

Philip C. Bevilacqua<sup>1,2</sup>

Trevor S. Brown<sup>1,2</sup>

Shu-ichi Nakano\*

Rieko Yajima<sup>1,2</sup>

<sup>1</sup> Department of Chemistry,  
The Pennsylvania State  
University,  
University Park, PA 16802

<sup>2</sup> The Huck Institutes of the  
Life Sciences,  
The Pennsylvania State  
University,  
University Park, PA 16802

Received 29 August 2003;  
accepted 29 August 2003

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## Catalytic Roles for Proton Transfer and Protonation in Ribozymes

**Abstract:** Utilization of proton transfer in catalysis, which is well known in the mechanisms of protein enzymes, has been described only relatively recently for RNA enzymes. In this article, we present a current understanding of proton transfer by nucleic acids. Rate enhancement and specificity conferred by general acid–base catalysis are discussed. We also present possibilities for electrostatic catalysis from general acids and bases as well as cationic base pairs. The microenvironments of a large RNA provide the possibility of histidine-like  $pK_a$ s for proton transfer, as well as lysine- and arginine-like  $pK_a$ s for electrostatic catalysis. Discussion on proton transfer focuses on the hepatitis delta virus (HDV) and hairpin ribozymes, with select examples drawn from the protein literature. Discussion on electrostatic catalysis also draws on these two ribozymes, and a postulate for electrostatic catalysis by a cationic base pair in the mechanism of peptidyl transfer in the ribosome is presented. We also provide a perspective on possibilities for phosphoryl transfer mechanisms involving phosphorane intermediates and unusual tautomeric forms of the bases. Lastly, a distinction is made between ground state and “transition state”  $pK_a$ s. We favor a model in which changes in pH lead to changes in the distribution of reactive and nonreactive ionizations of the ribozyme molecules in the ground state, and therefore suggest that “ $pK_a$  changes in the transition state” do not provide an acceptable explanation for observed pH-rate profiles. © 2003 Wiley Periodicals, Inc. *Biopolymers* 73: 90–109, 2004

**Keywords:** ribozyme mechanism; ribosome mechanism; general acid–base catalysis; electrostatic catalysis;  $pK_a$  shift; tautomer; phosphorane

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Correspondence to: Philip C. Bevilacqua; email: pcb@chem.psu.edu

Contract grant sponsor: NSF, a Camille Dreyfus, and Sloan (to PCB); NIH and Sloan (to TSB); and Natural Science & Engineering Research Council (NSERC) of Canada (to RY)

Contract grant number: MCB-9984129 (NSF)

\*Present address: High Technology Research Center, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan

*Biopolymers*, Vol. 73, 90–109 (2004)

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## INTRODUCTION

The two defining characteristics of enzymes are specificity and rate acceleration.<sup>1,2</sup> The focus of this article will be on the contributions that protonation events can make to the specificity and rate acceleration of RNA enzymes, or ribozymes. Most commonly, reaction rates are interpreted using transition state theory. Transition state theory states that a reaction rate is accelerated by lowering the activation free energy,  $\Delta G^\ddagger$ , of the reaction.<sup>3</sup> According to transition state theory, enzymes operate by either lowering the energy of the transition state or raising the energy of the ground state. Linus Pauling proposed that enzymes function by binding their transition states more tightly than their ground states<sup>4</sup>; indeed, RNA enzymes appear capable of doing this, as evidenced by a recent crystal structure of the hairpin ribozyme in complex with vanadate (see Ref. 5 and accompanying article in this issue<sup>6</sup>). There have been numerous mechanistic studies on RNA enzymes that are consistent with rate acceleration by ground state destabilization, as well.<sup>7–10</sup>

A multitude of catalytic strategies have been advanced for understanding how enzymes modulate the  $\Delta G^\ddagger$  gap including approximation, covalent catalysis, electrostatic catalysis, metal ion catalysis, desolvation, strain, orbital steering, and general acid–base catalysis.<sup>1,2</sup> Enzymologists have provided numerous illustrations of these mechanistic features in protein and nucleic acid enzymes alike (reviewed in Ref. 11). Extensive efforts in assaying the quantitative contributions of each strategy have also been made. However, because of the cooperative and nonadditive nature of energetics in biopolymers, it can be difficult to accurately assess quantitative contributions of a particular catalytic strategy. For example, it is difficult to make a change—typically a single mutation—that affects one catalytic process without perturbing another. This issue is discussed herein with regards to several ribozymes, and the interested reader is referred to two outstanding discussions on this topic.<sup>12,13</sup> Nonadditivity is a key issue to understanding the complexity of energetics in nucleic acids and can manifest itself in systems as simple as a stable DNA hairpin.<sup>14</sup>

The focus of this article will be on the unique roles that proton transfer by and protonation of the nucleobases can play in the reactions of catalytic nucleic acids. It is only recently that the direct involvement of the nucleobases in catalysis has been appreciated. In particular, a paradigm for direct involvement of  $\text{Mg}^{2+}$  ions in phosphoryl transfer has been established in the mechanisms of the much larger group I and II introns.<sup>15–17</sup> In contrast, divalent metal ions appear to

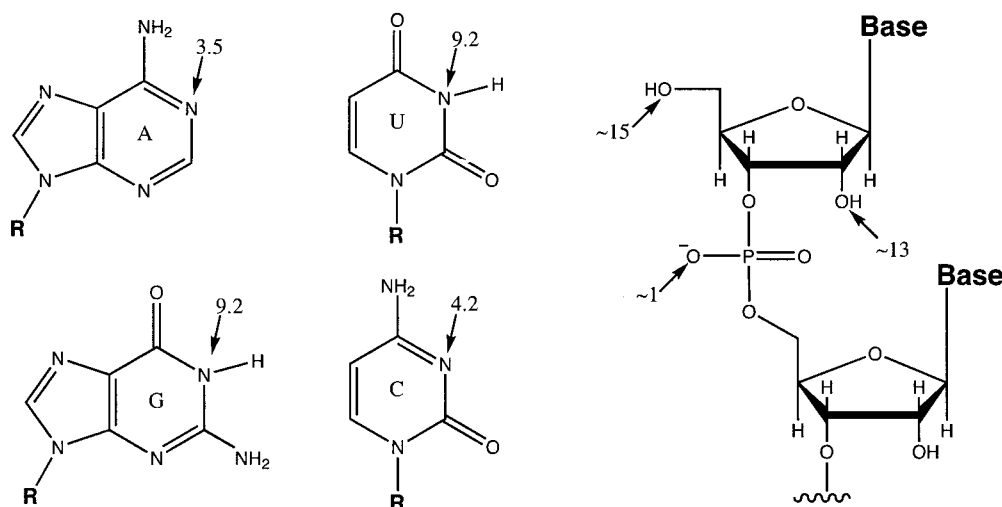
play much lesser roles in the mechanisms of small ribozymes. For example, the hairpin ribozyme reacts in the absence of divalent ions,<sup>18–20</sup> as do the hammerhead, VS<sup>21</sup> and hepatitis delta virus (HDV) ribozymes.<sup>9,22</sup> Quantitative studies have revealed only modest, 10- to 20-fold, contributions of divalent ions to rate acceleration for the HDV and hairpin ribozymes.<sup>22–24</sup> In addition, the structures of the reactant, transition state, and product forms of the hairpin ribozyme<sup>5,6,25</sup> do not show any metal ions at the active site. Likewise, the structure of the cleaved form of the HDV ribozyme does not show any ions strongly bound at the active site<sup>26,27</sup>—although a hydrated ion has been implicated to provide a modest contribution to catalysis under physiological ionic conditions.<sup>9,22,28–30</sup> Curiously, an ammonium ion may act as a general acid catalyst in the hammerhead ribozyme mechanism.<sup>31</sup> The absence of critical roles for divalent ions in the mechanisms of small ribozymes is suggestive of more direct catalytic roles for the nucleobases. In addition, no metal ions have been found near the active site of 23S rRNA in the ribosome, which suggests roles for the nucleobases in catalysis by the ribosome.<sup>32</sup>

Proton transfer as a catalytic strategy is of special interest since it can contribute to both rate acceleration and specificity. We argue that proton transfer, as well as protonation of the ground state itself, can make significant contributions to rate acceleration, although it can be difficult to quantitate these contributions because of the aforementioned nonadditivity. Perhaps more significantly, proton transfer may make ribozyme reactions more specific and even lead to new mechanisms. Last, we make a distinction between the effects of protonation on the distribution of ribozyme molecules over functional (i.e., reactive) and nonfunctional ionizations in the ground state, as compared to changes in the intrinsic reactivity of the functional state conferred by the presence of general acid–base and electrostatic catalysis. Before examining these issues, we will discuss some chemical and physical characteristics of RNA that provide a useful background.

## CONSIDERATIONS OF THE CHEMICAL MAKEUP OF RNA FOR PROTON TRANSFER AND PROTONATION

### Implications for Rate Acceleration

Initial consideration of the chemical nature of RNA might suggest that it is an unlikely candidate for



**FIGURE 1** Nitrogenous nucleobase and sugar–phosphate backbone  $pK_a$ s. (Left) The four nucleobases in their typical tautomeric forms that lead to Watson–Crick base pairing. The  $pK_a$  values for the imino protons and the protonation states dominant at pH 7.0 of adenosine (A), uridine (U), guanosine (G), and cytidine (C) ribonucleotides are shown.<sup>33,132</sup> (Right) Representative sugar–phosphate backbone. Average  $pK_a$ s of ionizable groups are marked. The  $pK_a$  of the 2'-hydroxyl is dependent on the base identity and has been estimated to be between 12.5 and 14.9 by NMR experiments and theory.<sup>57–59</sup> The negative charge on the nonbridging phosphate oxygens is delocalized, yielding an acidic  $pK_a$  of  $\sim 1$ , which is affected only slightly by oligomerization.<sup>33</sup> Due to its high  $pK_a$ , the 5'-oxygen is believed to require stabilization, such as by metal ions or a general acid, to assist its function as an oxyanion leaving group.

