N. GOOD NUCLEASE/BAD NUCLEASE

Background

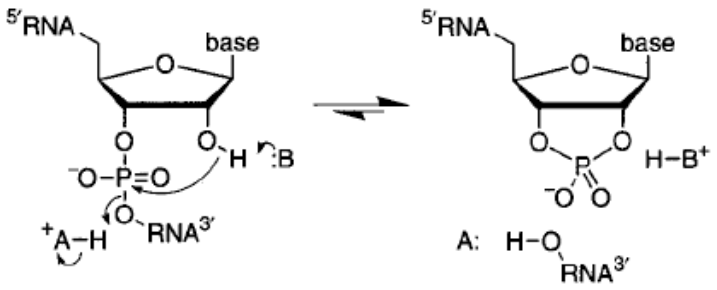

Figure N.1. Simplified scheme for the general acid/base-catalyzed hydrolysis of RNA. A pentavalent intermediate is formed along the pathway.

The hydrolysis of RNA is a pretty easy reaction to catalyze. A well-positioned nucleophile is steadily present at each position – the 2' OH group. All that is needed is a little encouragement and a transesterification reaction proceeds to yield a cyclic phosphate and a free 5' OH group following strand scission (Figure N.1). These notes will look at two catalysts for the process. One good (ribonuclease A). One bad (the HDV ribozyme).

Ribonuclease A

RNase A is the bane of every RNA biochemist's life. It is an incredibly good and non-specific catalyst for RNA hydrolysis and seems to find its way everywhere. The protein is also a rock. You can heat it to near boiling and it will refold to an active form. The common source of RNase A is beef pancrease. Due to their lifestyle, cows digest a lot of bacteria, which contain a lot of RNA.

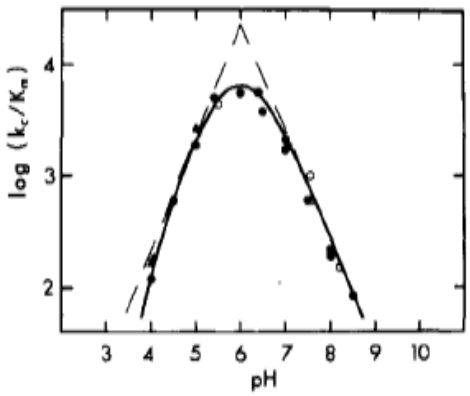

Figure N.2. Profile of k_{cat}/K_m of RNase A vs. pH. Note that the general acid and general base titrate to roughly the same pK_a. Adapted from Eftink & Biltonen (1983) *Biochemistry* **22**, 5123-5134.

The enzyme displays evidence of general acid base catalysis in its pH activity profile, with a peak in k_{cat}/K_m near pH 6. Both the general base (the acidic limb of the profile) and the general acid (the basic limb) titrate to a pK_a of roughly 6, indicating the presence of histidines in the mechanism. Kinetically, it's a pretty successful enzyme. The uncatalyzed reaction of hydrolysis takes place at pH 6 with a rate constant of 5×10^{-9} s^{-1}. The enzyme achieves a rate constant of 1400 s^{-1} and k_{cat}/K_m of 2.5×10^6 $M^{-1}s^{-1}$ (with UpA as a substrate).

The active site backs up the idea that histidines are involved in the mechanism (Figure N.3). Histidines 12 and 119 are well positioned to serve as the general base (12) and acid (119) implicated in the mechanism shown in Figure N.1.

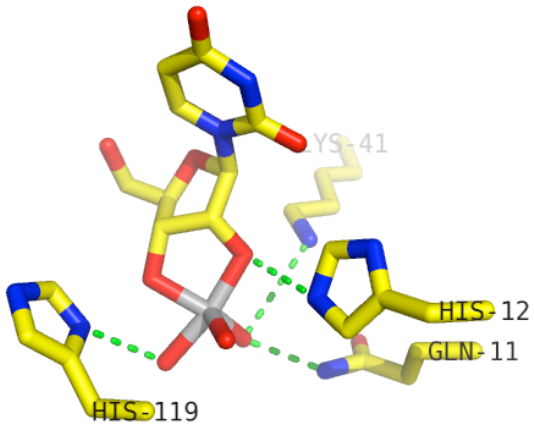

Figure N.3. Active site of RNAse A with vanadate inhibitor bound. The vanadate creates a pentavalent analog of the high energy phosphate intermediate. His12 is well positioned to act as a general acid and His119 as a general base. Gln11 and Lys41 provide important electrostatic stabilization of the intermediate via H-bonding.

