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A Transition-State Interaction Shifts Nucleobase Ionization toward Neutrality To Facilitate Small Ribozyme Catalysis

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Supporting Information

ABSTRACT: One mechanism by which ribozymes can accelerate biological reactions is by adopting folds that favorably perturb nucleobase ionization. Herein we used Raman crystallography to directly measure pK_a values for the Ade38 N1 imino group of a hairpin ribozyme in distinct conformational states. A transition-state analogue gave a pK_a value of 6.27 ± 0.05, which agrees strikingly well with values measured by pH−rate analyses. To identify the chemical attributes that contribute to the shifted pK_a, we determined crystal structures of hairpin ribozyme variants containing single-atom substitutions at the active site and measured their respective Ade38 N1 pK_a values. This approach led to the identification of a single interaction in the transition-state conformation that elevates the base pK_a > 0.8 log unit relative to the precatalytic state. The agreement of the microscopic and macroscopic pK_a values and the accompanying structural analysis supports a mechanism in which Ade38 N1(H)+ functions as a general acid in phosphodiester bond cleavage. Overall the results quantify the contribution of a single electrostatic interaction to base ionization, which has broad relevance for understanding how RNA structure can control chemical reactivity.

RNA plays a central role in several key biocatalytic reactions including noncoding RNA processing, protein translation, and RNA splicing.[1−6] Distinct strategies identified in the protein world have been posited to account for this catalysis, including electrostatic complementation and general acid−base catalysis.[7−10] These strategies require base ionization, which necessitates the generation of an imine with a pK_a near neutrality.[10] However, unlike proteins, there are no chemical groups in RNA that ionize near physiological pH, requiring the evolution of microenvironments that favorably perturb pK_a values.[11] Although methods have been developed for measuring nucleotide base pK_a values in a ribozyme, no study has quantified how specific stereochemical interactions modulate pK_a shifting.[12] These knowledge gaps hinder efforts to model RNA structure−function relationships, including rigorous computational treatment of folding and catalysis.[13] Here, we used Raman crystallography, X-ray diffraction, and mutagenesis to pinpoint how specific functional groups influence the pK_a of an essential imino group in the active site of an RNA enzyme known as the hairpin ribozyme (HPRZ), which is a model system for studying the architecture and function of RNA-mediated phosphoryl transfer.

The HPRZ is a member of the autolytic, small-ribozyme class.[8] It is derived from the negative-polarity strand of the satellite tobacco ringspot virus where it processes concatenated transcripts to unit length.[14] Like other small ribozymes, its chemical mechanism involves nucleophilic attack of the O2′ group from position −1 upon the scissile phosphorus at position +1 (Figure 1A). Unlike larger self-splicing ribozymes, the HPRZ does not require divalent ions for activity,[8] making it ideal for investigating nucleobase-assisted catalysis. Indeed, the HPRZ pH−rate profile shows an apparent pK_a of 6.2 ± 0.2, consistent with a single ionizable group.[8,15]

A synthetic variant of the HPRZ was developed in our lab for single-atom probing and structure−function investigations [Figures 1B and S1, Supporting Information (SI)]. Crystallographic analyses of this minimal construct in the context of precatalytic and transition-state analogues revealed Gua8 and Ade38 adjacent to the scissile bond (Figure 1B,C).[16] A functional analysis in which Gua8 was made abasic caused an 850-fold reduction in cleavage without altering the pH−rate profile.[17] By contrast, abasic replacement of Ade38 caused a 14 000-fold activity loss and a 3 log units shift of the apparent pK_a toward the basic.[15] The subtle exchange of Ade38 for N1-deaza-Ade also resulted in complete loss of detectable activity,[18] whereas similar active-site modifications at nearby Ade9 and Ade10 had modest effects.[19] These data indicate that the N1 imino of Ade38 has a critical role in catalysis, and an elevated N1 pK_a was inferred.

Because the Ade38 protonation state figures prominently in any proposed HPRZ mechanism, we previously used Raman crystallography to measure directly the N1 pK_a of Ade38 in the context of an inert precatalytic analogue (PCA) (Figure 1C). The results revealed a pK_a of 5.46 ± 0.04.[20] Although this value is elevated relative to AMP control measurements of 3.68 ± 0.06, it did not agree with the apparent reaction pK_a of 6.2 ± 0.2, which reports on transition-state proton transfer.[9] We hypothesized that an active-site conformation closer to the transition state would fine-tune the Ade38 N1 pK_a and bring the microscopic pK_a into better agreement with the apparent