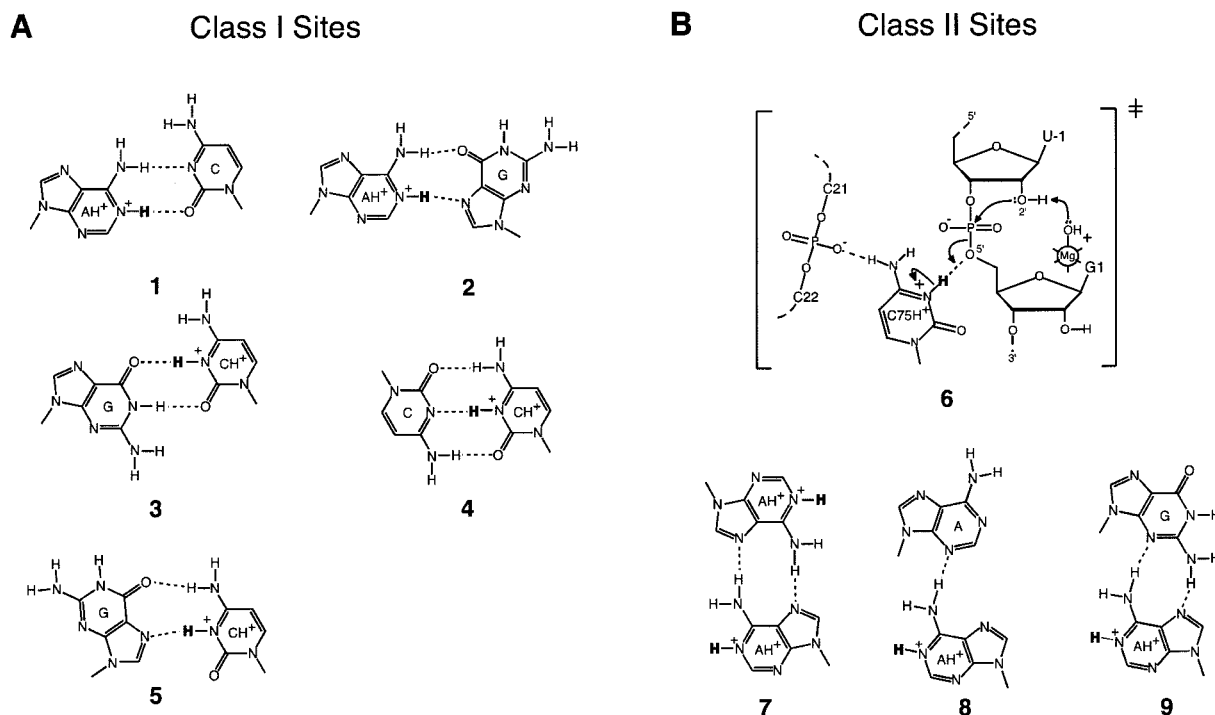
proton transfer. RNA consists of only four side chains, which are highly similar heterocycles with relatively limited functional group diversity (Figure 1). Moreover, the side chains, along with the sugar–phosphate backbone, do not have  $pK_a$ s near neutrality. The  $pK_a$ s for imino protons of A and C are near 4, and those for G and U are near 9 (Figure 1).<sup>33</sup> This property of RNA is often considered disadvantageous to proton transfer since  $pK_a$ s near biological pH strike an ideal compromise of having a good population of the functional form of the acid or base while maintaining reasonable acidity and basicity.<sup>1</sup>

However, these limitations may not be as severe as initially thought. In the absence of shifted  $pK_a$ s, comparison of the contributions of a guanine base to solvent water suggests that guanine is about 2000 times more effective as a general base at pH 7, assuming a Brønsted  $\beta$  value of 0.5.<sup>34</sup> In addition, theoretical considerations for the hairpin ribozyme using a model based on recent crystal structures<sup>5,25</sup> in which protonated A38 acts as a general acid and deprotonated G8 acts as a general base suggest that the hairpin ribozyme would be only 14 times less effective than RNase A in carrying out general acid–base catalysis at pH 7.4.<sup>34</sup>

Another possible strategy for facilitating proton transfer is for an RNA molecule to manipulate its

microenvironment to shift a  $pK_a$  towards neutrality. Indeed, protein enzymes often use amino acids other than histidine in general acid–base catalysis and covalent catalysis. For example, in hen egg white lysozyme the  $pK_a$  of Glu-35 is shifted *upward* from 4.5 to 6.5 in the presence of an inhibitor, and to 8.2 in the presence of its substrate glycol chitin; this shift allows Glu-35 to act as a general acid to protonate an alkoxide leaving group.<sup>35</sup> Likewise, in acetoacetate decarboxylase the  $pK_a$  of Lys-115 is shifted *downward* from 10.4 to 5.9, which allows this lysine to act as a nucleophile to form a Schiff base intermediate.<sup>36</sup> In this case, the  $pK_a$  shift arises because of electrostatic effects—Lys-115 is positioned near the positively charged Lys-116.<sup>36,37</sup>

RNA is also able to perturb the  $pK_a$  values of specific nucleobases. As first pointed out by Narlikar and Herschlag, it is important to distinguish between proton binding sites in which a proton is free to transfer and in which it is sequestered.<sup>11</sup> In this article, we divide shifted  $pK_a$ s of RNA into two classes (Figure 2): Class II sites are defined as those in which the proton is free to transfer, and therefore may act as a general acid or base. Class I sites, on the other hand, have the proton sequestered in hydrogen bonding, making proton transfer unlikely for three reasons: (1) steric exclusion would prevent easy access to the

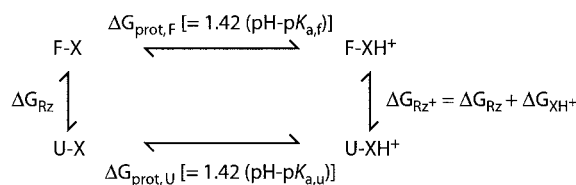


**FIGURE 2** Examples of structures involving ionized bases. All structures arise because of protonation of N1 of A or N3 of C and are due to  $pK_a$ s being perturbed toward neutrality. (A) Class I sites in which the added protons (red) are sequestered in base pairing. This class of shifted  $pK_a$  sites is termed “Class I” since they are structurally simpler than Class II sites and appear to be much more common. These motifs are unlikely to engage in general acid–base catalysis (see text), but they do have the potential for rate acceleration via electrostatic catalysis. These  $pK_a$  shifts arise because of coupling of protonation to structure formation. Structure 1 involves protonated adenine forming a wobble base pair with cytosine, as reported in DNA and RNA duplexes,<sup>133–135</sup> at the cleavage site of the hairpin ribozyme,<sup>43,136</sup> and a lead-dependent ribozyme, or leadzyme.<sup>42,44</sup> Structure 2 involves protonated adenine engaged in a Hoogsteen interaction with guanine, as reported in an oncogenic DNA duplex.<sup>137–139</sup> Structure 3 involves protonated cytosine forming a wobble base pair with guanine, as reported for the catalytic RNA from *Tetrahymena thermophila*.<sup>140</sup> Structure 4 involves a protonated cytosine forming a three hydrogen bond base pair with an unprotonated cytosine.<sup>141,142</sup> Structure 5 involves protonated cytosine forming a Hoogsteen base pair with guanine, as reported for DNA in a complex with an antibiotic.<sup>143</sup> (B) Class II sites in which the added protons (red) are not engaged in base pairing. These protons are free to transfer and therefore have the ability to engage in general acid–base catalysis, in addition to electrostatic catalysis. Structure 6 is the transition state proposed for cleavage of a phosphodiester bond in the HDV ribozyme.<sup>9</sup> The proton is donated to the leaving group oxygen making C75 a general acid catalyst for bond cleavage. Structure 7 involves two protonated adenines engaged in Hoogsteen interactions, as reported for polyadenylic acid.<sup>144</sup> Structure 8 involves a protonated adenine forming a base pair with an unprotonated adenine, as reported for a DNA duplex.<sup>145,146</sup> Structure 9 involves protonated adenine engaged in Hoogsteen pairing with guanine, as reported for a DNA duplex.<sup>146</sup>

proton inside of the base pair; (2) opening of the base pair—a presumed prerequisite to transfer—would require an especially large energetic driving force, especially if the  $pK_a$  of the site were highly shifted due to coupling to additional structure formation (see Scheme 1); and (3) once open, the base pair might lose its proton to solvent rather than transferring to the leaving group (see the section “A Role for Cationic

Nucleobases in Electrostatic Catalysis: A Postulate for the Ribosome” for further discussion).

Class II site  $pK_a$  shifts might be driven by local regions of high electrostatic potential. Since the  $pK_a$  of each phosphodiester linkage is near 1, it has essentially a full  $-1$  charge at biological pH.<sup>33</sup> Although this  $pK_a$  is probably too low to afford direct protonation of nonbridging oxygens in the phosphodiester



SCHEME 1

backbone, it does offer the possibility for the existence of local regions with enormous negative surface potential. Indeed, nonlinear Poisson–Boltzmann (NLPB) calculations have revealed a number of such regions in RNAs with tertiary structure, and several of these regions have been shown to correlate with  $Mg^{2+}$  binding sites.<sup>38–40</sup> It is also possible that regions of high negative potential could act as proton binding sites, as suggested by NLPB calculations on the HDV ribozyme.<sup>22</sup> These electrostatic properties endow RNA with the intrinsic ability to perturb the  $pK_a$ s of the A and C upwards towards neutrality. Using an analogy with proteins, adenines and cytosines with  $pK_a$  values near neutrality would be histidine-like and possible general acids and bases. At the same time, RNA has the ability to attract multiple classes of divalent metal ions to itself, especially hydrated and dehydrated magnesium ions.<sup>41</sup> This property might afford the stabilization of deprotonated, negatively charged nucleobases, leading to perturbation of the  $pK_a$ s of G and U downwards towards neutrality, providing another possible source of Class II sites and therefore of general acids and bases.

The cationic base pairs of Class I sites, although unlikely to participate in general acid–base chemistry, might be able to accelerate the reaction rate via electrostatic catalysis, stabilizing the development of negative charge in a transition state. Exceptionally high  $pK_a$  values for adenine N1, 8.5 or greater, would be optimal for electrostatic catalysis since >90% of the molecules would be in the functional, protonated form at biological pH. Again making an analogy with proteins, adenines and cytosines with such exceptionally high  $pK_a$  values would be lysine- and arginine-like, and therefore possible oxyanion holes. Possible mechanisms for achieving shifts of this magnitude are discussed in Scheme 1, and in the sections “A Role for Cationic Nucleobases in Electrostatic Catalysis: Background” and “A Role for Cationic Nucleobases in Electrostatic Catalysis: A Postulate for the Ribosome.”