The HDV Ribozyme – Bad Nuclease

Hepatitis delta virus is a human pathogen that possesses a single-stranded, circular RNA genome of about 1700 bases. It replicates itself inside the cell by the "rolling circle" mechanism. A cellular enzyme simply runs around the short circular piece of RNA indefinitely, creating an ever lengthening RNA strand that would ultimately, if not cleaved, contain many copies of the complementary sequence to the circular genome. However, the RNA molecule generated is capable of catalyzing its own cleavage and circularization at each copy to produce a large number of circular RNA molecules (Figure N.4). RNA catalysis was only identified in the 1980's but is now understood to be ubiquitous in nature.

The activity of the RNA in accelerating its own cleavage is not truly an enzymatic process since it only happens once per RNA molecule. However, a little clever engineering and the RNA can be separated into a catalyst strand (the ribozyme) and substrate (Figure N.5). By any other standard, the HDV ribozyme would not be considered much of a biological catalyst. It operates with a k_{cat} of

roughly 0.02 s^{-1} (six orders of magnitude lower than RNase A) and a k_{cat}/K_m of about 10^4 $M^{-1}s^{-1}$. But of course, in nature the ribozyme only needs to work once, so we shouldn't judge it too harshly.

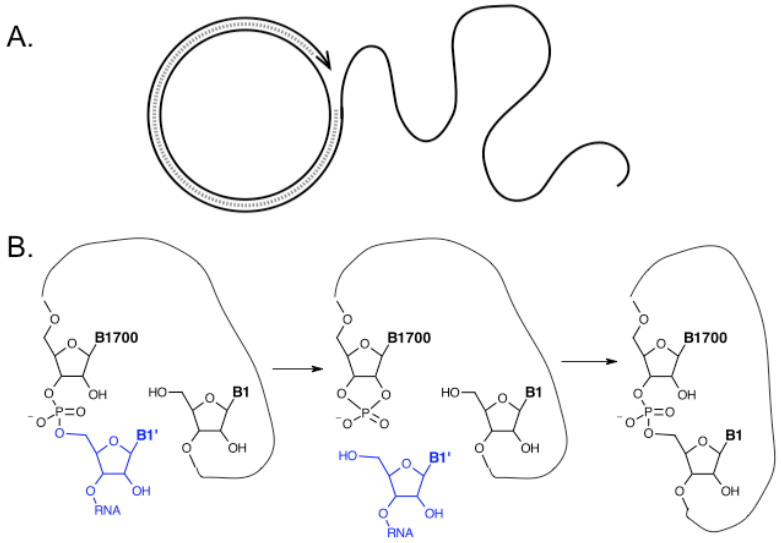

Figure N.4. (A) Rolling circle replication. Genome copying proceeds in a circular fashion creating multiple copies in an ever longer strand of RNA. (B) Transphosphorylation reaction leading to circular copies of the genome. The 3',5' phosphodiester bond between one copy of the genome (in black) and a second copy (in blue) is cleaved, leading to the formation of a cyclic phosphodiester at the 3' end of the black copy and freeing the 5' OH of the second copy (B1'). The first nucleotide of the black copy (B1) then performs nucleophilic attack, leading to the creation of a. phosphodiester linkage that closes the circular genome