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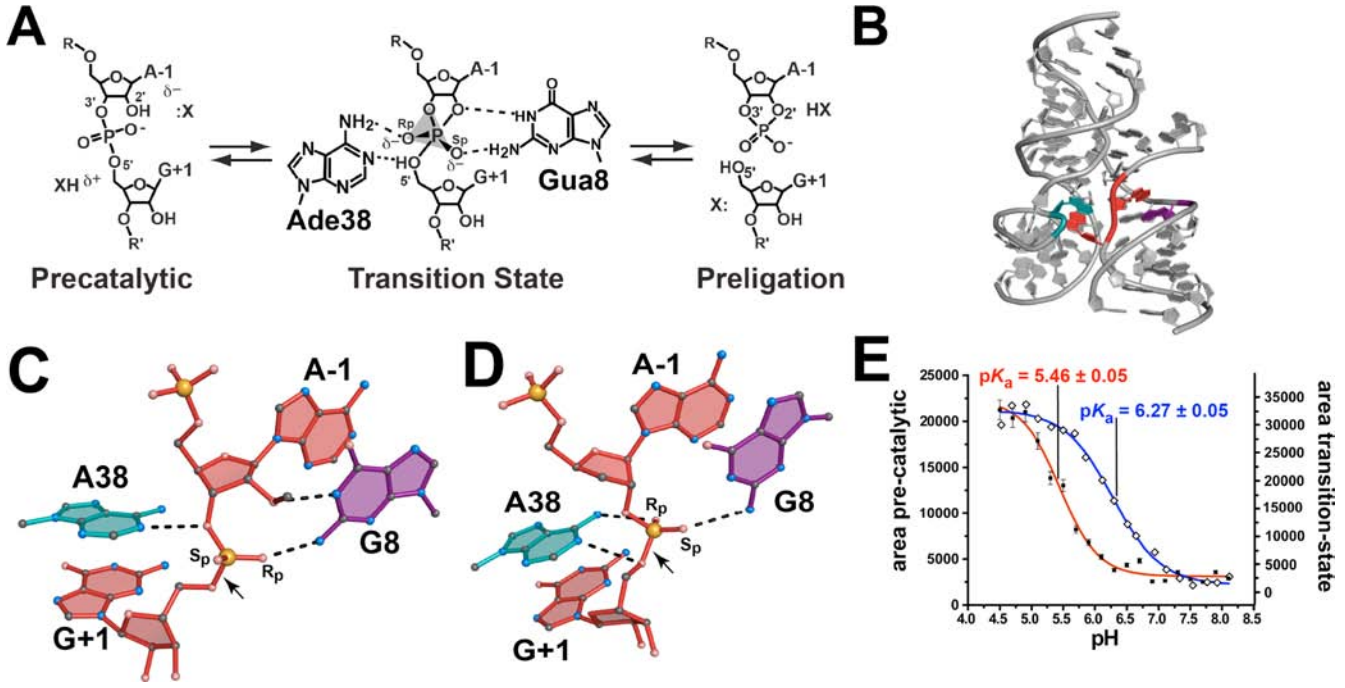

Figure 1. Chemical reaction of small ribozymes, HPRZ active site organization, and directly measured microsopcic pK_a values for the Ade38 N1 imine. (A) HPRZ reaction entails deprotonation of the Ade −1 2′-OH and attack on the neighboring phosphate of Gua +1. The reaction proceeds via a phosphorane transition state that is favored by protonation of the O5′ leaving group and electrostatic stabilization of the negatively charged phosphorane. The products are a cyclic 2′,3′-phosphodiester and a free 5′-hydroxyl. (B) Three-dimensional structure of a "minimal" synthetic HPRZ (PDB: 2OUE) with Ade −1 and Gua +1 in red; Ade38 is teal and Gua8 is maroon. (C) Ball-and-stick diagram of the active site of the HPRZ PCA reported previously in the presence of a 2′-*O*-Me cap that blocks activity[16] (PDB: 2OUE). Here and elsewhere a black arrow indicates the scissile bond. (D) Active site of a TSA of the HPRZ reported previously with a 3′-deoxy-2′,5′-phosphodiester linkage between Ade−1 and Gua +1[21] (PDB: 2P7F). (E) Raman crystallography results showing the area under the peak at 734 cm^{-1}, which reports on protonation of Ade38 N1, between pH 4.5 and 8.5. The red line shows the titration of a PCA HPRZ described previously;[20] the blue line shows the TSA Raman crystallography titration from the investigation herein.

pK_a of the reaction. To test this possibility, we utilized a transition-state analogue (TSA) to restrain the nonbridging oxygens of the scissile bond into a geometry consistent with the proposed transition state[21,22] (Figure 1D). Using Raman crystallography we measured a pK_a of 6.27 ± 0.05 for N1 of Ade38 in the context of the TSA, which represents a 0.81 log unit increase over the PCA value (Figure 1E and Table 1). This

