The Hydrolysis of RNA: From Theoretical Calculations to the Hammerhead Ribozyme-Mediated Cleavage of RNA

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I. Introduction

The mechanisms of the enzymatic and nonenzymatic hydrolysis of RNA have been of great interest for several decades. The discovery of catalytic RNA molecules in the early 1980s attracted many scientists to the field of RNA research.[1,2] Before the revolutionary discovery of catalytic RNA molecules, it was assumed for many years that protein enzymes were the sole catalytic moieties in the cell and that nucleic acids provided only genetic information and structural frameworks. DNA is transcribed into RNA, which is then translated into protein, and it was accepted dogma that all the intermediate reactions were catalyzed by proteinacious enzymes. However, in the 1980s, the finding that RNA molecules can catalyze their own chemical transformation and that of other RNA molecules revealed that some RNA molecules can function biochemically as enzymes.[3] Such ribonucleotides with enzymatic activity (catalytic RNAs) are generally known as ribozymes. Catalytic RNAs include group I and II introns; the RNA subunit of RNase P; hammerhead, hairpin and hepatitis delta virus ribozymes; and ribosomal RNA (Figure 1).[1−7] Among these catalytic RNAs, a group I intron and the RNA subunit of RNase P were the two ribozymes that were discovered first[1,2] and the hammerhead ribozyme is the smallest natural ribozyme known to date.[6−9] The group I intron cata-

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Kazunari Taira is a Professor of Biological Chemistry at the Institute of Applied Biochemistry, University of Tsukuba. He holds an adjunct Project Leadership position at the National Institute for Advanced Interdisciplinary Research and also holds an adjunct Group Leadership position at the National Institute of Bioscience and Human Technology. He was born in 1952 in Shimabara peninsula, raised in the Goto Islands in Nagasaki, and was educated in the Nagasaki area, receiving an Associate degree in Chemical Engineering from Sasebo Technical College in 1973. He worked as a research assistant at the Nagasaki University under the direction of Professor Junzo Sunamoto. He then obtained M.S. (1981) and Ph.D. (1984) degrees in Chemistry from the University of Illinois under the supervision of Professors David G. Gorenstein and William L. Mock. After three years of postdoctoral work at Pennsylvania State University with Professor Stephen J. Benkovic, he returned to Japan and joined the faculty of the National Institute of Bioscience and Human Technology in 1987. In 1994 he was appointed Full Professor at the University of Tsukuba. The main focus of his research is the use of the tools of physical organic chemistry and molecular biology to study biochemical reactions. Around 1990, he became interested in the mechanisms of action of ribozymes and their possible application to antiviral gene therapies.

lyzes the ligation of adjoining exons, using the 3′-OH group of a guanosine cofactor as a nucleophile to mediate transesterification. The group II intron also ligates adjoining exons but it uses the 2′-OH group of an adenosine moiety within the intron to mediate the transesterification. RNase P simply hydrolyzes the phosphodiester backbone of precursors to tRNAs, while the smallest hammerhead ribozyme, named on the basis of the secondary structure of the catalytic domain, can be engineered such that it cleaves other RNA molecules.[8,9] Although less than two decades have passed since the discovery of ribozymes, studies of ribozymes have become very exciting. This rapidly developing field is of interest not only because of the intrinsic abilities of these fascinating molecules but also because of their potential as therapeutic agents that can inhibit gene expression.[10−14] The recent discovery of DNA enzymes, created by a selection procedure in vitro, has added even more excitement to this area.[15−24]

In terms of the reaction mechanism, large ribozymes such as the ribozyme of *Tetrahymena* and the catalytic RNA of RNase P use external nucleophiles. By contrast, small ribozymes, such as hammerheads and hairpins, use an internal nucleophile, namely the 2′-oxygen at the cleavage site, with resultant formation of a cyclic phosphate. The large ribozymes do not require a 2′-OH group at the cleavage site, and the ribozyme of *Tetrahymena* can cleave DNA substrates in addition to RNA.[25,26] The mechanistic details of the hydrolysis of RNA remain obscure, even in the case of the nonenzymatic hydrolysis of RNA, despite extensive efforts over many years[27−29] and the mechanistic details of the more recently discovered ribozyme-mediated cleavage of RNA also need to be clarified. In this review, we shall focus on the most recent reports of theoretical studies on RNA hydrolysis and we shall compare the conclusions from theoretical calculations with the available experimental data. Since we have been involved predominantly in research on hammerhead ribozymes, we shall emphasize the mechanism of their actions in this review. Some data can be interpreted differently from the way that we choose to interpret them. Therefore, it is left to the reader to judge whether extrapolations from theoretical calculations to experimental results are valid. We admit that some of our proposals related to the mechanism of action of ribozymes need to be validated by further experiments.

II. General Reaction Mechanisms

The hydrolysis of RNA at a particular site is initiated by nucleophilic attack by the 2′-oxygen (**1** → **2** in Scheme 1). In this step, the 2′-OH is activated by a base and the phosphodiester can be activated by transfer of a proton either prior to or simultaneously with the nucleophilic attack by the 2′-oxygen.[27−29] The expulsion of the 5′-oxygen of the next nucleotide produces the cyclic phosphodiester **3**, cutting the RNA chain at that position. In the second step, the cyclic phosphodiester is hydrolyzed to yield a 3′-phosphate end and regenerates the 2′-OH group (as well as 2′-phosphate and 3′-OH groups).

