Involvement of a cytosine side chain in proton transfer in the rate-determining step of ribozyme self-cleavage

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Ribozymes of hepatitis delta virus have been proposed to use an active-site cytosine as an acid-base catalyst in the self-cleavage reaction. In this study, we have examined the role of cytosine in more detail with the antigenomic ribozyme. Evidence that proton transfer in the rate-determining step involved cytosine 76 (C76) was obtained from examining cleavage activity of the wild-type and imidazole buffer-rescued C76-deleted (C76Δ) ribozymes in D_2O and H_2O. In both reactions, a similar kinetic isotope effect and shift in the apparent pKa indicate that the buffer is functionally substituting for the side chain in proton transfer. Proton inventory of the wild-type reaction supported a mechanism of a single proton transfer at the transition state. This proton transfer step was further characterized by exogenous base rescue of a C76Δ mutant with cytosine and imidazole analogues. For the imidazole analogues that rescued activity, the apparent pKa of the rescue reaction, measured under k_{cat}/K_M conditions, correlated with the pKa of the base. From these data a Brønsted coefficient (β) of 0.51 was determined for the base-rescued reaction of C76Δ. This value is consistent with that expected for proton transfer in the transition state. Together, these data provide strong support for a mechanism where an RNA side chain participates directly in general acid or general base catalysis of the wild-type ribozyme to facilitate RNA cleavage.

General acid-base catalysis[†] is common in enzyme-catalyzed systems where proton transfer occurs in the transition state of the chemical step. General acid-base catalysis can accelerate chemical-catalyzed reactions 10- to 100-fold in solution (1); in enzyme systems, results from mutagenesis data indicate that the rate acceleration can be as high as 10^6-fold (2–4). With protein enzymes, general acid-base catalysis is commonly achieved by amino acid side chains with pKa values near neutral pH. However, RNA side chains have pKa values significantly higher or lower than neutral pH and, thus, would not generally be well suited for general acid-base catalysis. For an RNA side chain to participate directly in general acid-base catalysis, a significant shift in its pKa would appear to be required. Because most ribozymes require divalent cations for optimal activity, it has been proposed that hydrated divalent metal ions may act as a general acid or base in ribozyme catalysis (5–7). Divalent metal ions also can facilitate proton transfer through direct coordination to the attacking nucleophile (8–11). Thus, while there is precedent for the role of metal ions in catalyzing proton transfer in ribozymes, there is very little information on the direct involvement of RNA side chains in general acid-base catalysis.

Hepatitis delta virus (HDV) ribozymes, like other small ribozymes, catalyze a transesterification reaction of a phosphodiester linkage, generating a 2′,3′-cyclic phosphate and a 5′-hydroxyl. The proposed mechanism involves the adjacent 2′-hydroxyl group as an intramolecular nucleophile, a penta-coordinated dianionic transition state, and the 5′-oxyanion as a leaving group. In this mechanism, activation (deprotonation) of the 2′-hydroxyl nucleophile and stabilization (protonation) of the 5′-oxyanion could be achieved by general acid-base catalysis. The investigation of HDV ribozyme catalysis has been greatly facilitated by crystallographic studies of the genomic 3′ cleavage product (12). Although lacking the scissile phosphate, this structure is thought to closely resemble the precursor because most of the catalytic domain resides in the sequence 3′ to the cleavage site (13). An important residue, C75 in the genomic sequence (γC75), but no divalent metal ion, was observed within the active site of this structure; thus, a model of general base catalysis by an RNA side chain was suggested (12). A contribution of γC75 [or cytosine 76 in the antigenomic ribozyme (C76)] to HDV catalysis was consistent with mutagenesis studies (14, 15); a C to U (or G) mutation at this position resulted in a reduction of cleavage activity by $>10^6$. Only an adenine substitution for γC75/C76 retained detectable cleavage activity, but it was 10^3-fold less active than wild type (14, 16, 17). This result was consistent with a model where the ring nitrogen N1 of adenine fulfills a catalytic function similar to N3 of cytosine. General acid-base catalysis involving C76 also was supported by chemical rescue of cleavage of a C76u mutant with imidazole buffer (16). The imidazole rescue reaction followed a rate-law definition of general base catalysis and was consistent with a cleavage mechanism where C76 accepts a proton from the 2′-hydroxyl group. Nakano et al. (17) have proposed a kinetically equivalent mechanism, consistent with both biochemical and structural data, in which γC75 serves as a general acid by donating a proton to the 5′ oxyanion in the cleavage reaction.

Very recently a catalytic role for a conserved adenosine residue in 23S RNA of the large subunit of ribosome was proposed based on results from crystallographic studies and chemical modification experiments (18–20). The ring nitrogen N3 of the adenosine is positioned toward the reactive carbonyl carbon for peptide bond formation and is proposed to serve as both general base and general acid in the peptidyl transfer reaction. Because of the similarity in proton transfer involving a ring nitrogen of a nucleobase, results from characterization of the HDV ribozyme cleavage mechanism could have useful implications for understanding the catalytic mechanism of the ribosome.