Table 1. Direct Measurements of Ade38 N1 pK_a Values

sample	PCA	TSA
wild-type	5.46 ± 0.05	6.27 ± 0.05
Ade38Pur	5.00 ± 0.06 (measured)[a]	4.88 ± 0.06 (measured)[a]
	5.76 (corrected)[b]	5.66 (corrected)[b]
Gua8Ino	5.36 ± 0.08	5.45 ± 0.09

[a]Measured values are derived directly from Raman experiments. [b]Corrected value is obtained by adding 0.78 log units to the pK_a values of the respective Ade38Pur PCA and TSA variants. The value of 0.78 represents the difference between the solution pK_a values measured by Raman spectroscopy for the respective AMP control, with a value of 3.68 ± 0.06,[20] and the 2′-deoxynebularine control with a value of 2.92 ± 0.04 (Figure S3).

microscopic pK_a is identical to the apparent pK_a of the reaction,[15] thereby providing direct evidence that the N1 imino of Ade38 is the source of rate-limiting proton transfer.

To understand the molecular basis for the ∼1 log unit difference between the PCA and TSA ionization constants, we compared their corresponding structures, which were derived previously from crystals of the same space group as those used for Raman measurements in Figure 1E. Two main differences were apparent in the active site. Whereas the TSA exhibits hydrogen bonds between the *pro*-R_p oxygen equivalent and the N6 amine of Ade38, as well as the O5′ leaving group and the Ade38 N1 imine, these interactions are missing in the PCA (Figure 1D versus 1C). By contrast, both active sites showed interactions between one nonbridging oxygen of the scissile bond and the N2 amino group of Gua8. To determine the importance of hydrogen bonding between the *pro*-R_P oxygen of the scissile bond and the N6 group of Ade38 for pK_a shifting, we substituted a purine nucleotide at position 38 that is devoid of the exocylic amine, and the N1 pK_a was measured. The results revealed a PCA with a pK_a value of 5.00 ± 0.06 (Table 1 and Figure S2A). By contrast, the control pK_a for 2′-deoxynebularine, in which N6 is a hydrogen, was 2.92 ± 0.04 (Figure S3), indicating a 2.08 log unit shift toward the basic in the context of the HPRZ active site. Application of the latter shift to AMP gave a pK_a of 5.76, which is comparable to the wild-type PCA pK_a (Table 1). To assess how the Ade38Pur substitution influences the active site geometry, we determined its crystal structure (Table S1), which shows an active site conformation similar to wild-type (Figure 2A). The phosphate group at the scissile bond adopted two conformations: one is similar to wild-type, and the other is rotated such that the *pro*-R_p oxygen cannot hydrogen bond with N2 of Gua8. Neither of these conformations supports a hydrogen bond between the O3′ and N1 of position 38, as seen for the wild-type.

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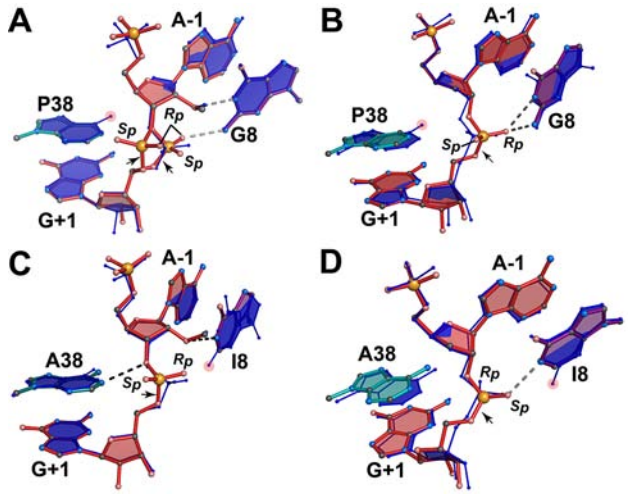

Figure 2. Superpositions of HPRZ active sites comparing wild-type structures to Ade38 or Gua8 variants in the context of PCA or TSA conformations. Structures of the base variants are depicted in red, green and purple with the wild-type structure in blue; for clarity, the atom present in the wild-type but absent in the base variant is highlighted in red. Hydrogen bonds (dashed lines) are indicated only for the Ade38 and Gua8 variants. (A) The PCA with an Ade38Pur substitution exhibits two scissile bond conformations. (B) The TSA with an Ade38Pur substitution. (C) The PCA with a Gua8Ino substitution reported previously[16] (PDB: IZFT). (D) The TSA with a Gua8Ino substitution.