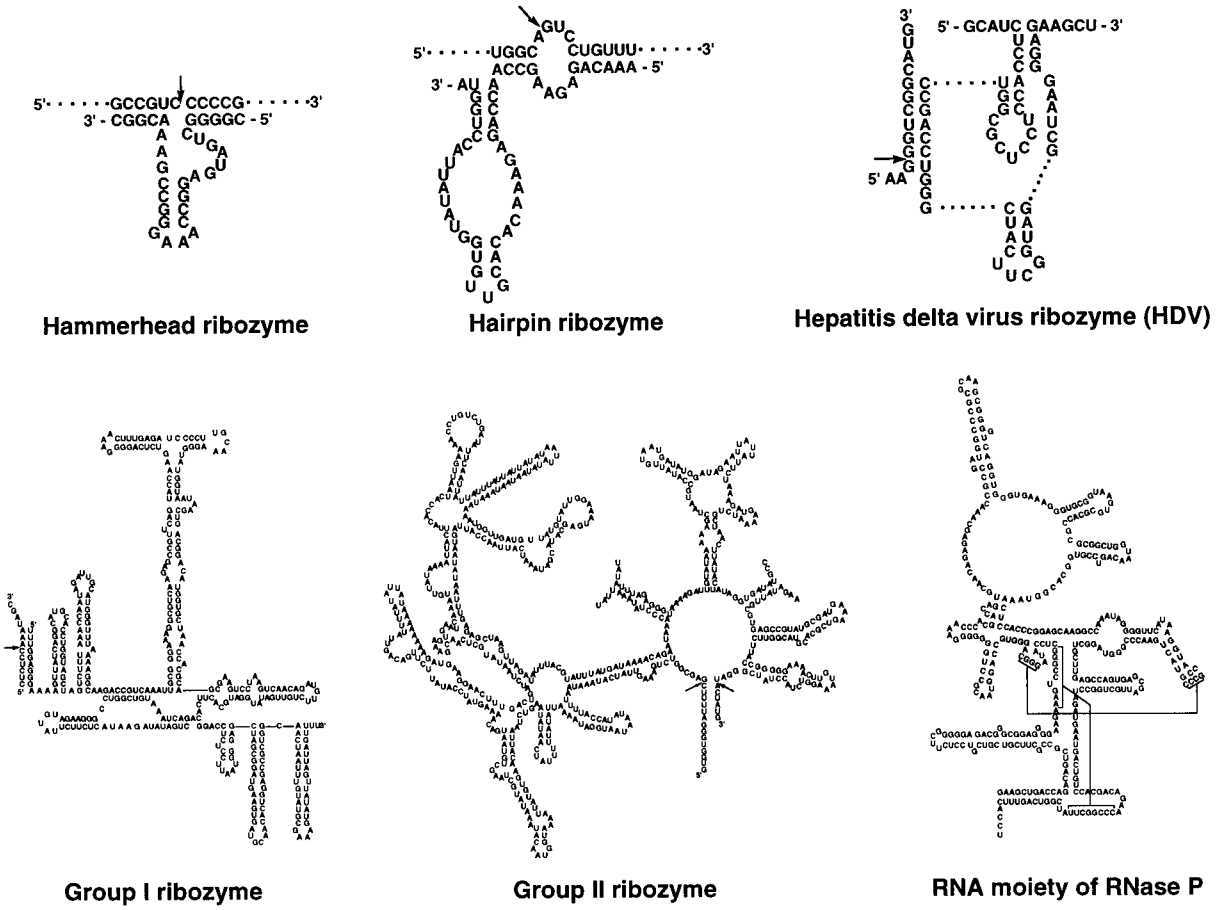
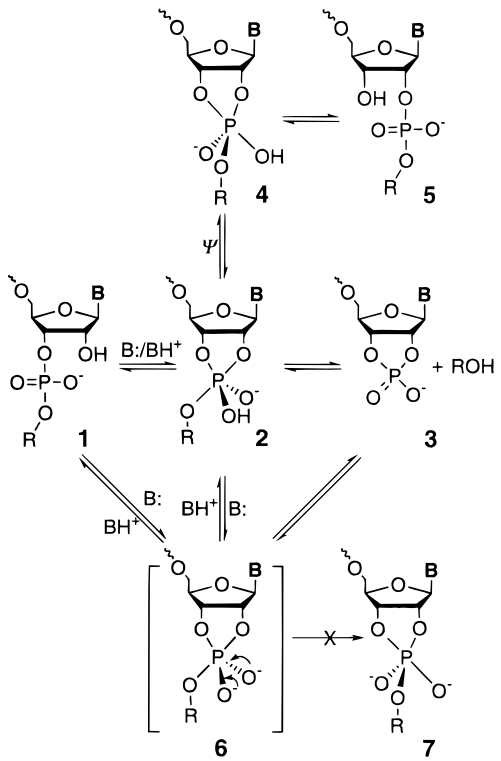


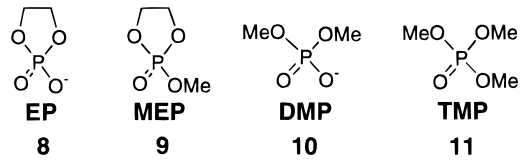
Figure 1. Secondary structures of ribozymes, namely, group I and group II introns, the RNA subunit of RNase P, and the hammerhead, hairpin, and hepatitis delta virus ribozymes.

Scheme 1



Under acidic conditions, the monoanionic structure **2** can undergo pseudorotation (Ψ) to give the pentacoordinate intermediate **4**, in which the electron-withdrawing 3′-oxygen and OH group occupy apical positions in accord with Westheimer's guidelines.[30] The presence of the pentacoordinate intermediate **4** under acidic conditions can be deduced from the identification of **5**, the migration product with 2′,5′-linkage. By contrast, under basic conditions, a dianionic intermediate or transition state **6** is formed. Even if **6** were an intermediate with a finite lifetime, pseudorotation to yield **7** would be prohibited because, in **7**, an electron-donating oxyanion must be placed at the apical position that would result in an extremely unstable species or transition state.[30,31] Unless **7** can be produced as an intermediate, no migration of the phosphodiester linkage (3′,5′-linkage → 2′,5′-linkage) can take place. Thus, the very high energy barrier for the pseudorotation (**6** → **7**) prevents the migration of the phosphodiester linkage under basic conditions. The absence of the migration product is often taken as evidence in support of the nonexistence of dianionic pentacoordinate intermediates, such as **6**. However, care must be taken in such an interpretation because, as mentioned above, even if **6** were to exist as an intermediate, the very high energy barrier for the pseudorotation (**6** → **7**) prevents the production of **7**, an intermediate or a transition state that is a prerequisite for the migration. As discussed below, we cannot completely exclude the possibility that dianionic species might be able to exist as pentacoordinate intermediates when they are well solvated.[32−34]

Model compounds for studies of the hydrolysis of RNA were investigated by Westheimer and Kluger et al. They examined the properties of five-membered cyclic phosphates, such as ethylene phosphate (EP, **8**) and methyl ethylene phosphate (MEP, **9**).[30,35,36] These cyclic phosphates are hydrolyzed 10^6-10^8 times faster than their acyclic counterparts, such as dimethyl (**10**) and trimethyl (**11**) phosphates, which are model compounds for studies of the hydrolysis of DNA. The differences in reactivity between the cyclic



and the acyclic compounds have been ascribed to ground-state destabilization that arises from ring strain in the cyclic phosphate, which is released in the trigonal-bipyramidal (TBP) transition state.[30,35-39] However, recent ab initio calculations suggest an alternative explanation, namely, that the difference in reactivity originates from differential solvation of the transition states.[40] In this review, we shall first discuss the properties of the cyclic phosphates.