In this study, general acid-base catalysis by cytosine is further explored by using the chemical rescue approach with exogenous bases and a C76-deleted mutant (C76Δ) of the HDV antigenomic ribozyme. The kinetic solvent isotope effect (KSIE) determined for self-cleavage of the wild type and chemical rescue of C76Δ ribozyme provides evidence for a single proton transfer at

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the transition state. In addition, a linear free energy relationship of log(rate constants) with pKa of exogenous bases was observed. The Brønsted coefficient, β, determined for the ribozyme-catalyzed phosphodiester bond cleavage was consistent with those obtained from model systems. These data support an involvement of a cytosine side chain, or an exogenous base, in general acid-base catalysis, and also provide a measurement of the approximate extent of proton transfer by general acid-base catalysis in the transition state of phosphodiester cleavage in an enzymatic system.

Materials and Methods

Reagents and Chemicals. T7 RNA polymerase was purified from an overexpressing clone (21). Restriction endonucleases, nucleotides, ^{32}P-labeled nucleotides, chemicals, reagents, and other enzymes were purchased from commercial suppliers.

Preparation of RNA. Self-cleaving ribozymes, PEX1 and its mutants, were derived from the HDV antigenomic sequence (16, 22). RNA was transcribed and isolated as described (16).

Self-Cleavage Reactions. RNA was stored at −20°C, and aliquots were heated to 95°C in 25 mM Tris·HCl (pH 7.5), 5 mM EDTA, and 1 mM spermidine for 1 min immediately before use and preincubated at 37°C for 10 min. The reactions were adjusted to the final pH with buffer systems containing 25 mM acetic acid/25 mM Mes/50 mM Tris (pH 4.0–8.0) or 50 mM Mes/25 mM Tris/25 mM 2-amino-2-methyl-1-propanol (pH 7.0–10.0). Self-cleavage reactions at 25°C were initiated by addition of divalent metal ion; chemical rescue reactions were at 37°C and initiated by addition of both divalent metal ion and exogenous base. Aliquots were quenched with an equal amount of stop mix containing 50% formamide and 50 mM EDTA and fractionated on denaturing polyacrylamide gels. Results were quantified by using a PhosphorImager (Molecular Dynamics).

Solvent Isotope Experiments. All solutions and buffers were prepared in 99.9 atom % D deuterium oxide (Sigma). The final pD of each reaction was determined with a standard glass electrode by adding 0.4 to the reading (23). The proton inventory experiments were done in nine compositions (23) of H_2O/D_2O at pL = 8.0 (plateau pH for both H_2O and D_2O reactions).

Data Analysis. Time courses of cleavage reactions were fit to a first-order single-exponential by using KALEIDAGRAPH data analysis/graphics application (Synergy Software, Reading, PA): $F_t = F_\infty \times (1 - \exp^{-k_{obs}t})$, where F_t and F_∞ are the fractions cleaved at time t and at the end point, respectively, and k_{obs} is the first-order rate constant. For the cleavage reactions of the C76 mutant ribozymes in the presence of exogenous base, the apparent second-order rate constant (k_{2nd}^{app}) with respect to base concentration is defined as $k_{2nd}^{app} = (k_{obs} - k_{uncat})/[\text{base}]_{total}$, in which k_{uncat} is the uncatalyzed first-order rate constant in the presence of divalent metal ion but absence of base. The second-order rate constant (k_b) with respect to the free base was determined as the slope from the plot of ($k_{obs} - k_{uncat}$) vs. $[\text{base}]_{free}$, by measuring k_{obs} at various total concentrations and pH of buffers.

Calculations of the Molecular Volume of the Bases. The molecular volumes of exogenous bases were calculated with ADVOL, developed by J. M. Word (Duke University), using coordinates generated by INSIGHT II (Molecular Simulations, San Diego).

