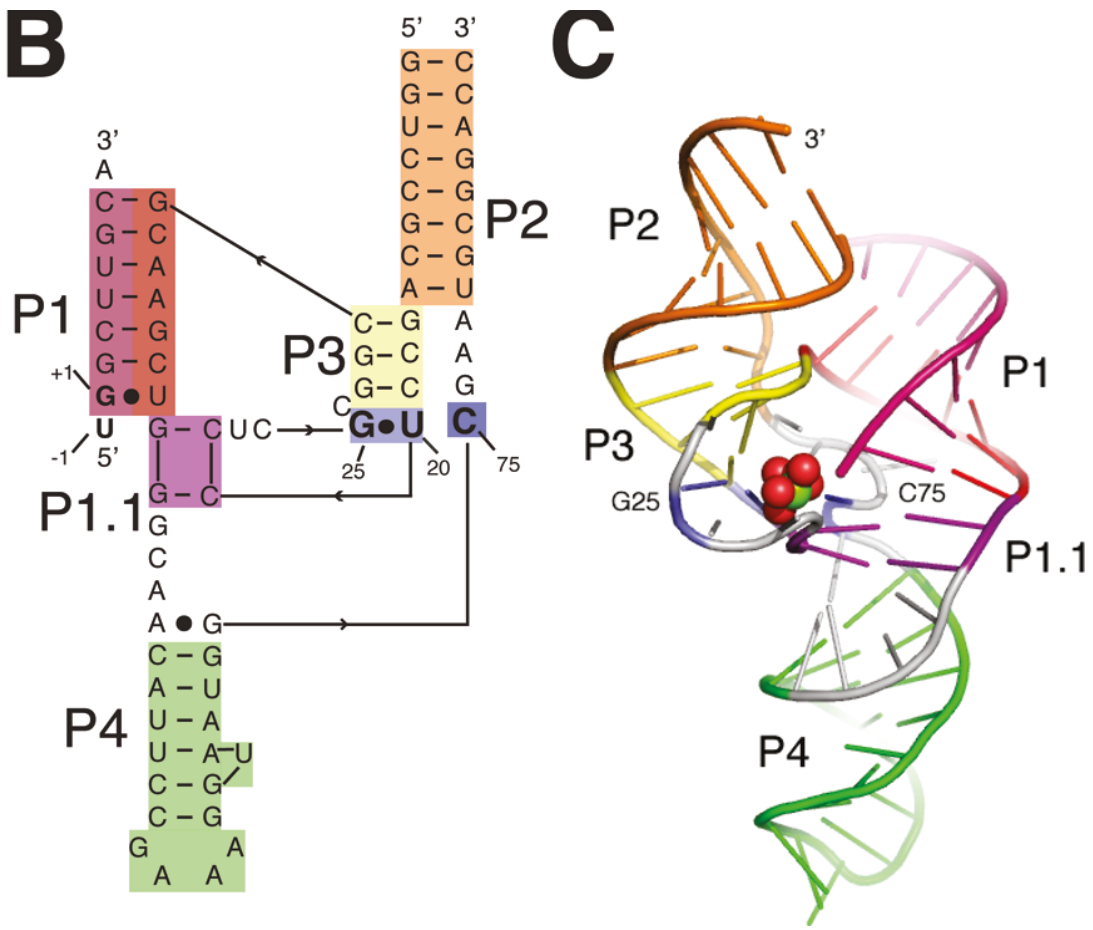


2F-A
$pK_a < 1.0$

n^8A
$pK_a = 2.2$

A38 cannot serve as the general acid when it is replaced by 2F-A or n^8A, providing chemical evidence (in contrast to crystal evidence) that it functions like a histidine in the cleavage/ligation reaction.

Biochemistry **2010,** *49,* 3723–3732



The Hepatitis Delta Virus ribozyme requires Mg^{2+} for cleavage.