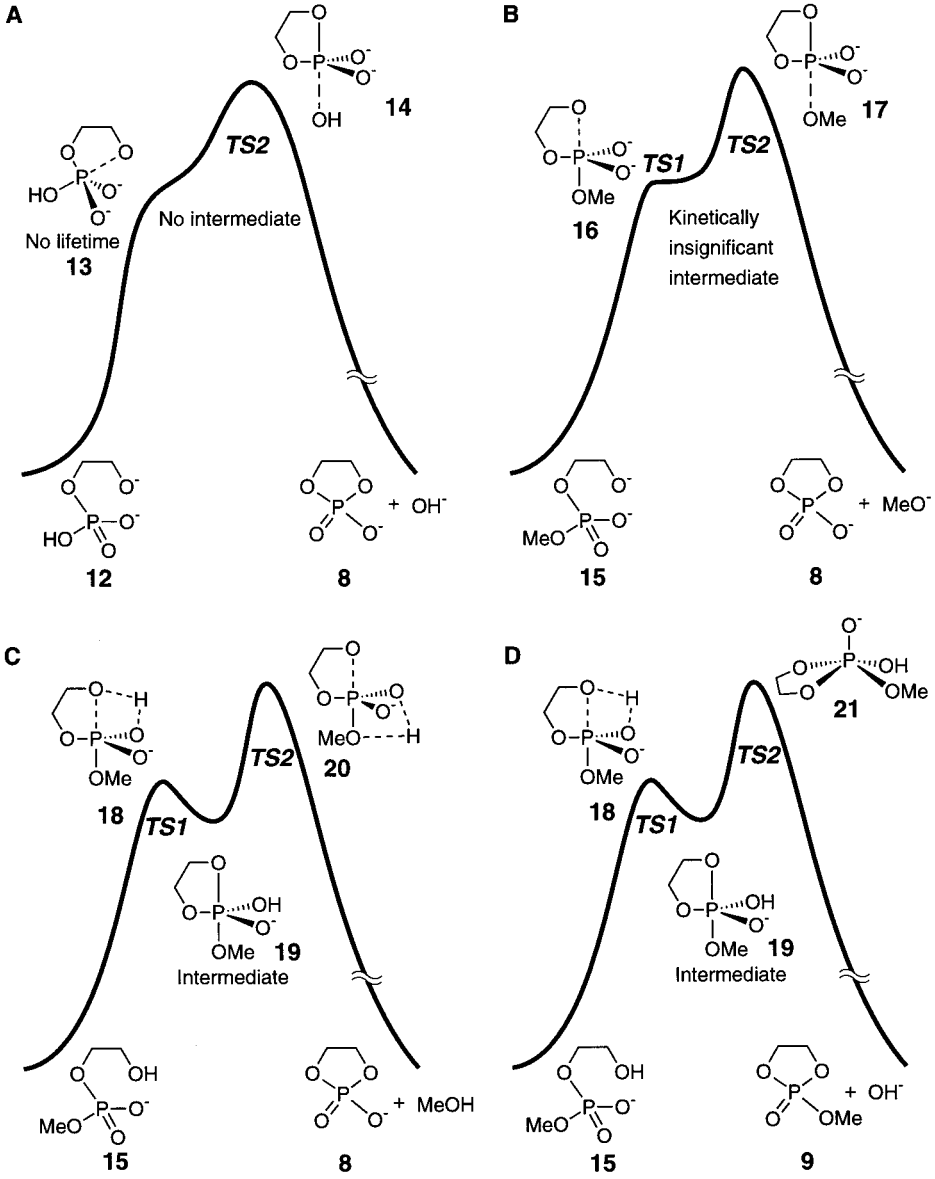
III. Theoretical Calculations

Over the last several years, numerous series of ab initio molecular orbital calculations[41] have been made on phosphoranes[31-34,40-64] and some of the results were used, by Karplus' and Lim's groups and also by our own group in attempt to elucidate mechanisms of RNA hydrolysis. The general conclusions drawn from the ab initio calculations on phosphates and phosphoranes can be summarized as follows (Scheme 2):

(1) The lifetime of phosphorane intermediates is dependent on the protonation state of the intermediates and on the molecular size of the phosphoranes, although phosphorane structures and energies are sensitive to the basis set used.[33,43,51,54,57,65]

(2) In the gas phase, dianionic phosphorane intermediates either do not exist or are only marginally

Scheme 2



stable and they are likely to be kinetically insignificant.[34,40,43,54] By contrast, monoanionic phosphoranes can exist as stable intermediates.[33,45,46,57]

(3) Solvation stabilizes phosphorane intermediates.[32,33]

(4) Regardless of the protonation state of the phosphorane intermediate, attack by the 2′-OH on the phosphorus atom [or cleavage of the P−O(2′) bond] is easier than cleavage of the P−O(5′) bond. (**TS2** is always higher in energy than **TS1**.) In other words, the structure of the rate-limiting transition state is susceptible to breakage of the P−O(5′) bond.[33,53]

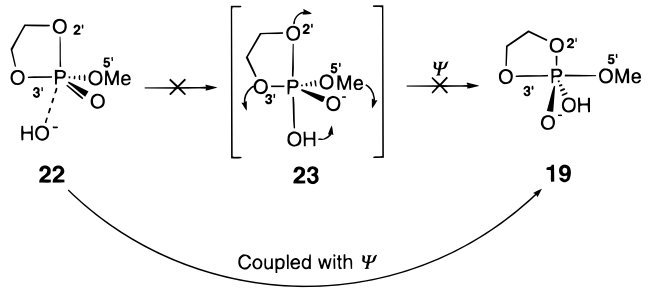
(5) Although the mode of cleavage conforms to stereoelectronic predictions,[53,54,66−69] the long-believed n−σ* orbital interaction [mixing of a lone-pair orbital (n) with the antibonding σ* orbital of an adjacent polar bond] is not the origin of the stereoelectronic effect.[49,60,62]

A. Relationship between the Protonation State and the Lifetime of the Pentacoordinate Intermediate

In theoretical studies of the hydrolysis of RNA, EP (**8**) and MEP (**9**) have been used as model compounds (Scheme 2).[33,34,40,42,44−48,53,57,61] For example, the base-catalyzed methanolysis of **8** (going from right to left in the energy diagram depicted in Scheme 2B) corresponds to the base-catalyzed hydrolysis of RNA in reverse (going from left to right). Early lower level ab initio calculations on the basic hydrolysis of dimethyl phosphate (DMP, **10**)[68,70,71] and on the base-catalyzed methanolysis of EP (**8**)[53] identified dianionic phosphorane intermediates with two separate transition states (**TS1** and **TS2**) with deep wells. However, higher level ab initio calculations showed that these stable intermediates were artifacts of the minimal basis set.[33,40,42,43,54] Although, in the reaction **15** → **8** in Scheme 2B, both a dianionic phosphorane intermediate and two transition states (**TS1** and **TS2**) were identified by higher level ab initio calculations in the gas phase,[33] the intermediate had a well depth of the order of k_BT, which is unlikely to be kinetically significant. In the reverse reaction (**8** → **15**), the transition state has to accommodate a charge of −2, while the reactants only have a charge of −1. It is possible that further improvements in the basis set or inclusion of better electron correlations might result in the complete disappearance of the dianionic intermediates.[40] A similar pentacoordinate dianionic **13** (Scheme 2A), which is a smaller molecule than **16** (OH vs OMe), does not exist as an intermediate at all.[42] Monoanionic phosphorane intermediates (**19**), which have been postulated in the acid-catalyzed hydrolysis,[30,35,37] have been identified as more stable species in the gas phase, even by higher level ab initio calculations (Scheme 2D).[45,57]

Pseudorotation (Ψ, Scheme 3) in pentacoordinate species is defined as an intramolecular process whereby a TBP (**23**), which might be short-lived, is converted into another isomer by deformation of angles such that the final TBP (**19**) appears to have rotated through 90° relative to the initial state.[30,72] The Berry pseudorotation process (Ψ) involves the

Scheme 3



simultaneous inward bending of bonds of both apical ligands (2′-O and OH in **23**) and the outward bending of two equatorial ligands (3′-O and 5′-OMe in **23**), with one equatorial ligand (O^-), called the "pivot", remaining at the equatorial position, to yield another TBP (**19**) in which the apical (3′-O and 5′-OMe) and equatorial (2′-O and OH) ligand pairs have exchanged positions. In the base-catalyzed hydrolysis of MEP (**9**), Lim and Tole identified a monoanionic phosphorane intermediate (**19**) in the gas phase (**9** → **19** in Scheme 2D and **22** → **19** in Scheme 3).[46,48] However, hydroxyl attack of MEP (**9**) occurred in concert with pseudorotation (through a square-pyramidal transition state, such as **21**) to yield the TBP intermediate **19** directly, with the hydroxyl group (OH) in an equatorial position, without formation of the monoanionic TBP intermediate **23** in which the hydroxyl group (OH) occupies an axial position.