Results

Rescue of Cleavage of C76 Mutants by Imidazole Buffer. Ribozymes used in this study were derived from the HDV antigenomic self-cleaving sequence (Fig. 1). The wild-type ribozyme, PEX1,
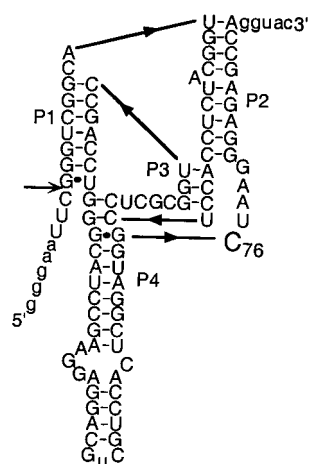

Fig. 1. Sequence and secondary structure of the HDV antigenomic ribozyme PEX1 with C76 emphasized. Numbering is from the cleavage site (indicated by an arrow) such that cleavage occurs between positions −1 and 1.

cleaves with a maximal first-order rate constant of 13.4 min^{-1} in 11 mM $MgCl_2$ and 1 mM EDTA at 25°C. Changing C76 to u (C76u) or deleting C76 (C76Δ) reduced cleavage activity ≈10[6]-fold from wild type in 11 mM $MgCl_2$ and 1 mM EDTA. Consistent with what was seen with C76u (16), cleavage activity of C76Δ was not stimulated by a higher concentration (50 mM) of divalent metal ions, but could be rescued by a combination of imidazole buffer and divalent metal ion (Mg^{2+} or Ca^{2+}). Also, a second-order plot of the cleavage rate constants k_{2nd}^{app} vs. fraction of imidazole free base showed that C76Δ cleavage in imidazole buffer followed the rate-law definition of general base catalysis (Fig. 2A).

Kinetic Solvent Isotope Experiments Revealed a Single Proton Transfer. To further establish that the ring nitrogen of C76 in the wild-type cleavage reaction and that of the imidazole base in C76Δ cleavage is involved in proton transfer at the transition state, the kinetic solvent isotope effect for both reactions was determined. The first-order rate constants ($k_{obs}^{H_2O}$ and $k_{obs}^{D_2O}$) for wild-type self-cleavage were measured over a pL range of 5.0 to 9.0 in H_2O and D_2O (Fig. 2B). For the C76Δ ribozyme, the apparent second-order rate constants ($k_{app,2nd}^{H_2O}$ and $k_{app,2nd}^{D_2O}$) were measured at a constant concentration of 0.4 M imidazole over a pH range of 6 to 9 (Fig. 2C). The KSIE was determined from the ratio of the two rate constants $[k(H_2O)/k(D_2O)]$ in the plateau region of the pH profile. KSIE for the wild-type cleavage and chemical rescue C76Δ cleavage was 2.3 (ΔpKa = 0.7) and 2.7 (ΔpKa = 0.5), respectively (Fig. 2 B and C). A ΔpKa ≈ 0.6 in both cases is the same as that predicted for an amino group involved in proton transfer (23). A similar KSIE seen with both the wild-type and the imidazole-rescued C76Δ reactions, with an apparent reaction pKa corresponding to the base, strongly supported the hypothesis that proton transfer at the chemistry step is directly involved with the cytosine side chain or exogenous imidazole buffer.

The partial KSIE (proton inventory) was determined for the wild-type cleavage reaction (23). The first-order rate constants, $k_{obs}^{H_2O/D_2O}$, were measured with varied compositions of H_2O and D_2O, and normalized to the rate constant in H_2O ($k_{obs}^{N_2O}$). A linear relationship ($n = 9$, $r = 0.996$) was obtained when the normalized rate constants ($k_{obs}^{H_2O/D_2O}/k_{obs}^{H_2O}$) were plotted against solvent isotopic composition n_{D_2O} [mol.(D_2O)/(mol.(H_2O) + mol.(D_2O))] (Fig. 2D). The lack of significant curvature in the plot suggested a single proton transfer at the transition state.

Shih and Been
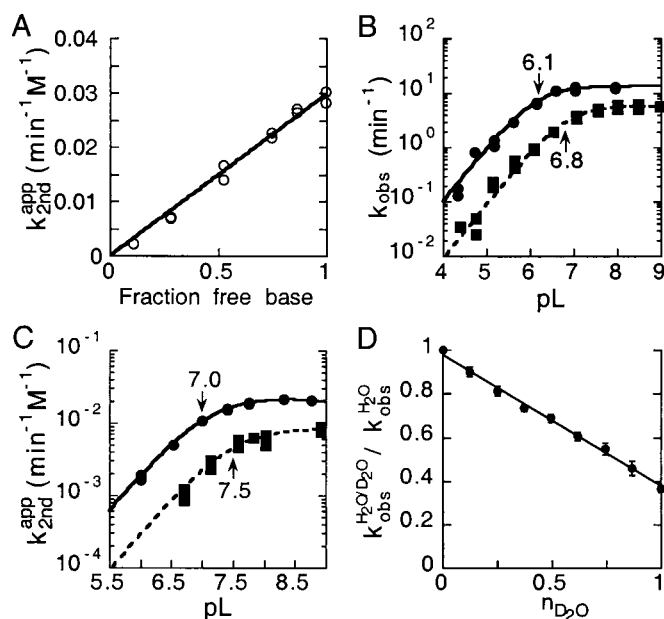
Fig. 2. Kinetic solvent isotope effect for wild-type and imidazole-rescue reactions of C76Δ. (*A*) Second-order plot of C76Δ cleavage by imidazole free base in H_2O. The apparent second-order rate constant (k_{2nd}^{app}) was plotted against fraction of free base. For wild type (*B*) and C76Δ (*C*), first-order (k_{obs}) and apparent second-order (k_{2nd}^{app}) rate constants in H_2O (○) and D_2O (□) were plotted against pL (L = H, D). A ΔpKa ≈ 0.6 was consistent with the expected value of 0.6 for an amino group. (*D*) Proton inventory of the wild-type PEX1 reaction. The first-order rate constants measured in varied solvent isotopic composition were normalized to the rate constant in H_2O ($k_{obs}^{H_2O/D_2O}/k_{obs}^{H_2O}$) and plotted against molar ratio of D_2O (n_{D_2O}). The data were fit to a linear dependence (*n* = 9; *r* = 0.99).