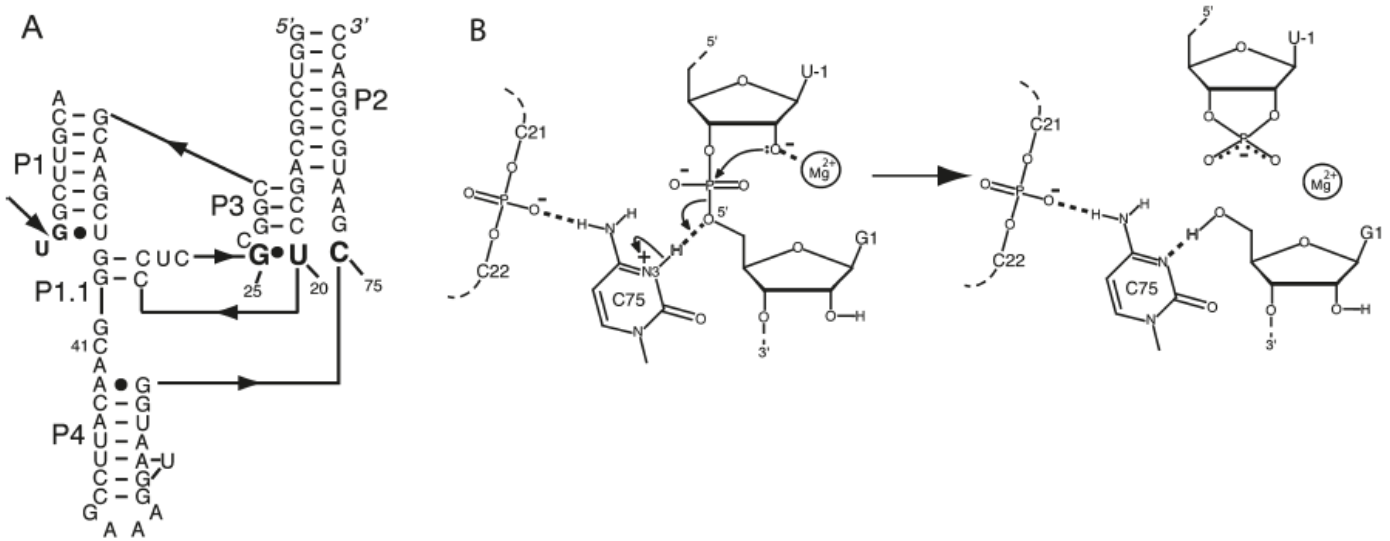


FIGURE 4. Structure and proposed mechanism of the HDV ribozyme. (A) Secondary structure of two-piece ribozyme. Arrow depicts cleavage site. (B) Proposed mechanism with Mg^{2+} acting as a Lewis acid and protonated C75 as a general acid. Reproduced with permission from ref 49. Copyright 2011 American Chemical Society.

C75 is particularly interesting. It serves as the general acid catalyst in the cleavage reaction.

In the crystal structure of the HDV ribozyme in its postcleavage state, C75 is proposed to make a hydrogen bond between its N3 atom and the protonated leaving group, the 5′-hydroxyl of G1. This conformation suggested that C75 might be protonated in the reactant state, donate its proton to the 5′-O leaving group, and thereby serve as a general acid in the cleavage reaction.

Removing the C75 base (creating an abasic site) inactivates HDV, but it can be rescued by addition of exogenous imidazole!