Except in the case of **23**, the general conclusions of the ab initio calculations are that (i) dianionic phosphorane intermediates either do not exist in the gas phase or are only marginally stable and are likely to be kinetically insignificant, and that (ii) monoanionic phosphoranes, by contrast, can exist as stable species in the gas phase.[45,57] Further analysis has indicated that the stability or the lifetime of phosphorane intermediates in the gas phase is also dependent on the molecular sizes of pentacoordinate species (in addition to the basis set used).[33] Recall that although the dianionic species **13** does not exist as an intermediate, a slightly larger molecule **16** does start to become a marginally stable intermediate, most probably because the transition state has to accommodate a charge of −2, while the reactants have only a charge of −1. Thus, those pentacoordinate species that are large enough to delocalize a charge of −2 can exist as TBP intermediates. This interpretation is supported by the observations that whereas the simplest possible dianionic pentacoordinate species **24** does not exist as an intermediate, the solvated form of the dianionic pentacoordinate species **24** with six water molecules, namely **25**, does exist as a TBP intermediate in the gas phase (Figure 2).[32,33] Intermediate **25** can exist because, in this molecule, a charge of −2 can be delocalized over the six solvated water molecules. Thus, the possibility exists that much larger RNAs can have a dianionic TBP intermediate during hydrolysis in alkaline solutions (under well-solvated conditions), although the question remains if the dianionic phosphoranes are long-lived enough to be protonated.

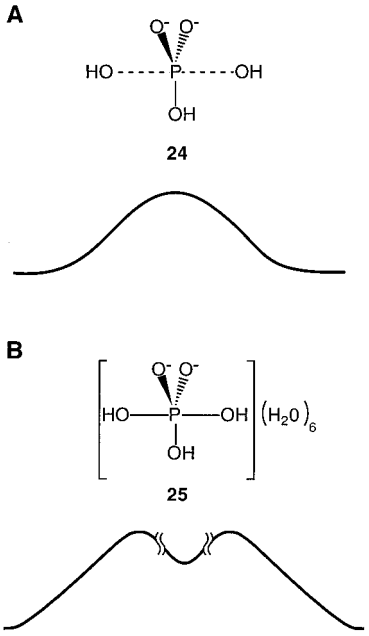


Figure 2. Schematic representations of reaction profiles for $OH^- + H_2PO_4^- \rightarrow [H_3PO_5^{2-}] \rightarrow H_2PO_4^- + OH^-$. (A) Ab initio calculations indicate that the dianionic TBP intermediate does not exist along the gas-phase reaction coordinate. Thus, the reaction takes place via a single-step mechanism in the gas phase. (B) By contrast, solvation with water molecules allows a dianionic oxyphosphorane species to exist as the TBP intermediate.

B. Structures of Transition States in the Gas Phase

As can be seen in Scheme 2, regardless of the protonation state of the TBP intermediates, cleavage of the exocyclic P−O bond (or intermolecular attack by the nucleophile on the cyclic phosphate in reverse) with the **TS2** transition state is more difficult than the cleavage of the endocyclic P−O bond (or intramolecular cyclization process) with the **TS1** transition state.[33] Therefore, the overall rate-limiting step in the reaction is governed by **TS2**. The relative stabilities of **TS1** and **TS2** are not sensitive to the basis set used and, thus, **TS2** is always higher in energy than **TS1** for all levels of calculation.[33,53] In contrast to the common belief that ring strain in cyclic phosphates is relieved upon formation of a TBP intermediate, Karplus' and Lim's groups found that the geometry of the TBP intermediate showed evidence of ring-strain in the gas phase.[40,44,48] Such strain causes the P−O (apical) bond in the ring to be weaker than the P−O bond outside the ring, which, in turn, results in a lower barrier for ring opening relative to cleavage of the exocyclic P−O(apical) bond.

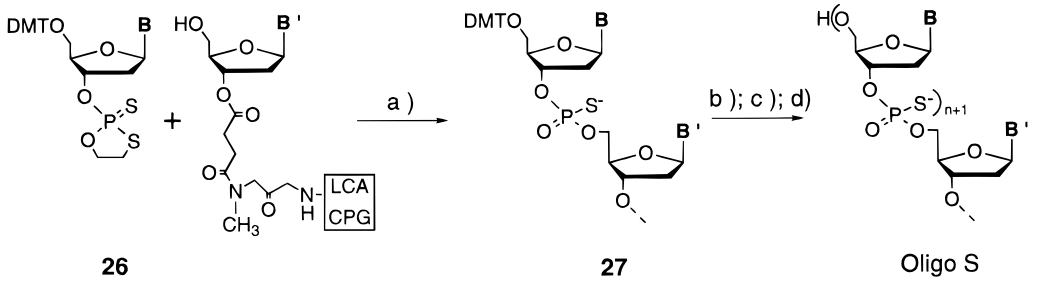
Theoretical calculations on cyclic as well as acyclic phosphoranes revealed that energies of phosphoranes are dependent on the conformation of the equatorial P−O bond and, moreover, that gas-phase reaction profiles are in accord with stereoelectronic effects, in that energetically favorable structures of transition states have lone pairs on their equatorial oxygens, which are antiperiplanar to the nucleophile or leaving group.[66−69] Nonetheless, the long-believed n−σ* orbital interaction has been found not to be the origin of the stereoelectronic effect (a detailed analysis can be found in ref 60).[49,60,62]

It is to be emphasized that, even in the case of concerted reactions without the formation of TBP intermediates as shown in Scheme 2A, the single transition-state structure is practically identical to **TS2**, in that the translating bond is the exocyclic P−O(apical) bond.[33] In other words, the imaginary frequency calculated by normal coordinate analysis of force constants corresponds to the atomic motion that indicates formation or cleavage of the exocyclic P−O(apical) bond (**TS2**), rather than that of the endocyclic P−O-(apical) bond in the ring (**TS1**). Therefore, the overall transition-state structure in the hydrolysis of RNA is **TS2**, regardless of whether the reaction proceeds via a concerted one-step mechanism or via a two-step mechanism with a stable phosphorane intermediate.

In summary, the ab initio energy profiles for phosphorane species (Scheme 2) indicated that it should be more difficult to cleave the exocyclic P−O(apical) bond than the endocyclic P−O(apical) bond, at least in the gas phase. This conclusion is applicable to the in-line processes for all cyclic phosphoranes examined, regardless of the protonation state and of the level of computation. Thus, **TS2** should be the rate-limiting overall transition state in the gas phase. We will demonstrate in the later section V.A that this conclusion is also valid for the hydrolysis of RNA in solution. It is noteworthy that this finding provides a reasonable interpretation for the results of mutagenesis of RNA-cleaving proteinacious enzymes, such as Barnase and RNase T_1. Those RNases require both acid and base catalysts, with stabilization of **TS2** and **TS1**, respectively, for the digestion of ribonucleic acids. Mutagenesis of Barnase to remove the general base (Glu73 → Ala73) resulted in some residual activity since the mutant enzyme still had a general acid (His102) to stabilize **TS2**. Removal of the general acid (His102 → Ala102) completely abolished the enzymatic activity.[73,74] Similar results of mutagenesis, indicating that the general acid catalysis is even more important than the general base catalysis, were also obtained in the case of RNase T_1, in which either Glu58 or His40 acts as a general base (stabilizing **TS1**), while His92 acts as a general acid (stabilizing **TS2**).[75,76] Thus, protonation of the 5′-leaving oxygen, which lowers the overall energy barrier of the transition state **TS2**, appears to be essential for enzymatic cleavage of a phosphodiester bond in an RNA molecule.