Table 1. Bases tested in rescue experiments and the apparent second-order rate constants k_{2nd}^{app}* for 76u and 76D cleavage at pH 7

	76u	76Δ
Cytosine analogues		
Cytosine (C)	50	120
Isocytosine	12	33
5-Methylcytosine	—[†]	—
5-Fluorocytosine	—	—
Pyridine	0.008	0.21
Purine		
Adenosine	—	—
Imidazole analogues		
Imidazole	32	15
1-Methylimidazole	—	—
2-Methylimidazole	—	—
1,2-Dimethylimidazole	—	—
4-Methylimidazole	4.5	10
4-Hydroxymethylimidazole	3.6	10
Histamine	0.069	2.5
Pyrazole	—	—
Histidine	0.049	0.82
3-Amino-1,2,4,-triazole	1.3	5.1

*k_{2nd}^{app} ($\times 10^3$ $min^{-1}\cdot M^{-1}$) was determined with reactions at pH 7 in 26 mM $MgCl_2$, with respect to the total base concentration, not to the free base component.
[†]— indicates no detectable rescue activity.

or base. For C76Δ cleavage in the presence of imidazole and imidazole analogues, where catalysis is contributed by the free base of the buffer, the second-order rate constant can be described as

$$k_{obs} = \frac{k_b}{1 + (10^{(pKa-pH)})} + k_{uncat}, \qquad [1]$$

in which k_{obs} and k_{uncat} are the first-order rate constants in the presence and absence of exogenous bases, respectively; k_b is the second-order rate constant with respect to the free imidazole base; pKa is the measured buffer pKa. The second-order rate constant, k_b, for each base was determined at various pH and total base concentrations and is listed in Table 2. Chemical rescue by imidazole and imidazole analogues (4-methylimidazole, 4-hydroxymethylimidazole, and 3-amino-1,2,4-triazole) was analyzed with a Brønsted plot based on the following equation

$$\log k_b = \beta \times pKa. \qquad [2]$$

Table 2. Reaction pKa and second-order rate constant k_b for 76u and 76D cleavage in the presence of 4-substituted imidazole analogues

			C76u		C76Δ	
	pKa	m.v.*	pKa	k_b	pKa	k_b
4-Methylimidazole	7.8	79	7.9	33	7.8	76
Imidazole	7.0	62	7.0	63	7.1	30
4-Hydroxymethylimidazole	6.5	84	6.6	5.0	6.6	14
3-Amino-1,2,4-triazole	5.5	67	5.4	1.3	5.5	5.3
Histamine	6.0	104	—	—	6.7	3.7

The second-order rate constants k_b ($\times 10^3$ $min^{-1}\cdot M^{-1}$) were determined from a plot of first-order rate constants against free base concentration. — indicates the numbers were not determined.
*Molecular volume of each base (m.v., Å³).

Chemical Rescue of C76 Mutants by Imidazole Analogues. To better define the participation of imidazole in HDV catalysis, several imidazole analogues were tested in cleavage of the C76 mutants. The ability of imidazole analogues to rescue cleavage of the C76 mutants correlated strongly with the position of substituents on the imidazole ring. All 1-, 2-, or 1,2-substituted imidazole analogues failed to rescue activity (Table 1). A subset of 4-substituted imidazole analogues were able to rescue activity of the C76 mutants and yielded ≈5 to 1,000-fold enhancement over the background rate (≈1 × 10⁻⁵ min⁻¹).

The concentration and pH dependence for cleavage of C76 mutants with 4-substituted imidazole analogues was similar to that of the imidazole-rescue reaction. For each of these analogues, the first-order rate constant (k_{obs}) showed a linear dependence on total base concentration up to 600 mM. The dissociation constant was estimated at >1.2 M (data not shown). Thus, all rates were determined under k_{cat}/K_M conditions with respect to imidazole analogue concentrations, and the apparent pKa of the C76u and C76Δ cleavage reactions corresponded to the pKa of the buffer (Table 2 and Fig. 3*A*). The second-order plot of k_{2nd}^{app} against fraction of free base exhibited a linear dependence with a *y*-intercept close to 0 (data not shown). These data suggested that rescue of C76u and C76Δ cleavage activity with 4-substituted imidazole analogues followed the rate-law definition of general base catalysis, with no acid catalysis from the buffer observed.