Scheme 1 shows a thermodynamic relationship between shifting of a low  $pK_a$  towards neutrality and stabilization of the ribozyme fold by the concomitant positive charge. This scheme applies equally to Class

I and II sites. For illustrative purposes, the ionizable atom is assumed to be the N1 of an A, and is represented as X; the entire remainder of the ribozyme is represented as U and F, for unfolded and folded states; the free energy for folding of the neutral, unprotonated ribozyme is given as  $\Delta G_{Rz}$ ; the free energy for folding of the protonated ribozyme is given as  $\Delta G_{Rz+}$ ; and the excess free energy for recognition of  $XH^+$  and any other accompanying structural changes is given by  $\Delta G_{XH+}$ . In addition, the  $pK_a$  of X in the unfolded state is given by  $pK_{a,u}$ , and the  $pK_a$  of X in the folded state is given by  $pK_{a,f}$ , where the free energy for protonation is a function of the pH and is given by  $\Delta G_{prot} = 1.42 (pH - pK_a)$ , in kcal/mol at 37°C; for this example,  $pK_{a,f} > pK_{a,u}$ . According to this model,  $\Delta G_{XH+} = 1.42 (pK_{a,u} - pK_{a,f})$ , or  $\Delta G_{XH+} = -1.42 \Delta pK_a$ , in kcal/mol.

If at a biological pH of 7.4 one desires 50% of the ribozyme to be in the functional protonated form (i.e.,  $pK_{a,f} = pH$ ), which is ideal for general acid–base catalysis, and given that the  $pK_a$  of N1 of A in the unfolded state is approximately 3.5, then  $\Delta G_{XH+} \approx -5.5$  kcal/mol. In other words, the favorable interactions of the ribozyme with protonated N1 of A would have to contribute about an extra 5.5 kcal/mol to folding. (To have 90% or more of the ribozyme molecules in the functional form, which is desirable for an oxyanion hole, the  $pK_{a,f}$  must be greater than the  $pH + 1$ , which decreases  $\Delta G_{XH+}$  by another 1.4 kcal/mol; see sections “A Role for Cationic Nucleobases in Electrostatic Catalysis: Background” and “A Role for Cationic Nucleobases in Electrostatic Catalysis: A Postulate for the Ribosome”) These  $pK_a$  shifts are similar in magnitude to the 4.5 unit shift found for acetoacetate decarboxylase due to positioning of charges.<sup>36,37</sup>

For Class II sites, the favorable interactions could likewise arise from the positioning of charges, for example, by positioning of the cationic nucleobase near the ribozyme phosphate backbone. For Class I sites, the favorable interactions could come in part from the new base pair that becomes possible upon protonation of the A, for example an A–C wobble pair. NMR studies indicate that in a typical AC wobble pair, the  $pK_a$  of the A is shifted from 3.5 to 6.0–6.5.<sup>42–44</sup> However, greater  $pK_a$  shifting might arise if the formation of other interactions was associated with the protonation (i.e. cooperative folding of secondary and tertiary structure occurred along with formation of the AC base pair). This illustration also serves to show that favorable *ground state* binding interactions between the ribozyme and the catalytic base involved in proton transfer or electrostatic catalysis are a necessary feature of catalysis by protonated

bases (see section “Can a  $pK_a$  Shift in the Transition State Account for  $pK_a$ s near 7 Observed from Kinetic Studies?”)

### Implications for Specificity

The relative lack of functional group diversity of the RNA side chains might suggest that RNA would be poor at carrying out reactions with high specificity. However, a number of studies suggest that this too is not the case. For example, numerous RNA and DNA aptamers have been selected that bind small molecules in a highly specific fashion.<sup>45</sup> RNA aptamers to theophylline have been selected that bind their target with 10,000 times higher affinity than caffeine, which differs from theophylline by the addition of a single methyl group.<sup>46</sup> Molecular discrimination is beginning to be understood on the structural level as well, owing in large part to numerous NMR studies on aptamers.<sup>47,48</sup> These studies reveal that specificity is achieved through multiple interactions, shape complementarity, and induced-fit mechanisms. Although much remains to be explored about the potential for RNA and DNA aptamers to discriminate for and against specific functionalities, it is clear that nucleic acids can achieve highly specific interactions that, in many cases, rival those of their protein counterparts.

If RNA can position its bases in defined and unique ways, then it should be able to achieve proton transfer specifically as well. Positioning can be obtained with high specificity at the secondary structural level through simple Watson–Crick base pairing, and at the tertiary level through electrostatic and hydrogen-bonding interactions. An extended set of recognition patterns, and therefore functional capabilities, for nucleic acids might also arise through the formation of unusual tautomeric forms of the bases. Tautomers, which are energetically unfavorable in the unfolded state, would have to be stabilized by interactions formed upon higher order folding, much like protonated bases described in Scheme 1 (see also the section “Kinetic Complexity and the Possible Involvement of Tautomers in the Mechanisms of Nucleic Acid Enzymes”).

Lastly, it should be mentioned that RNA is prone to occupying a multitude of nonreactive states, including alternative secondary structures, nonfunctional ionizations, and misfolded tertiary structure.<sup>49</sup> This behavior can be described by a partition function analysis in which the intrinsic reactivity of the fully functional ribozyme is modulated by the fraction of the ribozymes that are folded correctly,<sup>34,50</sup> although kinetic trapping requires nonequilibrium terms. This separation of concepts is important because changes

in nonactive site sequence and pH that redistribute the ribozyme population towards and away from maximal occupancy of the reactive state can be handled by ground state effects, while the presence of general acid–base catalysis and electrostatic catalysis, which change the intrinsic reactivity of the reactive state can be described by transition state theory. Consideration of the distribution of ribozyme molecules over non-reactive states has been recently discussed,<sup>34,50</sup> thus changes in the intrinsic reactivity of the reactive state is the primary focus of this article—only a brief discussion of ground state effects (see the section “Can a  $pK_a$  Shift in the Transition State Account for  $pK_a$ s near 7 Observed from Kinetic Studies?”) is presented in an effort to make this distinction clear.

## PROTON TRANSFER AND PROTONATION IN NUCLEIC ACID ENZYMES: RATE ACCELERATION

### Does Proton Transfer Need to be Facilitated?

Since the majority of enzyme reactions, including the most prevalent RNA-catalyzed reactions of phosphodiester bond cleavage and peptide bond formation, involve some transfer of protons,<sup>2</sup> it is worth asking whether such proton transfers need to be facilitated by well-positioned acids and bases in their functional forms. There are numerous catalytic strategies employed by protein enzymes besides general acid–base catalysis, suggesting that general acid–base catalysis is not an obligatory feature of enzymes. Indeed, enzymes can effect considerable rate acceleration simply by approximation. Jencks states, “*The most obvious means by which an enzyme might increase the rate of a bimolecular reaction is simply to bring the reacting molecules together at the active site of the enzyme.*”<sup>1</sup> The large magnitude possible for this effect was determined by comparing rates of bimolecular reactions to their unimolecular counterparts using model compounds in which the reactant functionalities were held together within a covalent framework.<sup>51,52</sup> The acceleration from approximation can be quite large, on the order of  $\approx 10^8$ – $10^{10}$ , and can be understood as the enzyme using the energy of substrate binding to decrease the rotational and translational entropy of the reactants prior to bond breaking and making.<sup>3,13,51,52</sup>

Since Watson–Crick base pairing can be used to bring reactants close together in a specific fashion, nucleic acids are adept at utilizing approximation to achieve rate acceleration. For instance, Orgel and

co-workers have carried out extensive studies on template-directed oligomerization of RNA and DNA.<sup>53</sup> More recently, Liu and co-workers have applied template-directed approximation to carry out small molecule organic syntheses in complex mixtures.<sup>54,55</sup> Thus, two of the most critical features of enzymes—rate acceleration and specificity—can be effected in nucleic acids by mere approximation. In addition, other catalytic strategies including covalent catalysis, electrostatic catalysis, desolvation and strain can also lead to significant rate acceleration in the absence of general acid–base catalysis. Apparently, it is not necessary that all enzyme-catalyzed reactions employ general acid–base catalysis in order to obtain rate acceleration; we might, therefore, ask whether *certain types* of enzyme-catalyzed reactions require facilitated proton transfer for rate acceleration.

Reactions in which there is a particularly poor leaving group might appear to be good candidates for general acid catalysis. For example, leaving groups with high  $pK_a$  values, such as the 5'-hydroxyl in RNA, which has a  $pK_a$  near 15,<sup>56</sup> generally require some form of stabilization to facilitate their release. Likewise, nucleophiles with high  $pK_a$  values, such as the 2'-hydroxyl in RNA, which has a  $pK_a$  between ~12.5–14.9 based on experiments and theory,<sup>57–59</sup> require assistance to facilitate their deprotonation. However, facilitation need not be from general acid–base catalysis, as other types of chemical catalysis can also stabilize the developing negative charges in these processes.<sup>3</sup> Indeed, metal ion catalysis has been shown to be operative in the phosphoryl transfer reactions of the Group I intron, wherein three  $Mg^{2+}$  ions stabilize the alcoholate ions that form in the transition state.<sup>16</sup> The ability of RNA to attract metal ions to itself makes metal ion catalysis equally, if not more, probable than general acid catalysis for stabilizing the transition state. Based on the multiplicity of mechanisms for explaining enzyme catalysis, one might wonder why proton transfer is worth considering. We believe that there are several reasons. First, proton transfer *can* effect a large degree of rate acceleration (see next section). Second, and more profoundly, direct involvement of the bases in the reaction opens the possibility for new types of reaction mechanisms. Third, RNA tends to bind protons more tightly than metal ions.<sup>30,41</sup> And, fourth, participation of the bases themselves, as opposed to the sugar-phosphate backbone, deepens the parallels between catalysis by protein enzymes and RNA enzymes, and sharply increases the number of reactions RNA is capable of facilitating.