In mechanisms in which departure of the leaving group is kinetically significant, a metal ion should improve the departure ability of the leaving group.[77] Such a role for a metal ion is analogous to the role of the proton in acid catalysis and it takes advantage of the metal ion's ability to function as a Lewis acid. Our ab initio calculations indicated that a divalent Mg^{2+} ion can act as a Lewis acid by directly coordinating to the 5′-leaving oxygen and, thus, it can stabilize the **TS2** transition state in reactions catalyzed by ribozymes.[61] The experimental evidence for the direct coordination of a metal ion with the 5′-oxygen has been recently published (see sections VII.D and VII.E for details).[78,79]

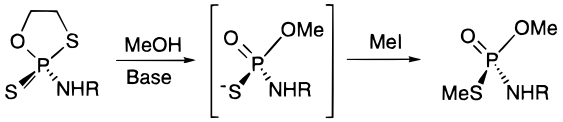
Scheme 4



C. Stabilities of Related Trigonal-Bipyramidal (TBP) Intermediates

The stereospecifically controlled synthesis of nucleic acids with phosphorothioate linkages is of particular current interest because of their potential utility as antisense molecules.[10−14,80] Recently, Stec's group developed an elegant method for the synthesis of diastereomerically pure nucleotides with 3′,5′-phosphorothioate linkages (Scheme 4).[80−84] Separated diastereomer **26** produced **27** with stereoselectivity above 98%. Model studies of **28** (an analogue of **26**) showed that the ring opening with MeOH/DBU occurs with retention of the configuration at the phosphorus atom to yield **30** (Scheme 5).[82] We

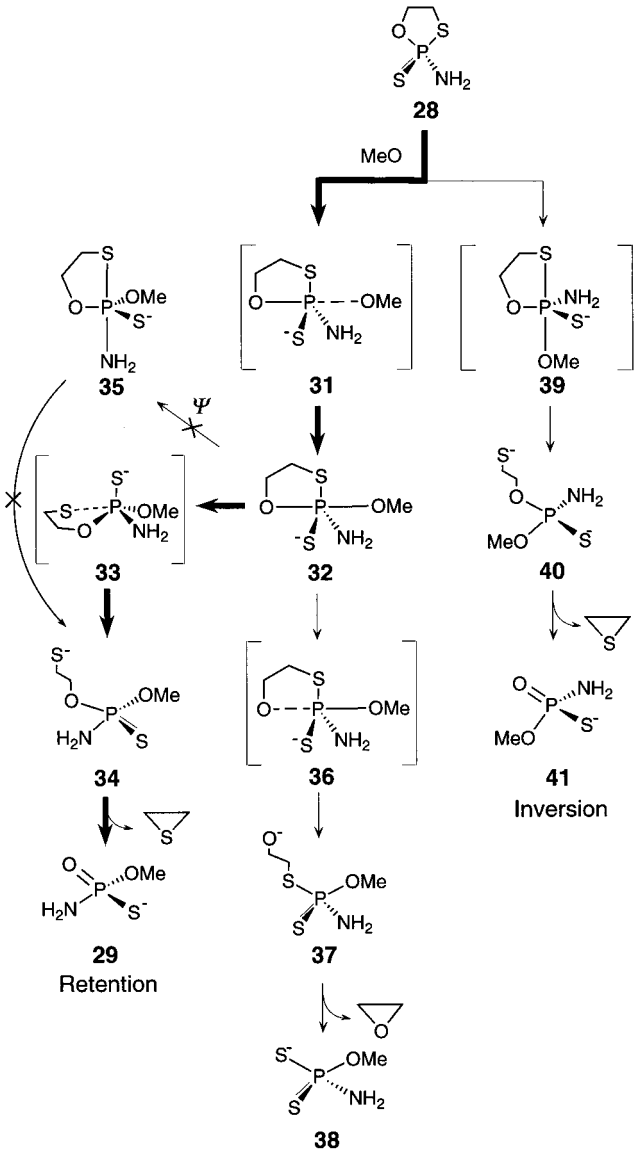
Scheme 5



performed ab initio calculations to examine the reaction pathway for the base-catalyzed ring opening (methanolysis) of **28**.[63,64] The calculated lowest energy reaction pathway is indicated by thick arrows in Scheme 6.

At first glance, according to accepted guidelines,[30] the reaction pathway **28** → **31** → **32** → **35** → **34** → **29** seemed plausible as an explanation for the high chemo- and stereoselectivity of the reaction. However, the drawback to this pathway is that the TBP intermediate **35**, which results from **32** after pseudorotation, would be expected to be significantly unstable as compared with the original TBP intermediate **32**. This is because, although the oxygen ligand is "apicophilic", sulfur and nitrogen ligands are more "equatoriphilic" [52] than the oxygen ligand. Application of Holmes' model for the calculation of conformational energies in pentacoordinate phosphorus compounds[85] suggested that the energy of **35** was 10 kcal/mol higher than that of **32**. Indeed, **35** could not exist as an intermediate and the lowest-energy reaction pass was found to be **28** → **31** → **32** → **33** → **34** → **29**, in which cleavage of the P−S bond was coupled with pseudorotation without formation of **35**.[63,64] This concerted pseudorotation and cleavage of the equatorial P−S bond is analogous to the concerted pseudorotation and formation of an equatorial P−OH bond, as depicted in Schemes 2D and

Scheme 6



3,[46,49] and it is also analogous to other related systems,[50] all of which provide a plausible mechanism for substitution via retention of the configuration at the phosphorus atom in the gas phase. In the case of the Stec reaction, namely, the base-catalyzed ring opening of **28** (Scheme 6), qualitative experimental data exists to support the calculated reaction pathway and proposed mechanism.[80−84]

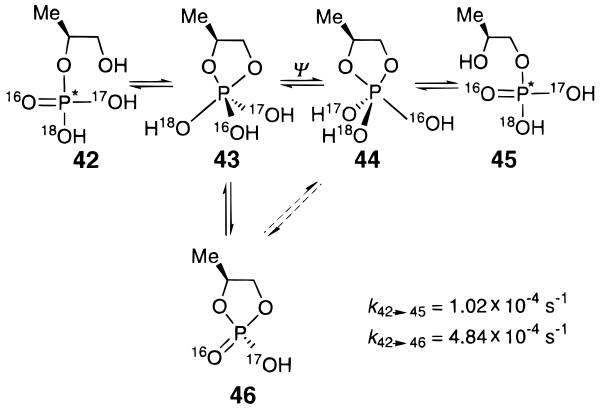
In Scheme 6, identified structures of transition states are indicated in square brackets. The energy of the TBP transition state **39** was found to be more than 6 kcal/mol higher than that of **31**, the difference between the two structures being the positions of endocyclic sulfur and oxygen atoms. Since the energy of **39** is higher than that of **31**, the reaction cannot proceed with inversion of the configuration via **39**. It must be emphasized that no TBP intermediates exist with sulfur at an apical position. Therefore, pentacoordinate species with an apical sulfur must be unstable. We will return to this point when we discuss nonenzymatic and ribozyme-catalyzed hydrolysis of 5′-thio substrates in sections V.A and VII.A.