Brønsted Coefficient for C76 Mutants. In general acid-base catalysis, the efficiency of catalysis correlates with the strength of the Brønsted acid or base; that is, the second-order rate constant shows a log-linear relationship with the pKa of the Brønsted acid

Fig. 3. Brønsted analysis of C76Δ cleavage activity with imidazole analogues. (*A*) The apparent second-order rate constant k_{2nd}^{app} as a function of pH. The apparent pKa of each reaction is labeled for 4-methylimidazole (squares), imidazole (circles), 4-hydroxymethylimidazole (diamonds), and 3-amino-1,2,4-triazole (triangles). (*B*) The Brønsted plot of C76Δ cleavage in the buffers shown in *A*; Brønsted coefficient β of 0.51 is determined from the slope ($n = 4$; $r = 0.96$).

The Brønsted coefficient, β, determined for cleavage of the C76Δ ribozyme was 0.51 ($n = 4$, $r = 0.99$) (Fig. 3*B*). It is worth noting that the Brønsted coefficient reported here was not corrected for molecular volume of the bases (Table 2).[‡]

Chemical rescue of C76u cleavage by imidazole analogues showed several similarities to that of the C76Δ ribozyme. For each 4-substituted imidazole derivative the apparent reaction pKa corresponded to the buffer pKa (Table 2), and the second-order rate constant, k_b, was slightly lower than, but of the same order as, that of C76Δ (Table 2). However, for imidazole rescue of C76u, k_b was higher than that of C76Δ. When the imidazole data were omitted, the Brønsted coefficient determined for C76u, using the remaining points, was 0.53 ($n = 3$; $r = 0.99$), consistent with that determined for the C76Δ mutant. This result suggested that, with respect to buffer accessibility or binding, the active site of C76u might be more crowded than that of C76Δ.

Chemical Rescue of C76 Mutants by Exogenous Cytosine Bases and Analogues. To link catalysis of C76 mutants in imidazole buffer to wild-type self-cleavage, exogenous cytosine base was tested for rescue activity with the C76 mutants. In contrast to rescue with imidazole and its analogues, cleavage activity of C76Δ showed a saturating dependence on the cytosine concentration, suggesting a possible binding site for exogenous cytosine with a K_d of ≈40 mM at pH 7. The apparent pKa determined from the pH profile of the cleavage reactions with 50 mM cytosine was 5.2, which suggested a shift in pKa of about ≈+0.8 from the measured pKa for cytosine in solution (24). Cleavage of C76u in the presence of cytosine exhibited the same pH and concentration dependence, but at slightly lower efficiency for rescue (data not shown).

Several other cytosine analogues were tested with the C76 mutants (Table 1). Only isocytosine, a close structural analogue of cytosine, was able to rescue cleavage activity. However, it was less effective than cytosine. The concentration and pH dependence of isocytosine-rescued cleavage of C76Δ was similar to that of cytosine-rescued cleavage, giving an apparent binding constant of ≈40 mM and an apparent pKa of 5.0 in 50 mM

isocytosine. Similar results were seen for C76u cleavage (data not shown). Cleavage activity of C76 mutants was not rescued to a significant extent in the presence of pyridine, 5-methylcytosine, or 5-fluorocytosine (Table 1). Moreover, these bases inhibited cleavage activity of C76 mutants in the presence of cytosine or imidazole with a K_i of 20–30 mM (data not shown), suggesting that these bases might bind at the active site of the ribozyme, but were not able to support catalysis.

Discussion

The data presented here provide strong evidence that the cytosine side chain is involved in proton transfer at the transition state in the HDV ribozyme cleavage reaction. Previous studies have suggested that the chemistry step is rate-limiting for PEX1 self-cleavage.[§] Kinetic solvent isotope and proton inventory experiments indicate that, in the presence of 10 mM divalent metal ion, the rate-limiting step of wild-type antigenomic PEX1 ribozyme cleavage involves a single proton transfer at the transition state. The KSIE of ≈2.7 and ΔpKa of 0.7 observed with PEX1 is consistent with what is seen with the wild-type genomic ribozyme under similar conditions (17). However, these experiments alone do not identify the functional group in the ribozyme responsible for this proton transfer. Similar KSIE seen in the chemical rescue of C76Δ, but with an apparent pKa corresponding to the buffer pKa, strongly implicate γC75/C76 in proton transfer in the wild-type reactions. In addition, chemical rescue of C76Δ demonstrates a linear free energy relationship with the strength of the base (pKa). The Brønsted coefficient for the general base of 0.51 is consistent with a model, in which the ring nitrogen of imidazole is involved in proton transfer at the transition state. Moreover, cleavage rescued with exogenous cytosine in place of imidazole supports the proposal that chemical catalysis by imidazole buffer in the C76 mutants is analogous to the catalysis by the cytosine sidechain in wild-type cleavage.