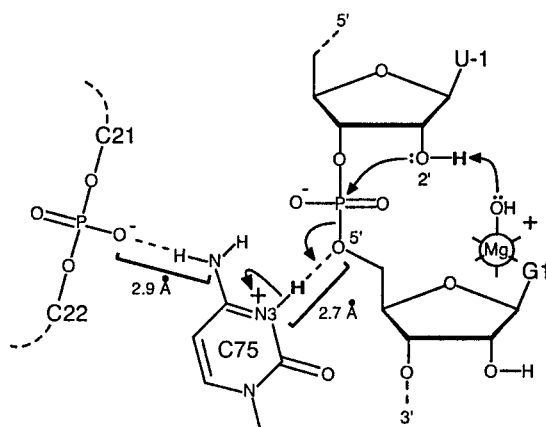
## Estimating the Magnitude of Rate Acceleration Afforded by General Acid–Base Catalysis

The standard approach for assessing the contribution of a catalytic strategy to rate acceleration is to mutate an important residue, measure the fold effect on rate, and convert this to a  $\Delta\Delta G^\ddagger$  value. This approach is invaluable for providing information about the importance of specific residues, as mentioned, but the non-additivity of energetics makes it difficult to assign this value to any one specific catalytic feature.<sup>12,13</sup> Thus, caution must be exercised when attempting to dissect the quantitative contributions of specific catalytic strategies, such as general acid–base catalysis. In general, one may assume that when the general acid or base is mutated, the observed fold effect on rate is probably an *upper limit* to the actual contribution of general acid–base catalysis, since the mutation might also interfere with other features of the ribozyme, such as the structural integrity of its active site or, in the case of single-turnover ribozymes, its folding pathway. In addition, it is not impossible that a mutation might *stimulate* another catalytic process as well.

The enzymes RNase A and phosphatidylinositol-specific phospholipase C use histidines as general acids and bases to protonate alcoholate and deprotonate alcohol functionalities.<sup>56,60</sup> Alanine mutants of each of the histidines revealed losses in  $k_{cat}/K_M$  and specific activity of  $10^3$ – $10^5$  per mutation. Studies by Li and Breaker on the specific-base catalyzed cleavage of ribose–phosphodiester linkages led to an upper limit for the possible contribution of general base catalysis to rate acceleration of  $\sim 10^2$ -fold.<sup>57</sup> Together, these studies suggest that general acid–base catalysis could contribute  $\sim 10^8$ -fold [=  $(10^4)^2$ ], or more, to catalysis. Clearly, the quantitative contributions of general acid–base catalysis can be very large.

Measurements of the contributions of general acid–base catalysis to phosphodiester bond cleavage by the HDV ribozyme have been made.<sup>9,61</sup> In both studies, C75, in the genomic HDV ribozyme,<sup>9</sup> and C76, its antigenomic counterpart,<sup>61</sup> were implicated as being either the general acid or base for bond cleavage, with a  $pK_a$  between ~6 and 7. Additional biochemical studies appear to be more consistent with a general acid role for C75 in cleavage, with a protonated N3 donating its proton to the 5'-bridging oxygen leaving group in the reaction (Scheme 2).<sup>9,62–64</sup>

In particular, general acid catalysis is consistent with the following observations: (1) the N3 of C75 is near the 5'-bridging oxygen in the crystal structure of



SCHEME 2

the cleaved form of the genomic ribozyme<sup>26,27</sup>; (2) negative linkage occurs between proton and  $Mg^{2+}$  binding in the ground state of the reaction<sup>9</sup>; (3) nucleotide analog interference modification (NAIM) experiments on the HDV ribozyme support general acid catalysis<sup>64</sup>; and (4) the pH profile for the reaction in the absence of a catalytic  $Mg^{2+}$  ion, which has been implicated as the general base in the reaction, is inverted (Scheme 2).<sup>9,22</sup> Moreover, physical organic studies of phosphodiester bond cleavage initiated by 2'-hydroxyl attack provide a  $\beta_{\text{leaving group}}$  value of  $\sim -1$  for the 5'-bridging oxygen,<sup>65,66</sup> which implicates substantial anionic character on this oxygen in the transition state. In addition, nonenzymatic hydrolysis studies on phosphodiester model compounds suggest that protonation of the leaving group is slower than deprotonation of the nucleophile, and this is further supported by experiments and calculations on the hammerhead and HDV ribozymes.<sup>67-69</sup> These studies lend credence to the importance of general acid catalysis, which can stabilize charge buildup in the transition state.<sup>70</sup> Nevertheless, either a general acid or base role remains formally possible for C75 based on complications from the principle of kinetic ambiguity.<sup>1,34</sup>

Replacement of C75/76 with a uridine, the most closely related base in size to cytosine, resulted in no detectable reaction in either the genomic or antigenomic ribozymes.<sup>9,61</sup> Perrotta and co-workers calculated an effect of greater than  $7 \times 10^5$  for the substitution,<sup>61</sup> which is larger than the  $10^5$  limit for general base catalysis provided by Li and Breaker,<sup>57</sup> and larger than the  $10^3$ – $10^5$  effects for alanine substitution for the catalytic histidines in protein enzymes<sup>56,60</sup>; a similar effect of  $\sim 2 \times 10^6$  was found for the C75U change in the genomic ribozyme (S. Nakano and P. C. Bevilacqua, unpublished data). The difference between these

effects and the limit predicted by Li and Breaker<sup>57</sup> is likely a manifestation of the aforementioned nonadditivity.<sup>12,13</sup> Indeed, the crystal structure of the genomic ribozyme<sup>26,27</sup> reveals that the exocyclic amine of C75, which is absent in uridine, donates a hydrogen bond to the phosphate of nearby C22 (Scheme 2); thus, substitution of a uracil for C75, in addition to causing a loss of general acid catalysis, may lead to partial or complete loss of the structural integrity of the active site.

In fact, substitution of C75/76 with an adenine, which does have an amine at an equivalent position as cytosine, does lead to reaction and gives a mechanistic  $pK_a$  value that is consistent with the  $pK_a$  differences between unperturbed A and C.<sup>9,61</sup> Quantitative considerations, however, revealed an unexpectedly large decrease in rate for C75/76A substitutions; estimates are 300- and 4000-fold in the genomic and antigenomic ribozymes, respectively.<sup>9,61</sup> These observations support a further source of nonadditivity in the effects of C75/76 substitutions. Clearly, this cytosine is important for more than just general acid–base catalysis. Despite these complications, quantitative consideration of general acid–base catalysis in the HDV ribozyme reveals the potential for rate acceleration that is similar in magnitude to that found for protein enzymes. It is remarkable to note that if the HDV ribozyme also employed effective general base catalysis, by having a general base with a  $pK_a$  of 7 rather than 11.4, that the rate of bond cleavage has been estimated to be similar to that for RNase A (see Ref. 9 for details of calculation), again emphasizing the potentially enormous rate acceleration that general acid–base catalysis can provide.

## A Role for Cationic Nucleobases in Electrostatic Catalysis: Background

The above considerations focused on quantitative contributions from proton *transfer*. Since proton transfer involves ionization of the nucleobases, general acid–base catalysis is accompanied by significant redistribution of charge. Charge redistribution changes the electrostatic potential of the active site and therefore has the ability to afford another type of chemical catalysis, electrostatic catalysis. Although electrostatic catalysis often accompanies general acid–base catalysis, it is classified differently since it does not involve the making and breaking of covalent bonds, and as discussed below, could operate in the absence of proton transfer altogether.

Electrostatics forces are described by Coulomb's law,

$$E = q_1q_2/(Dr)$$

where  $E$  is the energy of the interaction,  $q_1$  and  $q_2$  are the charges of the interacting ions,  $D$  is the dielectric of the medium, and  $r$  is the distance separating the charges. Because the energy is proportional to  $1/r$ , electrostatic interactions persist over much longer distances than other noncovalent interactions and can be very large in magnitude. For example, separation of a proton and an electron by 3.3 Å leads to a stabilizing interaction energy of  $-100$  kcal/mol in vacuo and  $-1.3$  kcal/mol in water, which has a dielectric constant of 79 at room temperature.<sup>3</sup> Moreover, doubling the distance between the charges to 6.6 Å, reduces the interaction energy by only a factor of two. Considerations of various catalytic strategies to enzyme catalysis has led Warshel to conclude that “*electrostatic effects provide the most important contributions to enzyme catalysis.*”<sup>71</sup>

Knowledge of the effective dielectric of the medium is critical for accurate quantitative considerations. In the case of proteins, the hydrophobic environment contributes to a lower effective dielectric, which can enhance electrostatic interactions.<sup>2,3,13,72–74</sup> The effective dielectric in the interior of proteins is heterogeneous, however, and its value depends on factors such as water penetration, which can cause the effective dielectric to be quite large ( $\geq 10$ ).<sup>72–74</sup> It is unclear what value to attribute to the dielectric of an RNA enzyme active site. Initial considerations suggest that the value may be higher in RNA due to the lack of substantial hydrophobic character of the bases,<sup>75–77</sup> and to the greater penetration of water into the less tightly packed active sites of RNA.<sup>11</sup> Even so, small (e.g., 2- to 3-fold) decreases in  $D$ , along with close and optimal positioning of charges, raise the possibility for substantial rate acceleration through electrostatic catalysis.

A classic example of electrostatic catalysis in protein enzymes occurs in the cleavage of a saccharide substrate by lysozyme. In this case, in a step after giving up its proton to the leaving group alkoxide (see the section “Implications for the Acceleration”), Glu-35 along with deprotonated Asp-52, form a linear  $- + -$  network with an oxocarbenium ion-like transition state and intermediate.<sup>3</sup> Theoretical calculations suggest that a significant fraction of the activation free energy ( $\sim 7$  kcal/mol for  $k_{\text{cat}}$ ) arises from stabilization of the ionic transition state by the alignment of charges and dipoles in the active site of the enzyme.<sup>78</sup> This has led to the concept of enzymes being “*super-solvents*” that bind ionic transition states better than bulk water.<sup>78,79</sup> This  $- + -$  arrangement of charges can be understood to be near ideal from simple Cou-

lombic considerations; namely, this arrangement leads to two highly favorable interactions and only one slightly unfavorable interaction—the unfavorable interaction is slight since the distance between the negative charges is further than the distance between the opposite charges.