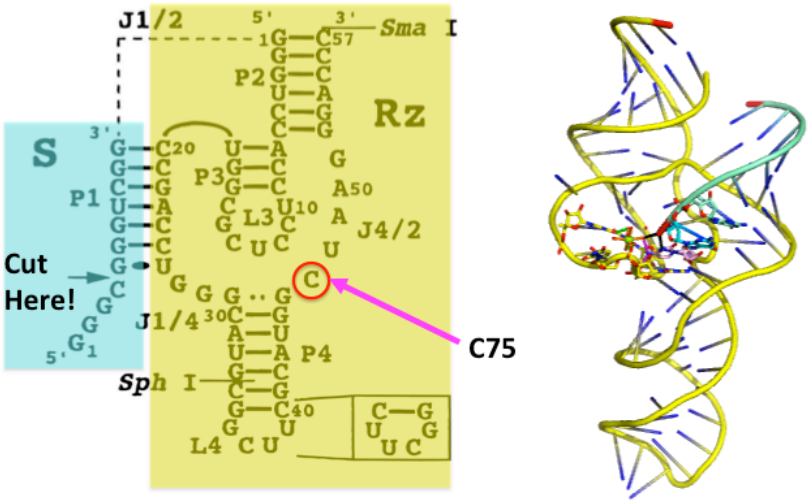

Figure N.5. Secondary and tertiary structures of the modified HDV ribozyme, showing substrate strand in light blue and the catalytic strand in yellow. The scissile phosphodiester is indicated in the substrate strand at left, and the catalytic cytosine (C75) is highlighted with a red circle, and is shown in pink in the 3D structure.

Crystal structures of the HDV ribozyme have been slow to reveal the secrets of catalysis, but there has long been evidence that the active site contains both a magnesium ion and a cytosine[1], both positioned to promote general acid/base catalysis (Figure N.6). The difficulty has been that a structure showing the pre-cleavage active site is ambiguous in positioning, leaving two competing mechanistic hypotheses. In one case N3 of cytosine is a general base and a water bound to Mg^{2+} (pK_a 11) is a general acid, and in the other the conjugate acid of cytosine (pK_a 4.2) is the general acid and a magnesium bound hydroxide is the general base (Figure N.7).

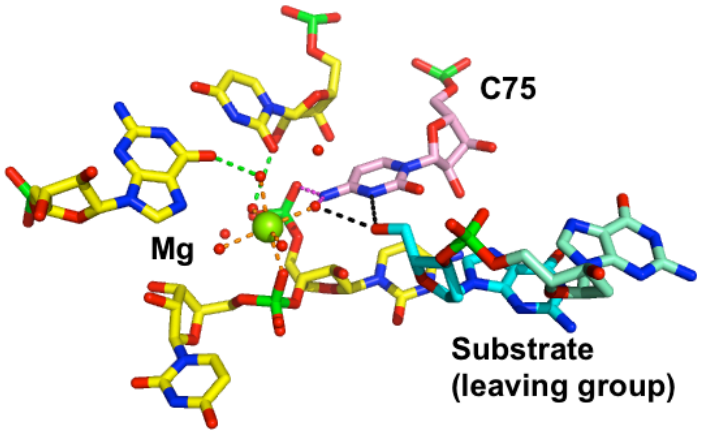

Figure N.6. Active site of the HDV ribozyme. The active site cytosine is shown in pink, and the active site Mg^{2+} is a green sphere with the coordinated waters shown as small red spheres. The portion of the substrate that is released following cleavage is shown in light blue. The portion forming a cyclic phosphate is not visible (hence the ambiguity in interpretation).

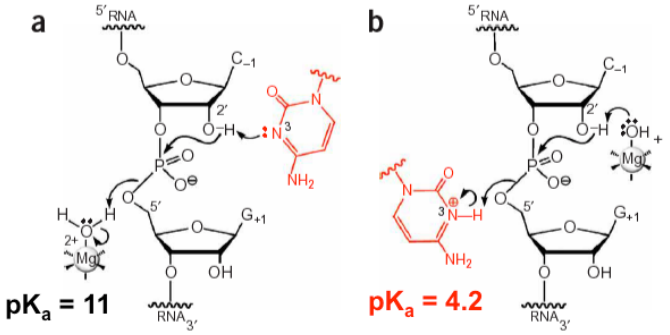

Figure N.7. Two mechanistic hypotheses for general acid base chemistry in the HDV ribozyme.

[1] The cytosine is either numbered 75 or 76 in the literature, depending upon the source of the ribozyme (for each genome there is an "anti-genome" that has its own ribozyme. You can look it up – it's more than I want to discuss here.