An important objective of a mechanistic study is to assign catalytic roles to specific residues. Self-cleavage of the wild-type and C76a mutant ribozymes both demonstrated an apparent pH dependence that would be consistent with a model where the unprotonated ring nitrogen (N3 for C and N1 for A) accepts a proton and thus acts as a general base. A pH profile suggestive of base catalysis also is seen in the rescue of the C76 mutants by imidazole and cytosine. Furthermore, the second-order plot for catalysis in each imidazole buffer exhibits a linear dependence with respect to the free base component of the buffer, but no acid catalysis from the protonated form of the buffer. From the proton inventory of the wild-type reaction, the linear dependence of the rate on solvent isotopic composition indicates that there is only one proton "in flight" in the transition state. These observations all would be consistent with a mechanism of general-base catalysis in which the unsaturated ring nitrogen of cytosine (or imidazole) base accepts a proton from the 2′-OH group at the transition state to facilitate nucleophilic attack (Fig. 4*A*). However, these data also can be explained by a kinetically equivalent cleavage mechanism involving specific base and general acid catalysis. In this model, the 2′-OH nucleophile is deprotonated at pre-equilibrium (specific base catalysis) followed by donation of a proton to the 5′-leaving oxyanion by a protonated cytosine side chain or imidazolium at the transition state (general acid catalysis) (Fig. 4*B*). Specific base catalysis might be achieved by hydroxide anion accompanied by direct metal coordination to the 2′-oxygen, or by a hydrated metal hydroxide.

[‡]Brønsted analysis in an enzymatic system can be complicated by the molecular volume (m.v.) of the exogenous bases. The relationship of k_b vs. the pKa of the bases can be corrected for the m.v. of the bases using the following equation: log $k_b = \beta \times (pKa) + V \times$ (m.v.), in which the value of the coefficient V is negative. For the set of 4-substituted imidazole analogues used in this study, the m.v. varied less than 25%. Assuming a V of −0.02 (2–4) the Brønsted coefficient β, obtained by using the equation above, is 0.45 with $r = 0.87$.

[§]The behavior of the antigenomic self-cleaving ribozyme PEX1 used in these studies suggests that chemistry, rather than a folding step, is rate determining for cleavage. The evidence is as follows: (*i*) when properly prefolded, it cleaves to near completion (>95%) with a first-order single exponential kinetics and the observed rate constants are log-linear with pH values below the apparent pKa (16), (*ii*) no increase in activity is seen with this form of the ribozyme upon addition of denaturants to the reactions (22), and (*iii*) a modification of the linkage at the scissile phosphate slows the rate of the reaction (38).

Shih and Been
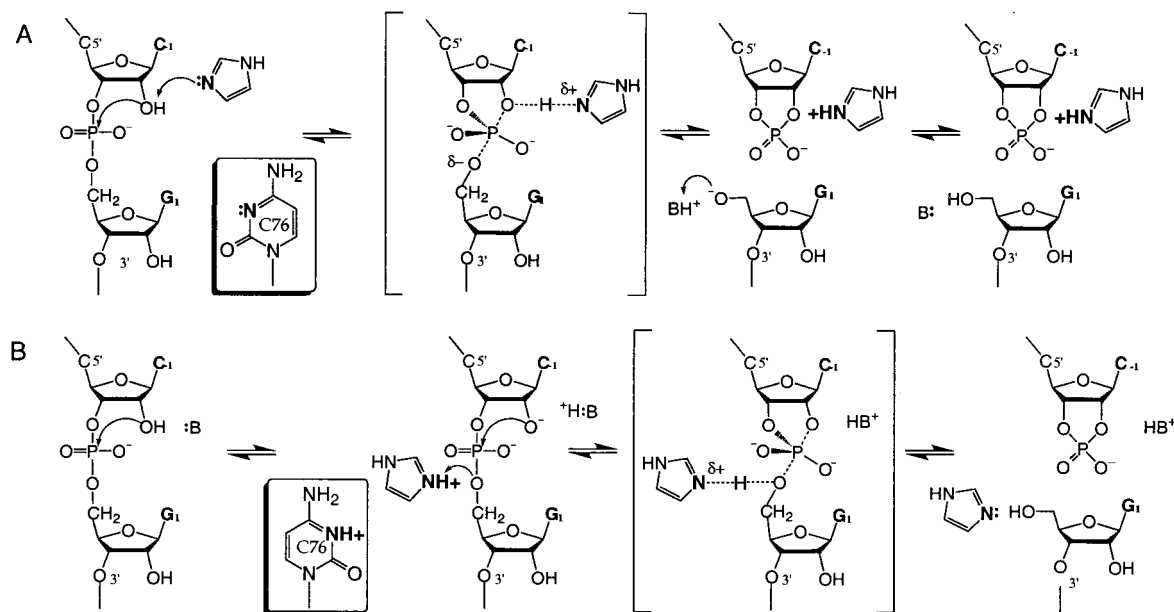
Fig. 4. Two kinetically equivalent mechanisms for general acid-base catalysis by RNA side chains or imidazole buffer. (*A*) In the C76u and C76Δ mutants, the unsaturated ring nitrogen of imidazole base accepts the proton from the nucleophilic group for general-base catalysis; in the wild type, cytosine serves a similar role. An unidentified functional group stabilizes the leaving group. (*B*) Imidazolium or protonated cytosine sidechain acts as a general acid by donating a proton to the leaving group. A specific base deprotonates the 2'-OH nucleophilic group at pre-equilibrium.