It is possible that a similar arrangement of active site charges could be effected by RNA and could be used to shift  $\text{p}K_{\text{a}}$  values, destabilize ground states, and stabilize transition states. For example, in the HDV ribozyme, protonated C75 is near the phosphate of C22<sup>26</sup> and must also be near the scissile phosphate, raising the possibility for a linear  $- + -$  arrangement of charges (Scheme 2). The significant stabilization afforded by this arrangement of charges could lead to significant upward shifting of the  $\text{p}K_{\text{a}}$  of C75. This possibility was suggested from NLPB calculations on the crystal structure of the cleaved ribozyme,<sup>22</sup> and specific hydrogen bonding schemes from N4 of C75 to a scissile phosphate nonbridging oxygen have been proposed,<sup>62,64</sup> although there is currently no direct support for this particular interaction.

The cationic nature of C75 has also been proposed to stimulate the rate through ground state destabilization.<sup>9</sup> The general base in the reaction has been proposed to be a hydrated magnesium hydroxide  $[\text{Mg}(\text{H}_2\text{O})_5(\text{OH})]^+$  (Scheme 2) based on competition studies with cobalt hexamine,  $[\text{Co}(\text{NH}_3)_6]^{3+}$ , comparisons to alkaline earth metal ions, pH-rate profiles, and melting studies.<sup>9,22,30</sup> Positioning of additional positive charge from a magnesium ion at the active site leads to a new electrostatic potential. Indeed, binding of proton and magnesium ions has been shown to be negatively coupled as expected for Coulombic repulsion,<sup>9</sup> and the  $\text{p}K_{\text{a}}$  for C75 is estimated to be  $\sim 1.5$  units lower in the  $\text{Mg}^{2+}$ -bound vs  $\text{Mg}^{2+}$ -free form of the active site (S. Nakano and P.C. Bevilacqua, manuscript in preparation). In addition, these interactions could cause the  $\text{p}K_{\text{a}}$  of magnesium hexahydrate to shift downward from its unperturbed value of 11.4.<sup>80</sup> As charge transfers from  $\text{C75H}^+$  to  $[\text{Mg}(\text{H}_2\text{O})_5(\text{OH})]^+$ , ultimately leading to a state of C75 and  $[\text{Mg}(\text{H}_2\text{O})_5(\text{HOH})]^{2+}$ , the electrostatic repulsion between these atoms will be lessened; thus, relief of ground state repulsion in the transition state might provide an additional driving force to catalysis by C75.<sup>9</sup> It should be noted that the scissile phosphate has also been shown to contribute to ground state destabilization of the HDV ribozyme.<sup>10,81</sup> Thus, the ground state of the active site (Scheme 2) appears to involve a complex positioning of at least four charges: two negative charges and two positive charges.

Electrostatic catalysis by oxyanion holes has been proposed in the mechanisms of the hairpin ribozyme

(by G8)<sup>82</sup> and the ribosome (by A2451 of *Escherichia coli* 23S rRNA).<sup>83</sup> Such a mechanistic strategy would parallel that of oxyanion holes in proteases.<sup>2,3</sup> It should be noted, however, that these roles in these RNAs are presently uncertain; in the case of the hairpin ribozyme, an alternative mechanism involving general acid–base catalysis has been suggested,<sup>34</sup> while in the ribosome, recent experiments testing the pH dependence of binding of a CCdA–phosphate–puromycin transition state analog, which was designed to mimic the tetrahedral carbon intermediate,<sup>83,84</sup> showed that there is no cationic residue with a  $pK_a$  between 5 and 8.5<sup>85</sup> (see next section for further considerations).

In closing this section, we note that the cationic charge required for “solvating” an anionic transition state need not arise from a general acid, or even be accompanied by general acid–base catalysis. For example, a developing negative charge in a transition state or high energy ground state (which typically resembles the transition state based on the Hammond Postulate<sup>86</sup>) could be stabilized by positioning of a permanent positive charge, an oxyanion hole, regardless of its source. Since electrostatic interactions are long range, a positive charge in a cationic base pair might be one way to exert a significant energetic effect on a developing negative charge, especially if the center of the base pair were pointed toward the incipient oxyanion. This point is nontrivial because  $pK_a$ s near, or greater than, neutrality in Class I sites are much more common and probably simpler to evolve than Class II sites (Figure 2). While a general acid or base is optimal with a  $pK_a$  near the pH of the reaction ( $pH_{rxn}$ ),<sup>34</sup> an oxyanion hole would be optimal with a  $pK_a > pH_{rxn} + 1$ , since this would allow >90% of the oxyanion holes to be in the functional protonated form. Thus, it is of interest to consider whether Class I sites with  $pK_a$ s of 8.5 or higher are feasible.

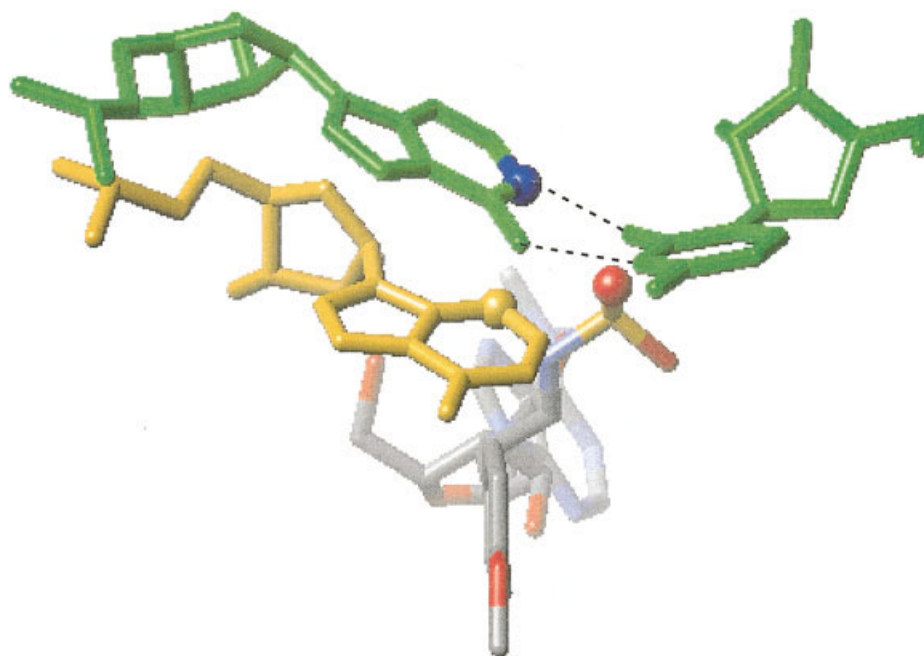
### A Role for Cationic Nucleobases in Electrostatic Catalysis: A Postulate for the Ribosome

Since the structure of the large subunit of the ribosome was solved at high resolution three years ago,<sup>32</sup> the mechanism of the peptide synthesis has been under intense scrutiny. It was originally suggested that A2451 of *E. coli* 23S rRNA might act as a general acid, a general base, or an oxyanion hole,<sup>83</sup> and the  $pK_a$  of its N3 was found to be near neutrality from the pH dependence of dimethyl sulfate (DMS) modification.<sup>87</sup> However, the importance of A2451 as well as

its  $pK_a$  have been called into question,<sup>88–92</sup> leaving the role of A2451 uncertain.

We provide an additional postulate for catalysis—namely, that the adjacent A2450, which forms a cationic base pair with C2063, might serve as an oxyanion hole. (Note that both of these residues are highly conserved.<sup>93</sup>) In the crystal structure, A2450N1 is near A2451N3,<sup>83</sup> putting A2450N1 in close proximity (5.2 Å) to the phosphate nonbridging oxygen that is the counterpart of the tetrahedral oxyanion in the CCdA–phosphate–puromycin transition state analog (Figure 3). Moreover, the nonbridging phosphate oxygen is pointed toward the center of the A2450–C2063 base pair. Of course, the CCdA–phosphate–puromycin compound is only an analog of the transition state and so the distance may be somewhat inaccurate. One particular concern is that the sugar pucker of the A76 P-site counterpart may be incorrect<sup>94</sup> since A76 in the inhibitor has a deoxyribose sugar.<sup>84</sup> Perhaps alteration of the sugar pucker or introduction of the 2'-hydroxyl would move the nonbridging phosphoryl oxygen even closer to A2450N1. Even so, with a value of 5.2 Å, the shallow distance dependence of electrostatic interactions suggests that A2450 might be able to act as an effective oxyanion hole. In principle, a role for A2450 as an oxyanion hole could be *mutually compatible* with general acid–base catalysis by A2451 since they are positioned adjacently (see below). It is also curious to note that there is another AC base pair, A2453–C2499, located below A2450–C2063, whose role is uncertain.<sup>95</sup>

Absence of stabilization of the CCdA–phosphate–puromycin transition state analog by lowering of the pH from 8.5 to 5.0<sup>85</sup> might seem to argue against this possibility. However, if the  $pK_a$  for the A2450–C2063 base pair is greater than 8.5, then this oxyanion hole would not have been detected. According to Scheme 1, the value of the  $pK_a$  shift for A2450 depends on the free energy associated with *all* of the structure that accompanies formation of the protonated AC base pair. If other components of the active site, such as the base triple involving A2451 and tertiary interactions between A2450N3 and A76 (see next paragraph),<sup>83</sup> did not fold in the absence of the AC base pair (i.e., folding of the active site is highly cooperative), then the  $pK_a$  shift for A2450 would be expected to be very high. Consistent with a high  $pK_a$ , the N1 of A2450 remained resistant to methylation by DMS from pH 5.5 to 8.5.<sup>95</sup> As mentioned in the previous section, a  $pK_a$  of 8.5 or higher would be ideal for providing a high fraction of the functional form of the oxyanion hole, conferring lysine- and arginine-like behavior to the base pair.