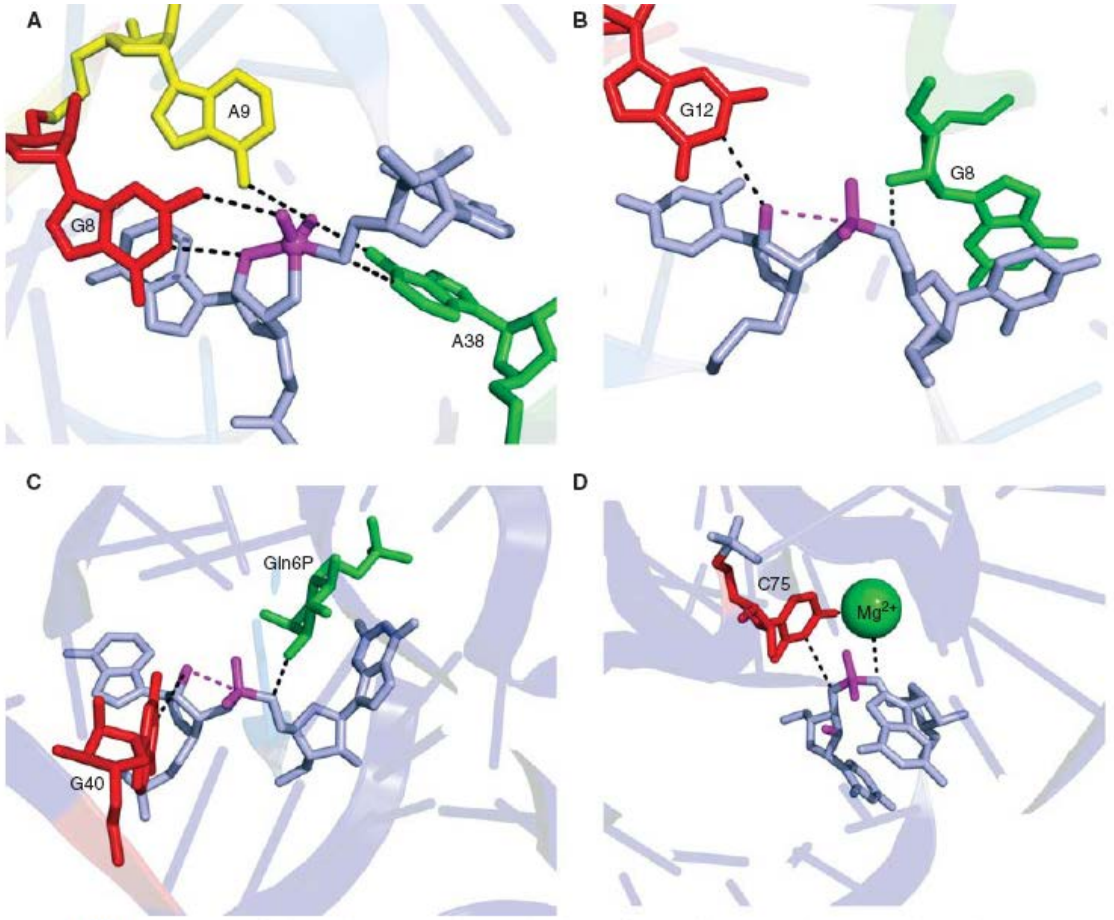


Figure 4. Active sites of the (*A*) hairpin, (*B*) hammerhead, (*C*) *glmS*, and (*D*) HDV ribozymes color-coding as in Figure 3. The moieties thought to contribute to catalysis are labeled.

In the hairpin (A), hammerhead (B), and *glmS* (C) ribozymes, the substrate is bound as part of a deformed helix that docks with other structures in the enzyme. The backbone is stretched to align it with residues that cleave it.

In the hairpin (A), G8 and A38 play the part of the histidines in RNase A: G8 is the base (pKa = 9.0), and A38 the acid (pKa=5.0). A9 is in the position of Lys41 where the exocyclic amines of A9 and A38 provide electrostatic stabilization of the transition state.

Conclusion:

These are small ribozymes, but they have evolved different global structures and sequences. In particular, three of the four use the same catalytic mechanism in different active sites.

The chemical contexts of the active sites allows bases and riboses to act as general acids/bases, which involves local changes of the pKa.

The structures flex: the active sites are in junction regions that are formed by docking of duplex regions through tertiary interactions. Docking is not stable, so the conformations fluctuate between an open inactive form and a closed active form.

In two of the ribozymes, the reactions can go forward and backward (cleavage and ligation), since product release can be delayed by salt and temperature.

Hammerhead ribozymes appear in all kingdoms in unexpected places!