IV. Hydrolysis of Cyclic Phosphates

A. Evidence for Phosphorane Intermediates in Acids

As indicated in Scheme 1, the hydrolysis of RNA under acidic conditions produces migration product **5**. A detailed kinetic analysis of this process was made by Lönnberg's group (for more details, see this group's review in this issue).[28,29,86−90] The migration proceeds via phosphorane intermediate **2**, which has to pseudorotate at least once (**2** → **4**) to yield **5**. The stereochemistry of a related migration process was investigated by use of a model compound with a chiral phosphate (Scheme 7).[91,92] The migration **42**

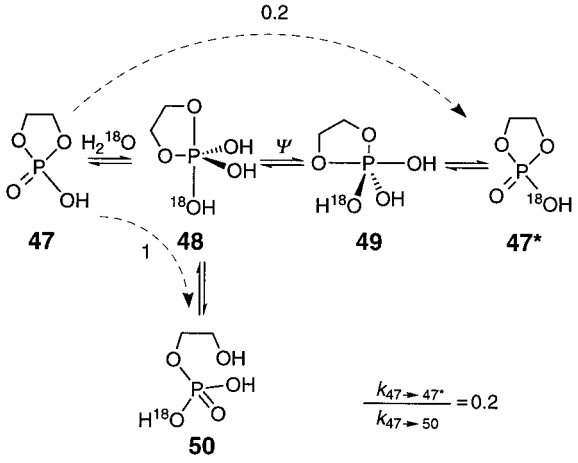
Scheme 7



→ **43** → **44** → **45** has been shown to proceed with retention of the configuration at the phosphorus atom, supporting the existence of phosphorane intermediates and a pseudorotation process. Under acidic conditions (0.5 N $HClO_4$), the ratio of the rate of migration (**42** → **45**) to that of hydrolysis (**42** → **46**) was approximately 1:5. This result demonstrates that the rate of pseudorotation (**43** → **44**) is slower than that of cleavage of the exocyclic P−OH bond (**43** → **46**) because, as discussed above, the rate of cleavage of the endocyclic P−O bond (**44** → **45**) should be faster than that of cleavage of the exocyclic P−OH bond (**43** → **46**) and, therefore, the former process (**44** → **45**) should not be the rate-limiting step in the migration of the phosphate group (**42** → **45**).

Similar results were obtained for the acid-catalyzed hydrolysis of ethylene phosphate (Scheme 8). When

Scheme 8



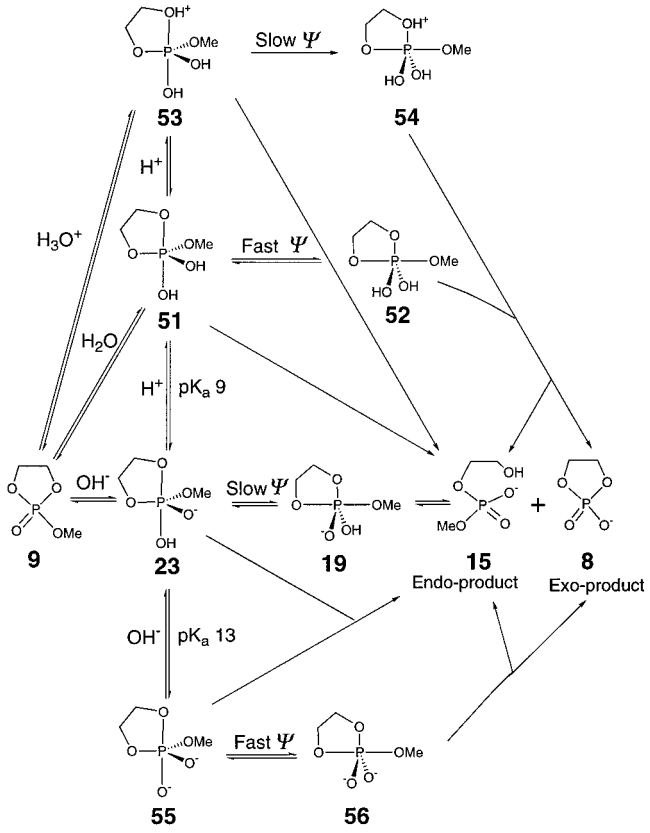
47 was hydrolyzed in isotopically labeled water ($H_2{}^{18}O$) under acidic conditions, hydrolysis to yield **50** was accompanied with oxygen (^{18}O) exchange in the reactant (**47** → **47***). Under acidic conditions, the ratio of the rate of ^{18}O exchange (**47** → **47***) to the rate of hydrolysis (**47** → **50**) was approximately 1:5, being almost identical to the ratio found in Scheme 7. This result again suggests that the rate of pseudorotation (**48** → **49**) is slower than the rate of cleavage of the endocyclic P−O bond (**48** → **50**) although, in this system, since the rate of cleavage of the endocyclic P−O bond (**48** → **50**) should be faster than the rate of cleavage of the exocyclic P−OH bond (**49** → **47***), we cannot exclude the possibility that the exocyclic cleavage (**49** → **47***) might be part of the rate-limiting step for the ^{18}O exchange (**47** → **47***). More detailed studies by Lönnberg's group demonstrated that, for RNA, hydrolysis is much slower than migration at pH 4−7, while, at pH < 2, hydrolysis and migration are comparable. At even more acidic conditions, hydrolysis becomes faster than migration.[86−90]

Results of Lönnberg's group and from an examination of Schemes 7 and 8 demonstrate that (i) the pseudorotation process can be slower than cleavage of the P−O bond under strongly acidic conditions and (ii) phosphorane intermediates exist under acidic conditions. We emphasize here that neither migation of the phosphoryl group nor ^{18}O exchange occurs under basic conditions: these phenomena are often taken as evidence to support the nonexistence of dianionic phosphorane intermediates, as discussed in section II under "General Reaction Mechanisms". We shall now examine the possibility of the existence of dianionic phosphorane intermediates under basic conditions.

B. Hydrolysis of Methyl Ethylene Phosphate (MEP) and the Possibility of Dianionic Phosphorane Intermediates

The pioneering work on the hydrolysis of MEP (**9**) by Kluger and Westheimer and their colleagues also suggested that the pseudorotation process is slower than cleavage of the P−O bond under acidic conditions, as well as under weakly basic conditions (Scheme 9 and Figure 3).[30,35−37] As depicted in

Scheme 9



Scheme 9, phosphoranes **23**, **51**, **53**, and **55** produce only the endocyclic product **15**. Formation of the exocyclic product **8** (together with **15**) is possible only after pseudorotation and from **19**, **52**, **54**, and **56**. In the graph of the extent of exocyclic cleavage vs pH (Figure 3), the decrease in formation of exocyclic product **8** in the region from pH 2 to strong acid was explained on the basis of the assumption that, although the hydrolysis of **9** is acid-catalyzed via the formation of **53**, pseudorotation to yield **54** is slower than the cleavage of the endocyclic P−O bond and, therefore, the former process becomes rate-limiting. Therefore, as the acidity is increased, a point is reached at which the rate of pseudorotation and, hence, of exocyclic cleavage fails to keep pace with the rate of ring opening, so that the yield of exocyclic products falls toward zero. Thus, in strong acid, the major reaction pathway is **9** → **53** → **15**.