**FIGURE 3** Postulate for electrostatic catalysis in the ribosome. View of the CCdA–phosphate–puromycin transition state analog<sup>84</sup> bound to the active site of 23S rRNA.<sup>83</sup> The CCdA portion of the analog is omitted for clarity. A2451 is shown in orange with its N3 in CPK, and the A2450–C2063 wobble pair is in green with the cationic N1 in blue CPK. Hydrogen bonding between A2450 and C2063 is shown with dashed lines. The phosphate oxygen of the CCdA–phosphate–puromycin transition state analog that mimics the oxyanion of the tetrahedral intermediate is shown in red CPK. The distance from A2451N3 to the nonbridging phosphoryl oxygen is 3.4 Å, while the distance from A2450N1 to this oxygen is 5.2 Å. A2450N1 and the tetrahedral oxyanion are proposed to have a favorable electrostatic interaction. Note that the nonbridging phosphoryl oxygen is pointed directly at A2450N1.

Several recent studies have found that large conformational rearrangements of the ribosome occur near a pH of 7, suggestive of a  $pK_a$  near 7.<sup>90,91,95</sup> Also, observation of a  $pK_a$  near 7 from rapid kinetic analysis of the reaction<sup>92</sup> supports a thermodynamic  $pK_a$  near 7. However, there are several candidates for  $pK_a$ s of 7 other than A2450, including A2451 or A2453. Moreover, even if this  $pK_a$  is from A2450, its value could very well be different in the presence of substrate, especially given that there are several interactions between A2450 and A76 of the substrate, including a potential hydrogen bond between the 2'-hydroxyl of A2450 and A76N1, as well as between A2450N3 and A76H2; the latter distance is only 2.9 Å and similar C–H hydrogen bonds have been noted before.<sup>96</sup> As mentioned in the section “Implications for Rate Acceleration” above, the  $pK_a$  of Glu-35 of lysozyme increases from 6.5 in the presence of an inhibitor to 8.0–8.5 in the presence of a substrate.<sup>35</sup>

We recently suggested that the pH-rate profile of the ribosome reaction could be accounted for by A2451 acting as a general acid in concert with a

general base with a high  $pK_a$ , such as a 2'-hydroxyl group.<sup>34</sup> The *additional* catalytic feature of electrostatic catalysis by an A2450–C2063 oxyanion hole discussed here does not negate a general acid–base role for A2451, assuming the  $pK_a$  for A2450 is 8.5 or greater, since its ionization state would not have been detected in kinetics experiments which explored a maximal pH of 8.5.<sup>85,92</sup>

An alternative role for A2450 was recently suggested in the rapid kinetic analysis of the peptidyl transfer reaction.<sup>92</sup> In this mechanism, A2450 was proposed to act as a general base in the first step of the reaction and as a general acid in a subsequent step. However, general acid–base catalysis by A2450 would require a large conformational change ( $\sim 7$  Å) to move it to the nucleophilic amino group; moreover, this model requires that A2450 be a Class II site, rather than the Class I site seen in the crystal structure.<sup>83</sup> A more complex scenario in which the proton is “stored” in the base pair after it is accepted and before it is transferred to the ribose 3'-oxyanion seems unlikely for several reasons: (1) steric exclu-

sion would seemingly prevent close approach of the leaving group and the proton inside the AC base pair, (2) opening of the AC base pair would require overcoming the stability associated with the base pair and other coupled structures, and (3) once open, the base pair might lose its proton to solvent rather than transferring to the leaving group.

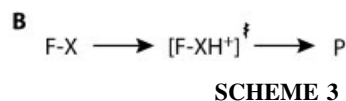
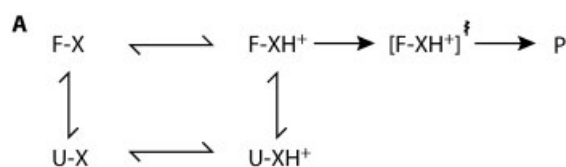
### PROTON TRANSFER BY NUCLEIC ACID ENZYMES: SPECIFICITY AND THE POSSIBLE INVOLVEMENT OF PHOSPHORANE INTERMEDIATES AND TAUTOMERS

The above considerations focused on the magnitude of rate acceleration attainable by proton transfer (general acid–base catalysis) and positioning of positive charges (electrostatic catalysis). While rate acceleration is certainly a critical aspect of catalysis, specificity is equally, if not more, important and is one of the most defining features of enzymes as catalysts.<sup>1</sup> We begin this section with a simple kinetic model of proton transfer and its implications for specificity in the ground and transition states of ribozymes. We then proceed to discuss mechanisms for phosphodiester bond cleavage by progressively more complex proton transfer catalysts: buffers, model compounds, and protein enzymes. Lastly, we consider how nucleic acid enzymes might be able to employ similar types of chemical catalysis, exploring the energetic and mechanistic feasibility of unusual tautomeric forms of the bases.

#### Can a $pK_a$ Shift in the Transition State Account for $pK_a$ s near 7 Observed from Kinetic Studies?

There has been an ongoing dialogue in the literature about the possibility of  $pK_a$ s of general acids and bases being perturbed in the transition state of the reaction. For example, it has been suggested that the  $pK_a$  of A2451 in the peptidyl transferase center of *E. coli* 23S rRNA might be perturbed toward neutrality in the transition state, and likewise for the  $pK_a$  of C75/76 of the HDV ribozyme. However, most of the  $pK_a$  values reported from biochemical experiments involving pH-rate studies require the  $pK_a$  change to occur in the *ground state*. This can be illustrated in several ways.

Recently, we offered a simple kinetic model for general acid–base catalysis by RNA that reproduced pH-rate profiles for the hairpin and HDV ribozymes.<sup>34</sup> One key feature of this model was that the shape of



the pH-rate profile arises because the pH of the solution redistributes the population of ribozyme molecules over four different ionized ground states, only one of which is functional. Importantly, values for the  $pK_a$ s of the general acid and base *were defined in the ground state*, and the only species that reacted was the fully functional ground state, and its intrinsic reactivity was independent of pH. The point to be made is that in the simulated rate–pH profiles, no transition state  $pK_a$  shifts were invoked. Thus, even though pH-rate profile experiments “sense” the transition state because covalent bonds are broken and made, the  $pK_a$  values they return reflect  $pK_a$  values in the ground state rather than the transition state. (This argument assumes that  $pK_a$ s reflect true ionization phenomena, as seems to be the case for the HDV ribozyme,<sup>9,61,97</sup> and are not kinetic  $pK_a$ s, which arise from a change in the rate-limiting step.<sup>98</sup>)

The point of requiring protonation in the ground state is further made in Scheme 3A (which is a modification of Scheme 1) in which protonation of residue X in the folded state of the ribozyme is required in order to give product, P (i.e., F-XH<sup>+</sup> is reactive, but F-X is not). It is this property of proton transfer, the ability to confer reactivity to one folded state and not another, that leads to specificity. As per above, the pH-rate profile associated with Scheme 3A would reflect changes in the relative populations of F-X and F-XH<sup>+</sup>, rather than the intrinsic reactivity F-XH<sup>+</sup>, meaning that the  $pK_a$  derived from such a profile would reflect ground state effects. The intrinsic reactivity of F-XH<sup>+</sup> can be changed by such factors as the presence and efficiency of general acid–base catalysis and electrostatic catalysis, however these are all independent of pH.

A scheme that envisions a  $pK_a$  change only in the transition state is also shown (Scheme 3B); however, this scheme can be regarded as the upper row in Scheme 3A, in which the protonation of the general acid is ignored. Scheme 3B really has (at least) two transition states, one for protonation of the general

acid, X, and the other for proton transfer to the leaving group. It is possible that the transition state for protonation of X has a greater activation free energy than for bond cleavage, causing protonation of X to be slow. Nevertheless, this scenario still implies that  $F\text{-XH}^+$  is a high energy intermediate, not a transition state. Doudna and co-workers raised this possibility in considering the absence of a shifted  $pK_a$  for C75 in their NMR studies of a precleaved HDV ribozyme.<sup>99</sup> However, observation of *ground* state destabilization between  $Mg^{2+}$  and protonated C75<sup>9</sup> suggests that the  $C75H^+$  ground state of the ribozyme is stable enough to make proton transfer to the leaving group at least partially rate limiting.

The distinction between a ground state, functionally protonated enzyme and a transition state-only protonated enzyme is an important one because it has implications for the mechanisms of ribozymes and for the prospect of isolating and studying fully functional protonated states of ribozymes by spectroscopic and crystallographic methods. Work from several laboratories has provided evidence that the precleaved HDV ribozyme, in particular, is dominated by conformations that do not resemble the crystal structure,<sup>50,100–102</sup> and that there is a large conformational change prior to cleavage.<sup>81,103,104</sup> However, an overwhelming amount of functional data on HDV ribozyme mutants also suggests that the reactive state of the precleaved ribozyme resembles the crystal structure of the cleaved ribozyme, including the presence of P1.1.<sup>105–108</sup> If  $F\text{-XH}^+$  is a ground state of the HDV ribozyme, and  $pK_a$  shifting of C75 requires interactions like those observed in the crystal structure,<sup>22,99</sup> then, in principle, this state, unlike a transition state, can be isolated and studied.