Each scenario in Figure N.7 is problematic. In N.7A, a very weak acid protonates the leaving group and a very weak base deprotonates the nucleophile. In N7.B both acid and base are stronger, but they are unlikely to exist at pH 7. An acid with pK_a near four is only present as 0.1% of the total pool of the ribozyme, and a base whose conjugate acid has a pK_a of 11 is only present in 0.01% of the total pool.

The Piccirilli lab investigated the general acid/base catalysis by the HDV ribozyme to ID which scenario (if either) is supportable.[2] The approach took advantage of a modified nucleotide in the substrate that permits strand cleavage without general acid catalysis. Cleavage of the regular substrate, with a hydroxyl leaving group shows a pH rate profile consistent with general acid base catalysis and with a general base whose conjugate acid's pK_a is about 7 and a general acid with a pK_a of 8. A 5'-thiodeoxynucleotide (Figure N.8A) can be cleaved efficiently at high pH without a general acid, because the thiolate leaving group is relatively stable. The two pK_a estimates derived from these data are interesting because they are each inconsistent with the groups involved – a Mg-bound water/hydroxide and cytosine 75.

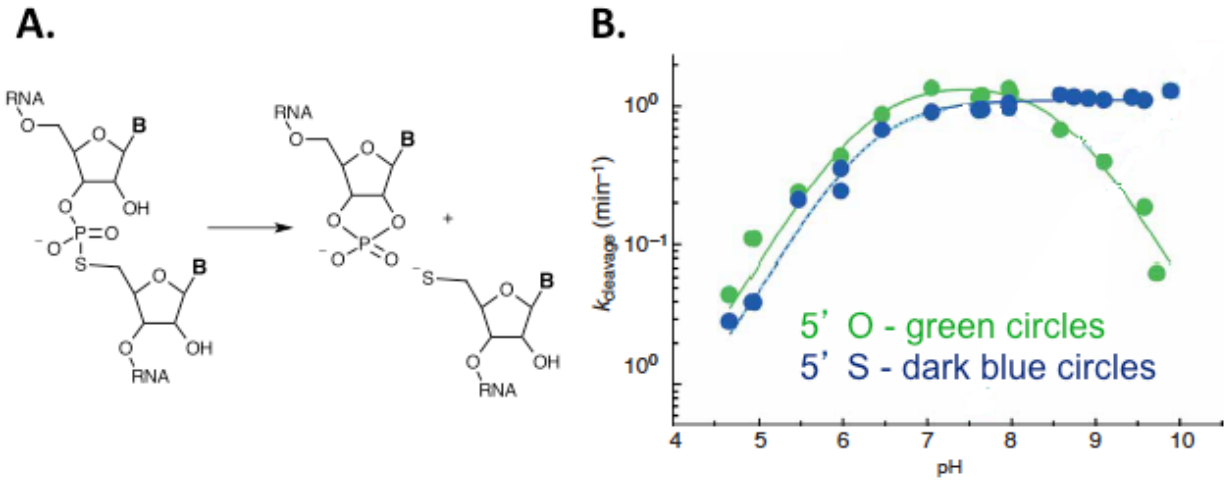

Figure N.8. (A) Thiosubstituted substrate that yields a thiolate leaving group. (B) pH rate profile. Note that cleavage of the oxy-substrate becomes slower at high pH, likely due to the loss of a general acid at high pH, whereas the thio-substituted substrate remains easily cleaved, since a general acid is not required.

Previous work had suggested that cytosine is the general acid (with a $pK_a > 8$?!). Substitution of C75 with uracil showed a similar loss of activity. But the problem is that uracil mucks with the catalytic site in multiple ways since the structure of uracil differs from cytosine at two positions (Figure N.9), and the keto group at C4 may disrupt the active site in unintended ways. So two substitutes were prepared that avoid the amino/keto swap between cytosine and uracil (Figure N.9).

[2] Das and Piccirilli (2005) *Nature Chemical Biology* **1,** 1-6.