Around pH 2, the maximum extent of exocyclic cleavage (∼50%) was observed to occur via the major pass **9** → **51** → **52** → **8**, where the rate of pseudorotation of **51** to yield **52** was assumed to be high (compared with the rate of cleavage). Since the extent of exocyclic cleavage was near zero in the

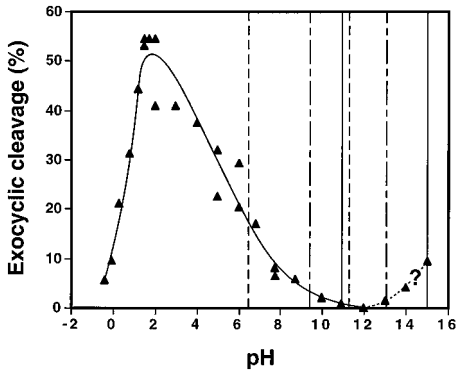
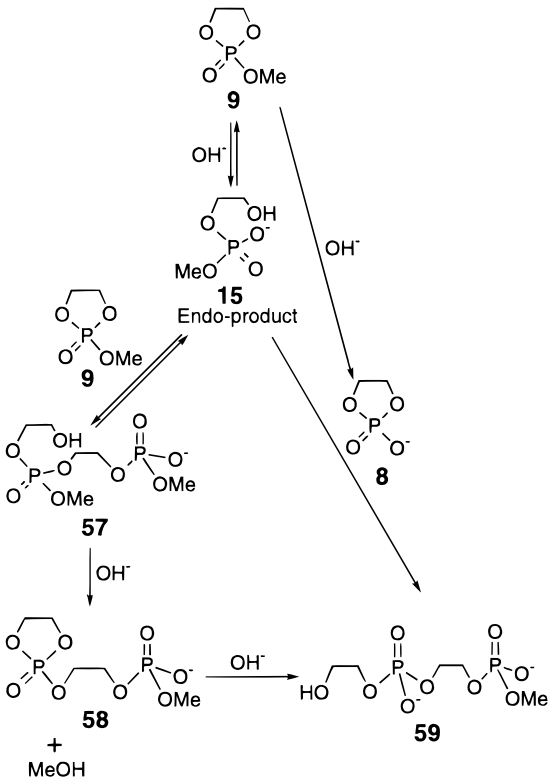


Figure 3. Plot of the % exocyclic cleavage of MEP, **9**, as a function of pH. The first and the second pK_a values for the phosphorane intermediate determined by Guthrie (− − −), by Westheimer et al. (— - —), and by Anslyn (—) are also indicated (adapted from ref 27). Measurements of the absolute amounts of the exocyclic products **8** and methanol in the region of strong alkali (indicated by - ? -) are technically difficult because of the high reactivity of **9**. The products of hydrolysis of **9** produce acids in the microenvironment. Analysis based upon more recent data from ^{31}P NMR spectroscopy indicates that the extent of exocyclic cleavage of **9** during hydrolysis in 1−9 M NaOH is ∼3 ± 1.5%.[95]

region from pH 8 to pH 13, where monoanionic phosphoranes **23** and **19** are important species, the rate of cleavage of **23** was assumed to be very much greater than the rate at which **23** pseudorotates to yield **19**. Therefore, from pH 4 to pH 11, the fraction of product formed as a result of exocyclic cleavage decreased from about 50% to close to zero because the reaction pathway changed from **9** → **51** → **52** → **8** to **9** → **23** → **15**.

The increase in the extent of exocyclic cleavage above pH 13 can be explained by the rapid pseudorotation of **55** to yield **56**. In the case of the dianionic phosphorane **55**, since one of the electron-donating oxyanions occupies the apical position, the compound is not stable and, thus, it pseudorotates to produce the more stable dianionic phosphorane **56**. Measurements of the absolute amounts of the exocyclic products **8** and methanol in strong alkali are technically difficult because of the high reactivity of **9**. The products of hydrolysis of **9** produce acids in the micro environment.[35,36,68,93−98] An increase in concentration of **9**, to increase the detectability of the products, causes formation of the dimer **59** (Scheme 10).[68] The dimer is formed by the reaction of product of the endocyclic cleavage **15** and unreacted **9**. The initial dimer **57** contains one diester phosphoryl group and one triester phosphoryl group. Whereas the diester phosphoryl group is hydrolyzed very slowly, as is **15** in alkaline solution, the rate of hydrolysis of the triester phosphoryl part is much higher. In addition to the higher intrinsic reactivity of the triester phosphoryl group, as compared with the diester phosphoryl group, the rate of hydrolysis of **57** is significantly enhanced by the anchimeric acceleration by the β-hydroxyl group. With anchimeric acceleration by the β-hydroxyl group, **57** cyclizes to generate methanol and the cyclic triester dimer **58**. Further rapid hydrolysis of **58** finally yields the phosphodiester dimer **59**.[68] The final phosphodiester dimer **59**

Scheme 10



can, in principle, be formed by an alternative pathway, namely, by the reaction of **15** with the initial product of the exocyclic cleavage **8**.[36] However, the former mechanism appears to be more attractive and more likely to be operative because (i) the neutral triester **9** is much more reactive than the monoanionic diester **8** and (ii) during most of the reaction, the concentration of **9** is much higher than that of **8**.[68] If the extent of exocyclic cleavage in alkaline solution were followed by quantitation of methanol, which would also be generated by a route other than the exocyclic cleavage of **9**, namely, by hydrolysis of the triester dimer **57**, the analysis would yield an erroneous overestimate of the amounts of products of exocyclic cleavage. Analysis based upon more recent data, obtained by ^{31}P NMR, indicated that the extent of exocyclic cleavage of **9** during hydrolysis in 1−9 M NaOH was ∼3 ± 1.5% (Figure 3).[95]

The formation of the product of exocyclic cleavage **8** at a level of ∼3 ± 1.5% in strong alkali requires the existence of dianionic phosphorane intermediates, such as **55** and **56** in Scheme 9, unless the hydroxide attack, followed by deprotonation, is coupled with pseudorotation with a single transition state to yield **56** directly. As discussed in Scheme 3, ab initio calculations indicated that **19** can be formed directly by hydroxide attack on **9**, without formation of **23**, since the nucleophilic attack is coupled with pseudorotation (**22** → **19**).[46,48] According to the calculations, attack by a hydroxyl ion on the phosphorus atom of **9** occurs in concert with pseudorotation to yield a TBP intermediate, **19**, with an equatorial hydroxyl group. As the distance from P to OH in **22** is decreased (see Scheme 3), a stable TBP intermediate, **23**, with an apical hydroxyl group can no longer be found. Instead, pseudorotation with the P−O$^-$ bond as pivot occurs to place the hydroxyl group in an equatorial position and the methoxy group in an apical position, to yield an intermediate **19** directly. This proposed mechanism predicts that both endo- and exocyclic cleavages occur with retention of configuration. Unless pseudorotation (**23** → **19**) is very rapid, the validity of this mechanism can be probed by examining the stereochemistry of **15** with a chiral MEP, namely, **9** (Scheme 9).[48] A possible explanation of why monoanionic phosphorane **19** exists and **23** does not exist as a stable intermediate in the gas phase is that while the larger apical methoxy group in **19** can accommodate the negative charge, the smaller apical hydroxyl group in **23** cannot, so that the latter species is unstable in the gas phase. Then, under well-solvated conditions, **23** might exist as a stable intermediate. Such a possibility is being examined in our laboratory. The difficulty with the coupling mechanism shown in Scheme 3 is the rationalization of relationship in Figure 3. If **19** were to be formed directly from **9**, we would have to explain why the extent of exocyclic cleavage changes with changes in pH.

