Biochemical detection of adenosine and cytidine ionization within RNA by interference analysis

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ABSTRACT
Perturbation of active site functional group pKₐs is an important strategy employed by protein enzymes to achieve catalysis. There is increasing evidence to indicate that RNAs also utilize functional group pKₐ perturbation for folding and reactivity. The two best candidates for a functionally relevant pKₐ perturbation are the N3 of C (pKₐ = 4.2) and the N1 of A (pKₐ = 3.5), either of which could be sufficiently raised to allow protonation near physiological pH. Here we report the synthesis and use of a series of α-phosphorothioate tagged cytidine and adenosine analogs whose altered pKₐs make it possible to efficiently detect functionally relevant protonation events by Nucleotide Analog Interference Mapping. This approach has been used to detect ionization events in several catalytic RNAs, including the group I intron, the Hepatitis Delta Virus (HDV), the hairpin and the Varkud Satellite (VS) ribozymes. The active site residue of the VS ribozyme appears to be ionized in the course of the reaction pathway, which may be indicative of a general acid or base mechanism for catalysis by this RNA.

INTRODUCTION
The Neurospora Varkud Satellite (VS) ribozyme is a naturally occurring self-cleaving RNA found in the mitochondria of certain isolates of the species Neurospora crassa (1). It catalyzes a reversible self-cleavage reaction that generates 5'-OH and 2', 3'-cyclic phosphate products (2). The reaction involves nucleophilic attack of the scissile phosphate by the neighboring 2'-oxygen and displacement of the 5'-oxygen. The active structure of the VS ribozyme is contained within 154 nucleotides that fold into six helices (I-VI) (3). A seventh helical element (VII), formed by complementary nucleotides 5' of the cleavage site and 3' of the ribozyme sequence, is important for the ligation activity of the RNA (4). Mutational studies show that the 730 loop, an asymmetric bulge within helix VI, is a critical component of the ribozyme active site (5). Of particular importance is nucleotide A756, which is the nucleotide most sensitive to mutation or modification. Substituting A756 with any base (C, G or U) results in catalytic rate reductions greater than 300-fold (6; 7). Deletion or modification of the base significantly reduces the catalytic rate (>900 fold), though removal of the 2'-OH group from A756 reduces the rate of cleavage only 10 fold (8). While mutation of A756 reduces the reaction rate, it does not appear to affect folding or substrate binding (7). These results suggest an important catalytic role for A756 in VS RNA activity.

Other catalytic RNAs, which perform phosphotransfer reactions equivalent to that of the VS ribozyme, are proposed to utilize a general acid base mechanism involving ionization of active site residues (9-11). It is possible that the VS ribozyme may also employ an ionized base to facilitate catalysis, but there is little or no evidence in support of this hypothesis. To explore this possibility, we employed a series of adenosine and cytidine analogs with N1 and N3 perturbed pKₐ values, respectively, in nucleotide analog interference mapping (NAIM). These analogs make it possible to simultaneously, yet individually, assay for functionally important base ionization at every A or C residue in an RNA. Each of the analogs is prepared as a triphosphate for transcriptional incorporation into an RNA and tagged with an α-phosphorothioate linkage, a bond that can be cleaved with iodine to reveal the position of analog incorporation within the RNA polymer. These analogs were instrumental in the identification of a protonated C (C300) important for folding of group I introns, ionization of a C (C75) in the active site of the genomic HDV ribozyme, and the ionization of an A (A10) in the active site of the hairpin ribozyme (9; 12; 13). They are ideally suited to assay for base ionization within the VS ribozyme.

RESULTS AND DISCUSSION
Seven nucleotide analogues were used to explore base ionization within the ribozyme. This included four adenosine and three cytidine analogues, specifically: 8-aza-adenosine (n₈A, pKₐ = 2.2), formycin A (FormA, pKₐ = 4.4), purine riboside (Pur, pKₐ = 2.1), and 7-deaza-adenosine (7dA, pKₐ = 5.2) for adenosine; 6-azacytidine (n₆C, pKₐ = 2.6), 5-fluorocytidine (FC, pKₐ = 2.3) and
pseudouridine (ΨC, pKₐ=9.4) for cytidine. We employed a ligation-based NAIM assay using the self-ligation construct VSE, to probe important functional groups throughout the VS RNA. Of the 171 nucleotides in this construct, 142 nucleotides (G640-A781) were informative for the assay.

Each of the As and Cs within the VS sequence were assayed for ionization using this series of nucleotide analogues. None of the Cs within the VS sequence displayed an interference pattern consistent with ionization. However, the interference pattern using the adenosine analog series indicated that A756 undergoes ionization during the ligation reaction. The data to support this were as follows. At pH 7, n⁴A and Pur incorporation caused interference at A756. Both of these nucleotides have acidic pKₐs compared to adenosine. By contrast, both nucleotides with elevated pKₐs, FormA and 7dA, caused enhanced ligation activity when incorporated at A756. To further test if the interferences at A756 was due to base ionization, we performed the NAIM assays at pHs ranging from 8.0 to 5.4. n⁴A interference at A756 persisted at pH 7.0 and 6.0, however, at pH 5.4 the interference was essentially eliminated. Similarly, the A756 Pur interference observed at pH 7.0 was rescued at pH 5.4. Thus, both the pKₐs perturbed adenosine analogs demonstrated a pH dependent interference pattern at A756. The specificity of the effect was tested using dA, an analog that does not have a reduced pKₐ, but causes interference at A756. dA interference at pH 7.0 was rescued at pH 5.4. Thus, both the pKₐs remain too low to have much effect on a reaction proceeding efficiently at neutral pH (15).

If A756 is ionized, as suggested by these data, then how might protonation of this adenosine promote the reaction of the VS ribozyme? There are several lines of evidence that suggest that A756 protonation plays a catalytic, rather than a structural role in the VS reaction. First, mutation of A756 to any other base results in drastic reductions in self-cleavage activity (6; 7), but structural comparison of wild type and mutant VS constructs by FRET indicate that mutation of A756 does not affect the global folding of the VS RNA. Second, A756 variants bind substrate efficiently (16). Third, 4-thioU cross-linking experiments pinpoint A756 as being in close proximity to the cleavage site (17). Fourth, a FRET-derived structural model of the VS ribozyme generated by Lilley and coworkers places A756 near the substrate helix, where it can make significant tertiary interactions with nucleotides. Of the 171 nucleotides in the active site and to its ability to be ionized at some point in the reaction pathway.

REFERENCES