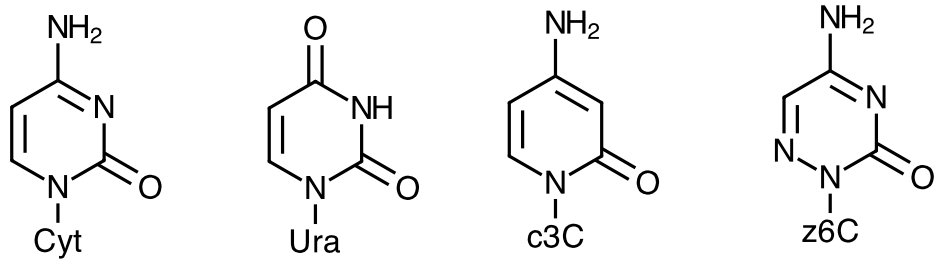

Figure N.9. Structures of cytidine, uracil, 3-carbacytosine (c3C) and 6-azacytosine (z6C). Note that c3C cannot act as a acid or base, while z6C is interesting because its conjugate acid has a lower pK_a (2.6) than cytosine's conjugate acid (4.2).

The use of c3C supports the role of C75 as a general acid. The thio-substituted substrate continues to be cleaved with a pH rate profile similar to that in Figure N.8, but the natural, 5'-hydroxy substrate is not cleaved by the ribozyme at any pH, suggesting that c3C is failing to protonate the leaving group, rather than failing to deprotonate the nucleophile.

The use of z6C is even more interesting. Its conjugate acid has a pK_a (2.6) lower than that of the conjugate acid of cytosine (4.2). The pH rate profile with this modification is significantly different between the two substrates. The activity with the hydroxyl substrate is significantly depressed, while the thio-substrate shows rising activity from about pH 4-10. Note that with the WT cytosine at position 75, both the thio and hydroxyl substrates show comparable activity up to about pH 7 (Figure N.8). However, with z6C at position 75, the curves depart from one another at a lower pH (Figure N.10). A simple explanation for this observation is that an inhibiting titration (loss of an acidic group as pH increases) is taking place at a lower pH that previously.

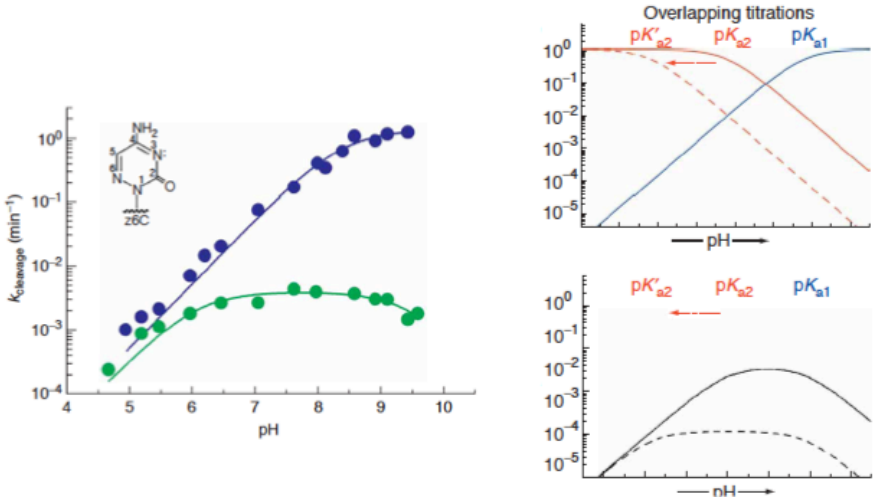

Figure N.10 pH rate profiles with thio (blue dots) and hydroxyl (green dots) substrates. The Decline in activity of the hydroxyl substrate with the z6C substitution can be ascribed to the lower pK_a of the general acid (see panels at right). Note, this assumes that the pK_a of the general acid is lower than the pK_a of the conjugate acid of the general base.

Note that these results are consistent with a mechanism in which C75 is the general acid (with a pK_a of about 4) and the Mg^{2+} bound hydroxide is the general base (conjugate acid with pK_a about 11). That means at pH 7, few molecules of the ribozyme are fully active, since very few (0.1% of 0.01%) have both species in the desired protonation state. As Jennifer Doudna has comments, ribozyme catalysis is not different – just worse.