### Mechanisms for Phosphodiester Bond Cleavage Catalyzed by Buffer, Model Compounds, and Protein Enzymes

In this section, we provide a brief overview of several mechanisms for phosphodiester bond cleavage in RNA. These studies include detailed mechanistic studies on RNase A, which uses two histidines in its mechanism,<sup>109</sup> the effects of imidazole groups tethered to a cyclodextrin framework,<sup>110,111</sup> and imidazole unconstrained and free in solution.<sup>112,113</sup> Implications of the imidazole studies in Breslow's lab include the involvement of a phosphorane intermediate in the reaction (Figure 4).<sup>110,112,113</sup>

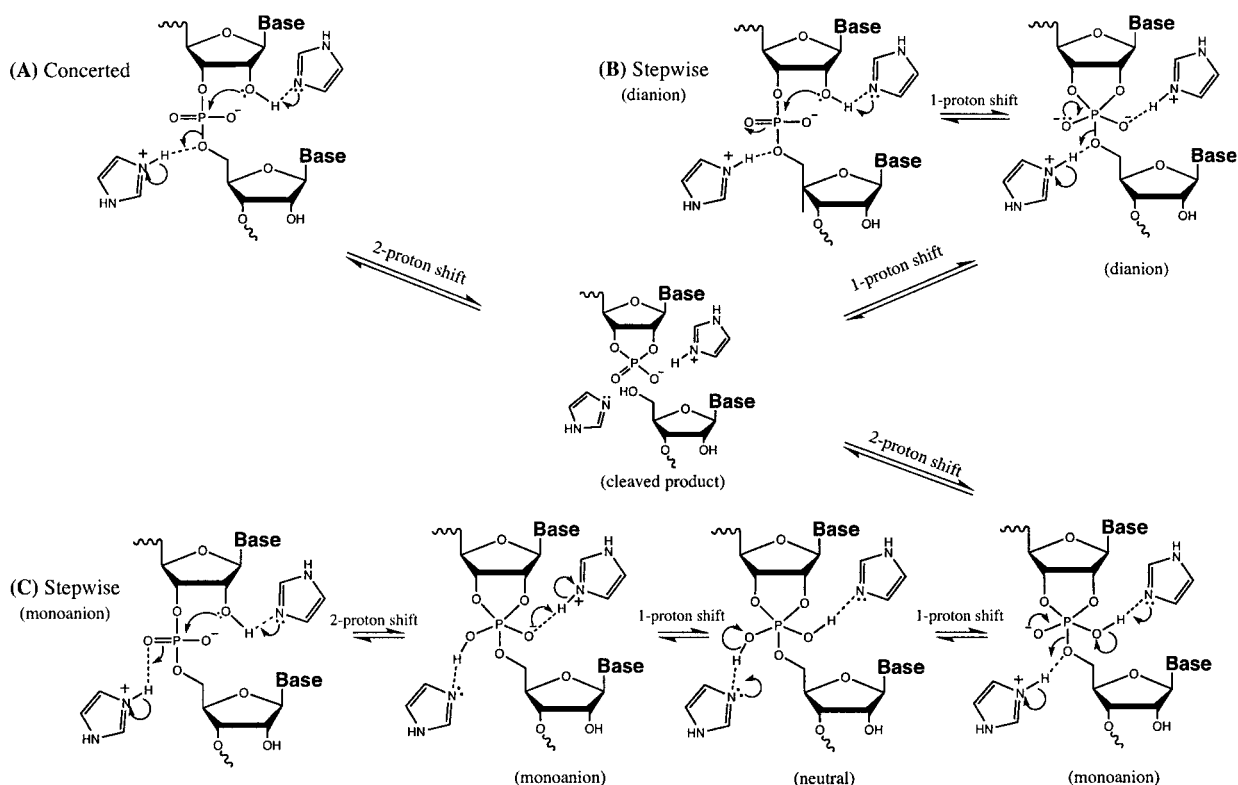
Debate on the mechanism of RNase A has been lively and arguments in favor of a concerted mechanism<sup>56,114,115</sup> and a stepwise mechanism<sup>116</sup> have been made. Considerations of heavy atom isotope effects<sup>115</sup> and thio-effects<sup>114</sup> have suggested that a

phosphorane intermediate does not occur in the mechanism of RNase A. This has been challenged,<sup>116</sup> however, in part because many of the RNase A studies were done with an unnaturally good leaving group in the reaction, *p*-nitrophenol,<sup>56,115</sup> which may have changed the mechanism and thereby abrogated the requirement for a phosphorane intermediate.<sup>66</sup> Proton inventory experiments on RNase A, which detect the number of protons “in-flight” or “shifting” in the mechanism, give a value of two,<sup>117</sup> consistent with a concerted mechanism. However, this too turns out to provide little insight since one or more of the individual steps in a multistep phosphorane intermediate-containing reaction may proceed in concert with 2-proton shifts (Figure 4).<sup>111,113,116</sup>

One possibility is that the mechanism for phosphodiester bond cleavage changes depending on the number and positioning of the imidazole groups in the catalyst. On the one hand, RNase A, which has its general acid and base histidines positioned precisely for proton transfer, may take advantage of a one-step concerted mechanism.<sup>56,114,115</sup> On the other hand, detection of imidazole-catalyzed isomerization of UpU dinucleotide phosphodiester linkages from 3',5' to 2',5' constitutes proof that an intermediate can occur in phosphodiester cleavage, with the intermediate most likely being a neutral or monoanion phosphorane that partitions between P—5'—O bond cleavage and pseudorotation followed by 3'—O—P cleavage. Without an intermediate, the formation of these two products would violate the principle of microscopic reversibility.<sup>66,113</sup> Perhaps the positioning of functional groups guides the transition state towards the lowest energy reaction trajectory, which may or may not have intermediates depending on the catalyst. The point to be made is that even if we consider RNase A to operate by the classical concerted reaction mechanism,<sup>56,114,115</sup> this does not mean that other phosphodiester cleavage catalysts—such as imidazole buffer,<sup>112,113</sup> cyclodextrin–imidazole compounds,<sup>110,111</sup> other protein enzymes such as RNase T1,<sup>118</sup> and catalytic nucleic acids—necessarily operate by the same mechanism, much in the same way that not all proteases use the same mechanism as serine proteases.

### Kinetic Complexity and the Possible Involvement of Tautomers in the Mechanisms of Nucleic Acid Enzymes

Proton inventory experiments on the hairpin ribozyme provide a 2-proton inventory,<sup>119</sup> while those on the HDV ribozyme provide a 1- or a 2-proton inventory depending on reaction condi-

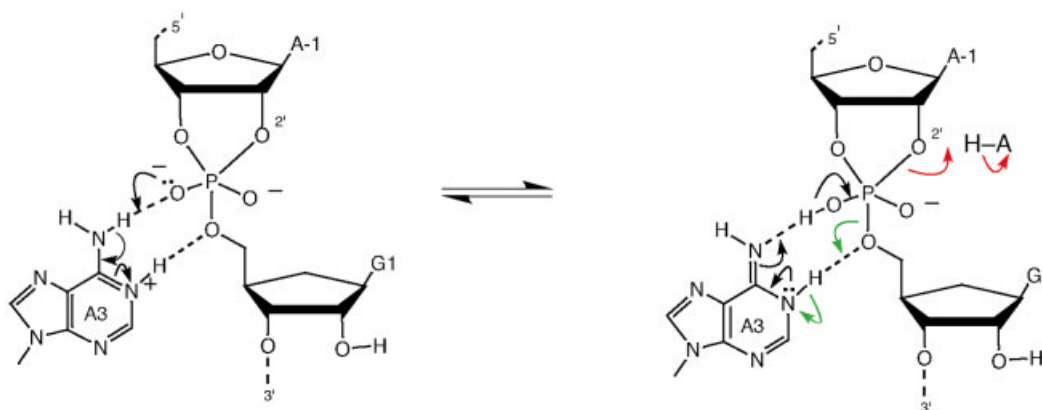


**FIGURE 4** Concerted vs stepwise reaction mechanisms for phosphodiester bond cleavage in RNA via general acid–base catalysis by imidazole. Each mechanism results in products with 2',3'-cyclic phosphate and 5'-hydroxyl termini. Mechanisms are based on those from Breslow and Anslyn,<sup>66,113,116</sup> and the charge of the phosphorane species is noted in the figure. (A) The concerted mechanism is represented by concurrent 2'-hydroxyl deprotonation, 2'-O—P bond formation, P—5'-O bond cleavage, and 5'-O—leaving group protonation. (B,C) Complex, stepwise mechanisms have also been proposed involving a phosphorane intermediate. After nucleophilic attack of the 2'-hydroxyl on the phosphorus, (B) the dianion or (C) monoanion phosphorane intermediate is formed. The pentavalent intermediate then collapses and releases the 5'-oxanion, which is concurrently or subsequently protonated by the general acid. In mechanism (C), there are several alternative combinations of 1-proton or 2-proton shifts following the initial formation of the phosphorane monoanion and leading to the formation of the imidazole general acid that protonates the 5'-O—leaving group. One of the features of mechanism (C) is the avoidance of the phosphorane dianion, which has a very high  $pK_a$  and is therefore likely nonexistent.<sup>66,121,122</sup>

tions.<sup>97,120</sup> As mentioned in the previous section, proton inventories of 2 provide little insight into the difference between concerted and stepwise reaction mechanisms. An inventory of 1, however, is suggestive of a more complex, stepwise mechanism in which one of the 1-proton shifts is rate limiting (Figure 4). The inventory for the HDV ribozyme tends towards 1 in higher concentrations of magnesium chloride.<sup>97,120</sup> Since hydrated magnesium hydroxide has been proposed to be the general base in the reaction, one possibility is that in saturating concentrations of magnesium ions, deprotonation of the 2'-hydroxyl, which may be accompanied by a 2-proton shift if it is coincident with protonation

of the nonbridging oxygen (Figure 4), is no longer partially rate limiting. A different step having a 1-proton shift, such as protonation or deprotonation of nonbridging oxygens, or protonation of the leaving group may become rate limiting. This would be consistent with the aforementioned studies in which the rate-limiting step for cleavage of a natural phosphodiester bond has been shown to be breaking of the P—5'-O bond.<sup>67–69</sup>

Herschlag and co-workers have pointed out that RNA enzymes, especially smaller ones, are less rigid than their protein counterparts.<sup>11</sup> Taking this point to its extreme, one might ask whether certain small RNA enzymes are more like imidazole buffer rather than



SCHEME 4

RNase A, having difficulty simultaneously positioning functional forms of proton donors and acceptors. If so, this might open up the possibility of more kinetically complex, multistep reaction mechanisms, such as seen for imidazole buffer-catalyzed cleavage of UpU.<sup>113</sup>

One way in which a smaller ribozyme might increase the number of interactions at the active site and thereby confer rigidity and specificity is through the use of tautomers and protonated tautomers. Rare tautomers present different hydrogen bonding patterns for the bases, which could increase the limited diversity of their typical tautomeric form and lead to enhanced molecular recognition capabilities and new reaction mechanisms. In fact, imino tautomers of G and A have been invoked in the mechanism of peptide synthesis by the ribosome.<sup>83</sup> Also, Burke and co-workers recently suggested the involvement of a guanine tautomer in the mechanism of phosphodiester bond cleavage by the hairpin ribozyme.<sup>119</sup> In their mechanism, G8 is proposed to act as a concerted general acid–base, with G8O6 deprotonating the 2'-hydroxyl and G8N1 acting as a general acid to protonate the 5'-bridging oxygen, resulting in an overall conversion of guanine from the keto to enol tautomer. Concerted general acid–base catalysis by a guanine (or a uracil) is appealing since it would avoid the presumably unfavorable state of being anionic within of a negatively charged RNA active site.