These two mechanisms, general base catalysis and specific base/general acid catalysis, are indistinguishable with the kinetic data presented here. However, it has been argued that the rate-determining step for cleavage of a RNA phosphodiester linkage is not formation of the 2'O-P but breaking of the P-5'O bond (see ref. 25 and references therein). Thus, general acid catalysis by the protonated cytosine or imidazolium to stabilize the 5'-leaving group in the transition state is an attractive model. Biochemical data in support of general acid catalysis was suggested from self-cleavage reactions of the wild-type genomic ribozyme in the presence of high concentrations of monovalent cation with no added divalent metal ion (17). Under these conditions, self-cleavage demonstrated a pH profile essentially reverse of that of the "standard" cleavage reactions containing divalent metal ions. The interpretation for this observation was that in the absence of base catalysis involving a hydrated metal hydroxide, the general acid mechanism of γC75 is unmasked. The apparent pKa of 5.7 determined from this reverse pH profile corresponds well to the apparent pKa of 6.1 determined under standard reactions conditions. However, whereas a γC75a mutant is active under standard conditions, there was no detectable activity with γC75a in the absence of added divalent metal ion. Therefore, it has not been possible to more definitively link the apparent pKa for cleavage activity in the absence of the added divalent metal ion to γC75. Wadkins *et al.* (26) recently have found that cleavage with a reverse pH profile in high concentrations of monovalent cation requires the presence of a specific trinucleotide sequence, γC41-A42-A43. This sequence is present in the wild-type genomic ribozyme but absent in the antigenomic ribozyme. Thus, the reverse pH profile alone does not justify assignment of a role of general acid to γC75/C76. However, a persuasive argument for a role of γC75/C76 as proton donor comes from the crystal structure of the 3' cleavage product of the genomic ribozyme; in the structure, the 5'-OH leaving group is within hydrogen bonding distance of N3 of the γC75 residue (12). Assuming that there is no major conformational change between structures of the transition state and product, this geometrical arrangement is strongly suggestive of a mechanism of acid catalysis involving γC75/C76 (17).

Difference in the concentration dependence of cytosine and imidazole analogues in the rescue reactions may reflect features of the active site. With cytosine and isocytosine, rescue of the C76 mutants demonstrated saturating concentration dependence. Moreover, the cytosine analogues that cannot rescue inhibit the cytosine-dependent reactions. These results would be consistent with a binding pocket for cytosine in these mutants. A higher second-order rate constant k_b for cytosine than imidazole (Tables 1 and 2) suggested that interactions of cytosine (or isocytosine) with other functional groups in the putative binding pocket might allow a more favorable orientation for the exogenous cytosine and hence increases efficiency of the chemical rescue. Interactions in the binding pocket for cytosine also could lead to better discrimination against cytosine analogues and might explain the observation that 5-methylcytosine, which has a pKa close to neutral pH, cannot rescue the C76 mutants.

With cytosine rescue of C76Δ, the reaction pKa (5.2) was higher than the pKa of cytosine in solution (4.4), but lower than the apparent pKa of the intact antigenomic ribozyme reaction (6.1) (16). In the crystal structure of the genomic 3' cleavage product, the N4-amino group of γC75 is involved in a hydrogen bonding triad with the 2'-O of γU20 and the nonbridging phosphate oxygen of γC22 (12). If this interaction forms at the transition state, it would increase electronegativity of the cytosine ring and contribute to a pKa shift of γC75. A very similar structure is likely to be found in the antigenomic ribozyme and would be consistent with a shift in the reaction pKa in the rescue reaction with exogenous cytosine. If, in the cytosine-rescue reaction, the apparent pKa reflects the pKa of bound cytosine, the shift in reaction pKa would be greatest when the binding site is saturated (k_{cat} conditions), whereas the apparent pKa should equal the solution pKa of cytosine under k_{cat}/K_M conditions. The cytosine concentration used in this study (50 mM) was at the practical upper limit under these conditions, thus these reactions were carried out at a concentration of cytosine that was sub-saturating but exceeding k_{cat}/K_M conditions ($K_M \approx 40$ mM).