We then asked how loss of the N6 amine at position 38 would affect its N1 pK_a in the context of the TSA. The Ade38Pur variant produced a pK_a of 4.88 ± 0.06 (Table 1 and Figure S2B), representing a 2.0 log units shift toward the basic relative to wild-type. When this shift is applied relative to the controls, the Ade38 N1 pK_a is 5.66, which is significantly less than the pK_a of 6.27 ± 0.05 measured for the wild-type TSA (Table 1). To understand how the Ade38Pur variant influences the HPRZ active site in the context of the TSA, we determined its crystal structure (Table S1). The results showed that the *pro*-R_P oxygen of the scissile bond alters its conformation to be within hydrogen bonding distance of the N1 and O6 groups of Gua8 (Figure 2B). This represents a significant loss of scissile bond localization, and documents the extent to which the interaction between the *pro*-R_P oxygen and the N6 amine of Ade38 contributes to imine pK_a shifting. Our pK_a measurements and structural results are consistent with reports of Ade38Pur activity loss ranging from 10^2- to 10^3-fold.[15,23]

We next asked whether the N2 group of Gua8 influences Ade38 ionization. Structures of the wild-type HPRZ indicated that hydrogen bonding occurs between the exocyclic amine of Gua8 and the scissile bond in both PCA and TSA conformations (Figure 1C,D). To assess the importance of these interactions we replaced Gua8 with inosine, which lacks an exocyclic amine. In the context of the PCA, the N1 pK_a for Ade38 was 5.36 ± 0.05 (Figure S2C), which is nearly identical to wild-type (Table 1). A prior PCA structural comparison of the Gua8Ino variant to wild-type revealed only minor structural changes localized to the scissile bond[16] (Figure 2C) even though the hydrogen bond is absent between the *pro*-R_P oxygen and the N2 amine of Gua8.

By contrast, the Gua8Ino substitution in the context of the TSA had a significant impact on Ade38 ionization via an indirect effect that repositioned the scissile phosphate. Our results revealed a pK_a of 5.45 ± 0.09, which represents a 0.82 log unit decrease compared to wild-type (Figure S2D and Table 1). We determined the crystal structure of this variant in the context of the TSA (Table S1) and compared it to wild-type. The superposition indicates very subtle changes in the overall structure but a significant change in the hydrogen-bonding pattern to the scissile bond. In particular, the *pro*-S_P oxygen—unable to form a hydrogen bond to N2 of Ino8—forms a new hydrogen bond to N1 (Figure 2D). This change shifts the *pro*-R_P oxygen location so that it is unable to hydrogen bond with N6 of Ade38. Consequently, the O5′ leaving group is misoriented so that it does not interact readily with the Ade38 imine, consistent with the >10-fold loss in k_{cat} for the Gua8Ino variant.[24]

Here we have shown that the microscopic pK_a of Ade38 N1, in the context of a TSA, is identical to the apparent pK_a measured for the cleavage reaction for the most active, four-way helical junction form of the HPRZ.[15] This agreement provides strong evidence that the N1 imine of Ade38 is involved in rate-limiting proton transfer. Independent evidence that the protonated form of Ade38 is operative in catalysis is supported by substitution of the base with 2-fluoro adenosine (pK_a < 1), which significantly reduces activity.[25] Simulated pH−rate profiles that assumed a pK_a-shifted Ade38 and an unperturbed Gua8 pK_a appeared similar to experimental pH−rate profiles, supporting the plausibility of general acid/base catalysis.[10] Herein, we also visualized HPRZ active sites that retained functional characteristics of the overall reaction, thereby allowing us to draw stereochemical inferences about the mechanism. Significantly, TSA crystal structures containing either vanadate[21,22] or a 2′,5′ linkage at the scissile bond[21]—identical to that employed here—do not show water localized at the N1 imine of Ade38, as proposed in a prior N1 imino pK_a analysis.[26] As such, our observations strongly support a general acid role for Ade38. Similar roles have been ascribed previously to base Cyt75 of the hepatitis delta virus ribozyme[27] and Ade756 of the Varkud satellite ribozyme.[28] We anticipate that when TSA structures become available for other small ribozymes, pK_a-shifting interactions will be exhibited that are similar to those observed here. Our experimental analysis also provides a quantitative result that should be useful for computational biologists seeking ways to benchmark pK_a prediction algorithms.

■ ASSOCIATED CONTENT

Supporting Information

Methods, the diffraction table, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED IN PROOF

While this manuscript was in review, a complementary work was published that describes the use of phosphorothioate leaving groups and base mutants, such as Ade38Pur, which strongly supports our conclusion that Ade38 (H)+ functions as a general acid catalyst in cleavage.[29]