After the publication of the study from the Burke group,<sup>119</sup> the crystal structure of the hairpin ribozyme was solved with a transition state mimic (see Ref. 5 and accompanying article in this issue<sup>6</sup>), which provided new clues about the mechanism, including the possibility for general acid–base catalysis by G8 and A38.<sup>34</sup> Inspection of this structure reveals that although G8, the putative general base, and A38, the putative general acid, do have their imino nitrogens

positioned to transfer protons from and to the 2'-hydroxyl and 5'-bridging oxygen groups, respectively, they also have their amino groups positioned to interact with the two nonbridging phosphoryl oxygens. One possibility is that the amino groups bind to and stabilize the transition state, as suggested.<sup>5</sup> An additional possibility is that the amino protons are actively involved in the chemistry, being transferred to and from the nonbridging oxygens in the formation of a short-lived phosphorane intermediate. (Note that in both possibilities, the extra interactions with the amino protons would provide rigidity to the active site.)

Scheme 4 presents such a possibility, focusing on a possible role for the putative general acid for cleavage, A38. The left-hand structure is based on hydrogen bonding in the vanadate crystal structure,<sup>5</sup> while the right-hand structure is a possible phosphorane monoanion. The monoanion could decompose in two ways beginning electron pushing at N1 and branching (red arrows) to afford either the 3',5' starting material (ligation reaction) or (green arrows) the 2',3'-cyclic phosphate plus 5'-hydroxyl products (cleavage reaction). If such a phosphorane monoanion forms, it would also require A38 to assume an imino tautomeric form (right-hand structure). Next, we judge the thermodynamic feasibility of the phosphorane monoanion species using energetic considerations, in particular  $pK_a$  values for the proton transfer and an equilibrium constant for tautomer formation.

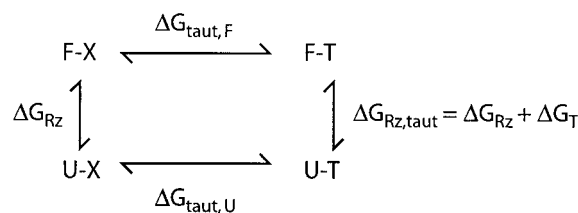
Recent calculations from the Karplus and Kirby groups<sup>121,122</sup> suggest that the  $pK_a$  of the phosphorane dianion is quite high at approximately 14.3, while the  $pK_a$  of the monoanion is lower, at approximately 7.9–8.6 (Figure 4); also, see Ref. 66 for review of prior calculations. These values can be understood qualitatively, on the basis of close positioning of the two anionic oxygens in the dianion. The  $pK_a$  for the N1 of

A38 does not appear to be shifted any higher than 5,<sup>34</sup> providing an  $\sim 9$  unit  $pK_a$  difference (or  $\approx 13$  kcal/mol), which would favor proton transfer to the dianion. However, because of adenine's highly favorable electronic structure, the energy needed to populate the imino tautomer is quite large and has been calculated to be on the order of 10–12 kcal/mol.<sup>123</sup> (See below for a discussion on tautomer calculations.) Nevertheless, the high  $pK_a$  difference between the N1 of A and the dianionic phosphorane may be sufficient to shift the equilibrium to the right, deprotonating the A, protonating the phosphorane dianion, and forming the imino tautomer of A (Scheme 4). Alternatively, the proton could be donated to the other nonbridging oxygen from the amino of G8, leading it to adopt an imino tautomeric form. Note that the dianionic phosphorane, which is thought to be extremely unstable, may be avoided altogether if protonation of the nonbridging oxygen occurs simultaneously with or prior to nucleophilic attack by the 2'-hydroxyl, as demanded for dianions by Perreault and Anslyn (see Figure 4C).<sup>66</sup>

Whether the neutral phosphorane forms should depend both on the  $pK_a$  of the neutral phosphorane, 7.9–8.6,<sup>121,122</sup> and whether formation of the neutral phosphorane requires further tautomerization of the bases. Formation of the neutral phosphorane is probably unlikely given that the monoanion is less basic than the dianion. Requirement for further tautomerization may depend on whether the nonbridging oxyanion is free to accept a proton from water, or if it instead interacts with the amino group of another nucleobase, such as seen in the hairpin ribozyme.<sup>5</sup>

Although the position of the scissile phosphate in the HDV ribozyme is not known, it has been suggested that C75 would interact with it, perhaps at one of the nonbridging oxygens,<sup>22,62,64,99</sup> opening the possibility for phosphorane formation in this ribozyme as well.<sup>9,97</sup> This possibility is also qualitatively consistent with theoretical considerations for the HDV ribozyme.<sup>69</sup>

A great deal of theoretical work has been carried out in recent years on the energetics of tautomers and protonated tautomers in both the gas phase and aqueous solution. Implementation of increased basis sets of atomic orbitals and electron correlation effects, which are critical for capturing stacking interactions, has led theoreticians to claim more accurate and realistic values for tautomeric equilibria.<sup>123–126</sup> Typically, these studies give free energies that disfavor rare tautomers by  $\sim 5$ –10 kcal/mol under aqueous conditions, with protonated tautomers disfavored by an additional 5 kcal/mol or so at neutral pH. Although these values are likely to get adjusted as methods are



SCHEME 5

refined, the fact that they cluster suggests that they may be used as reasonable estimates of the energetics that a ribozyme would have to provide in order to have a reasonable population of a tautomer. The nature of the energetic shift is illustrated with Scheme 5, which is similar to Scheme 1 with the following changes: the tautomeric base is represented as “X” in its normal tautomeric form and “T” in its rare tautomeric form; the free energy for folding of the tautomeric ribozyme is given as  $\Delta G_{Rz,taut}$ ; and the excess free energy for recognition of T is given by  $\Delta G_T$ . In addition, the free energies for forming the tautomer in the unfolded and folded states are given by  $\Delta G_{taut,U}$  and  $\Delta G_{taut,F}$ , respectively. According to this model,  $\Delta G_T = \Delta G_{taut,F} - \Delta G_{taut,U}$ . Using similar arguments as for Scheme 1, if one desires 50% of the ribozyme to be in the tautomeric form (i.e.,  $\Delta G_{taut,F} = 0$ ), then  $\Delta G_T = -\Delta G_{taut,U}$ ; in other words, the favorable interactions of the ribozyme with the rare tautomeric form of the base would have to contribute between 5 and 10 kcal/mol for the tautomer to populate appreciably. Can RNA provide the energy needed to shift these tautomeric equilibria?

For the case of the phosphorane dianion to monoanion equilibrium (Scheme 4), it appears that the free energy in proton transfer itself may be sufficient to drive tautomer formation (see above). In other cases, formation of the tautomeric base might be coupled to formation of a novel tautomeric base pair, which might be worth as much as 3 kcal/mol in  $\Delta G_{37}^\circ$  based on nearest-neighbor values for formation of the most stable Watson–Crick base pairs.<sup>127,128</sup> Moreover, if interactions with the tautomeric base make possible additional interactions, such as flanking base pairs or tertiary interactions, then  $\Delta G_T$  could be worth considerably more. Thus, tautomer formation appears to be thermodynamically feasible, assuming it is coupled to highly favorable proton transfer processes, or to the formation of new secondary or tertiary structures.

In a similar fashion, a thermodynamic cycle for formation of a protonated tautomer can be envisioned using Scheme 5 and replacing T with  $TH^+$ . By analogy to Scheme 4, it is necessary to fulfill the condition,  $\Delta G_{T^+} = -\Delta G_{taut,U^+}$ , in order to achieve an

appreciable population of a protonated tautomer. Formation of a protonated tautomer would thus require favorable interactions with the ribozyme on the order of 10–15 kcal/mol. Despite the extra energy required to form a protonated tautomer, it may be more feasible than a neutral one because of the possibility for strong electrostatic interactions with the phosphate backbone. Indeed, favorable electrostatic interactions can be used by protein enzymes to populate deprotonated tautomers; for example, the equilibrium of a ketone and an enolate is shifted towards the enolate by positioning of the oxyanion near a cationic amino acid.<sup>129–131</sup>

## CONCLUSIONS AND PROSPECTS

In this article, we described how protonation equilibria might be perturbed to aid catalysis of nucleic acid enzymes. Two classes of shifted  $pK_a$ s were identified, and mechanistic possibilities for each class were provided. General acid–base catalysis takes advantage of Class II protonation sites and provides the opportunity for large values of rate acceleration ( $>10^8$ ), as well as specificity to the outcome of reactions. Electrostatic catalysis may take advantage of Class II or Class I protonation sites, and also provides the opportunity for significant rate acceleration and specificity. Because of their generality, Class I sites may be simpler to evolve. Moreover, the potential for high cooperativity in RNA folding raises the possibilities for highly shifted  $pK_a$ s ( $>8.5$  for As and Cs), which would form effective oxyanion holes. Consideration of the mechanism of the ribosome suggested that the cationic A2450–C2063 base pair might act as an oxyanion hole, perhaps in the presence of general acid–base catalysis by A2451. As the characterization of ribozyme mechanisms continues, we anticipate that additional examples of proton transfer and electrostatic catalysis by cationic base pairs may emerge, including the possible involvement of rare tautomeric forms of the bases.

We thank Rachel Green, Mike Harris, Juliette Lecomte, and Jon Lorsch for helpful discussion and comments on the manuscript. We also thank members of the Bevilacqua lab for comments on the manuscript and for stimulating conversations. This work was supported by NSF Grant MCB-9984129, a Camille Dreyfus Teacher-Scholar Award, and a Sloan Fellowship to PCB; an NIH predoctoral fellowship and Sloan Scholarship to TSB; and a Natural Science & Engineering Research Council (NSERC) of Canada postgraduate scholarship to RY.

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*Reviewing Editor: Dr. David E. Wemmer*