The self-cleavage reaction of the HDV ribozyme, with formation of a 2',3'-cyclic phosphate and a 5'-hydroxyl, is very

similar to the first step of RNase A-catalyzed cleavage. In both cases the adjacent 2′-OH group serves as an intramolecular nucleophile. The classical mechanism for RNase A cleavage is concerted general acid/general base catalysis involving two histidine side chains (His-119 and His-12) that catalyze protonation of the 5′-oxyanion leaving group and deprotonation of the 2′-OH group. Based on this mechanism, a dianionic penta-coordinated phosphorane is proposed for the transition state, but mechanisms involving protonation of a nonbridging phosphate oxygen also have been proposed¶ (27–29). Consistent with the concerted mechanism proposed for RNase A, proton inventory of the RNase A reaction showed a quadratic dependence on isotope composition, indicating that two protons are transferred in the transition state (30). In contrast, the linear dependence on isotopic composition for the HDV ribozyme reaction suggests that a single proton is transferred in the transition state. This result would be inconsistent with a mechanism of concerted general acid/general base catalysis, but, given other data in this paper, would be consistent with a kinetically indistinguishable mechanisms of general base catalysis or sequential specific base/general acid catalysis. The latter mechanism would be very similar to the mechanism of general acid/general base catalysis proposed for the genomic ribozyme by Nakano *et al.* (17), except that deprotonation of the 2′-OH group would occur at pre-equilibrium, thus accounting for the proton inventory data.

Numerous studies have been directed at elucidating the details of general acid-base catalysis and the nature of the transition state for the class of reactions typified by RNase A cleavage. Charge distribution of the transition state in both enzymatic and nonenzymatic systems has been probed by using a linear free energy relationship. For a poor leaving group, such as the 5′-oxyanion in RNA cleavage, several model compounds yielded a highly negative $\beta_{\text{leaving group}}$ (β_{lg}) of −0.9 to −1.3 (25, 31), suggesting that the rate-determining step for this cleavage pathway would be breakdown of the penta-coordinated structure. Consistent with this idea, a β_{lg} of −0.54 to −0.59 was determined for an aryl phosphate diester that bears a good leaving group (32). It has been shown that the β_{lg} for a series of aryl-phosphate diester compounds is even less negative ($\beta_{\text{lg}} = -0.2$) in the active site of RNase A, suggesting a considerable contribution of electrophilic catalysis in the active site (33). Brønsted analysis using aryl-phosphate diester compounds gave a Brønsted coefficient, β, of 0.67 (α of 0.33) (32, 34). In the study presented here, a Brønsted coefficient was obtained by using a natural RNA phosphodiester as the substrate: for a mechanism of general base catalysis, β is 0.51, or for the kinetically equivalent mechanism, specific base/general acid catalysis, α is 0.49. These numbers suggest a proton transfer in the transition state with approximately half of the charge shared between the donor and acceptor atoms. This value agrees well with the value determined in the nonenzymatic system and is consistent with a model where the proton is "in flight" at the transition state (35). The β value for the HDV ribozyme is also comparable to that of other reactions in enzymatic systems with general acid-base catalysis by histidine and/or lysine side chains (β = 0.3–0.8) (2–4).

Chemical rescue experiments presented here suggested a linear free energy relationship between the cleavage rate and the strength of bases involved in general acid-base catalysis. Although additional work is required for more detailed understanding of the catalytic role of γC75/C76 in the HDV ribozymes, Brønsted analysis and proton inventory strongly support the hypothesis that the cytosine side chain is involved in the single proton transferred in the transition state of the wild-type cleavage. Using the linear dependence between the second-order rate constants of 4-substituted imidazole analogues and buffer pKa and the apparent reaction pKa of cytosine, the effective molarity of the C76 side chain in the antigenomic HDV active site is estimated to be $\approx 10^3$ M. This is comparable to 10^3–10^4 M for the intramolecular general acid catalysis observed in a model compound (36). In addition, Brønsted analysis for phosphodiester bond cleavage in RNA in the HDV ribozyme lends itself to detailed understanding of the structure of the penta-coordinated transition state in an enzyme-catalyzed system.

¶The alternative model proposed for RNase A involves additional protonation and deprotonation on nonbridging oxygens with a more stable monoanionic phosphorane intermediate (27–29).

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