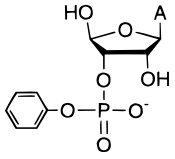
The base-catalyzed hydrolysis of **8** is not accompanied by ^{18}O exchange, by analogy to the results for the acyclic counterpart DMP (**10**).[37] As mentioned in section II, the absence of ^{18}O exchange in the base-catalyzed hydrolysis is often used as evidence in support of the nonexistence of dianionic phosphoranes. However, its absence can be explained either by a high energy barrier for pseudorotation or by the nonexistence or the very brief existence (too brief to allow psedorotation) of dianionic phosphorane intermediates. If and only if the coupling mechanism is not operative, the observation of the product of exocyclic cleavage **8** in strong alkali at ∼3 ± 1.5% requires the existence of dianionic phosphorane intermediates (**55** → **56**), as suggested by ab initio calculations under well-solvated conditions.[32,33] Whether the dianionic phosphorane is stable enough to become protonated needs further examination. It is to be mentioned that no experimental data are available that directly support the existence of dianionic phosphorane intermediates.[27−29] Only the observation of the product of exocyclic cleavage **8** in strong alkali at ∼3 ± 1.5% in the context of the relationship in Figure 3 of the extent of exocyclic cleavage versus pH demands (unless the relationship can be rationalized by the coupling mechanism) the existence of the dianionic phosphorane intermediates (**55** → **56**).

V. The Rate-Limiting Step in RNA Hydrolysis

A. Experimental Evidence for the Rate-Limiting Cleavage of the P–O(5′) Bond

In the nonenzymatic hydrolysis of RNA, there are two putative transition states, designated **TS1** and **TS2**. **TS2** is always a higher-energy state than **TS1**, at least according to our molecular orbital calculations (Scheme 2). In attempts to identify the overall transition state, several RNA analogues were synthesized and their properties were characterized. The initial model studies, in which the phenyl ester of

adenosine 3′-phosphate [Ap(3′-phenyl); **60**] was used as the probe, indicated that it is the second state (**TS2**) that corresponds to the overall rate-limiting step.[99] This conclusion was reached because (i) if the



60

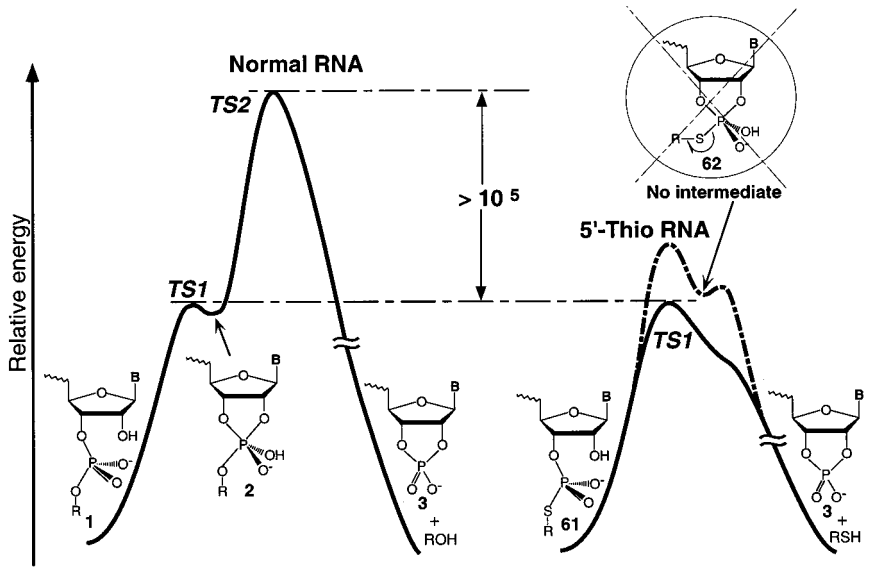
formation of the pentacoordinate intermediate were the rate-limiting step (if **TS1** were a higher-energy state than **TS2**), the probe should be hydrolyzed at a rate similar to the rate of cleavage of an adenosine dimer (ApA). By contrast, (ii) if the decomposition of the intermediate were the rate-limiting step (if **TS2** were a higher-energy state than **TS1**), the probe should be hydrolyzed much more rapidly than ApA. Kinetic measurements revealed that Ap(3′-phenyl) **60** was hydrolyzed much more rapidly than ApA, supporting the hypothesis that formation of **TS2** is the overall rate-limiting step. This system, although interesting, does not give an unequivocal answer because, although the phenyl group of **60** surely accelerates the second step (formation of **TS2**), it could also accelerate the first step (formation of **TS1**) by making the phosphorus atom more electrophilic as a result of the electron-withdrawing ability of the phenyl group.

Better substrates for use in attempts to identify the rate-limiting step seem to be RNA analogues with a 5′-thio leaving group since sulfur is typically considered to be a conservative substitution for oxygen. Eckstein's, McLaughlin's, Reese's and Usman's groups synthesized RNA analogue **61**, in which the 5′-oxygen was replaced by sulfur (Scheme 11).[100−108] Measurements of the rate of hydrolysis revealed that **61** was hydrolyzed at least 10^5 times more rapidly than the counterpart RNA with a natural phosphodiester linkage. If the formation of the TBP intermediate were rate-limiting (that is, if **TS1** were to be a higher energy state than **TS2**), **61** should be hydrolyzed at a rate similar to the rate of the hydrolysis of the natural RNA because the 5′-bridging phosphorothioate linkage would not be expected to enhance the attack by the 2′-oxygen,[109] unless the anionic transition state could be stabilized to a significant extent by the polarizable sulfur leaving group (see section V.C). By contrast, if the decomposition of the intermediate were the rate-limiting step (that is, if **TS2** were at a higher energy state than **TS1**), we would expected that **61** would be hydrolyzed much more rapidly than the natural RNA because the pK_a of a thiol is more than 5 units lower than that of the corresponding alcohol.[110,111] Examination of the rate of hydrolysis of **61** and of the rate of hydrolysis of the corresponding RNA with a native phosphodiester linkage revealed that **61** was hydrolyzed more than 10^5 times more rapidly than its counterpart.[100−108] Since a 5′-sulfur moiety, which is less electronegative than oxygen, is not expected to accelerate the first step of the reaction (formation of **TS1**), the higher reactivity of **61**, as compared with that of the counterpart RNA with the regular phosphate linkage, demonstrates unambiguously that **TS2** is at a higher energy state than **TS1**, as shown in Scheme 11. Therefore, all the available results of hydrolysis studies support the hypothesis that the decomposition of the intermediate [cleavage of the P−O(5′) bond] is rate-limiting in the nonenzymatic hydrolysis of RNAs with native phosphodiester linkages.

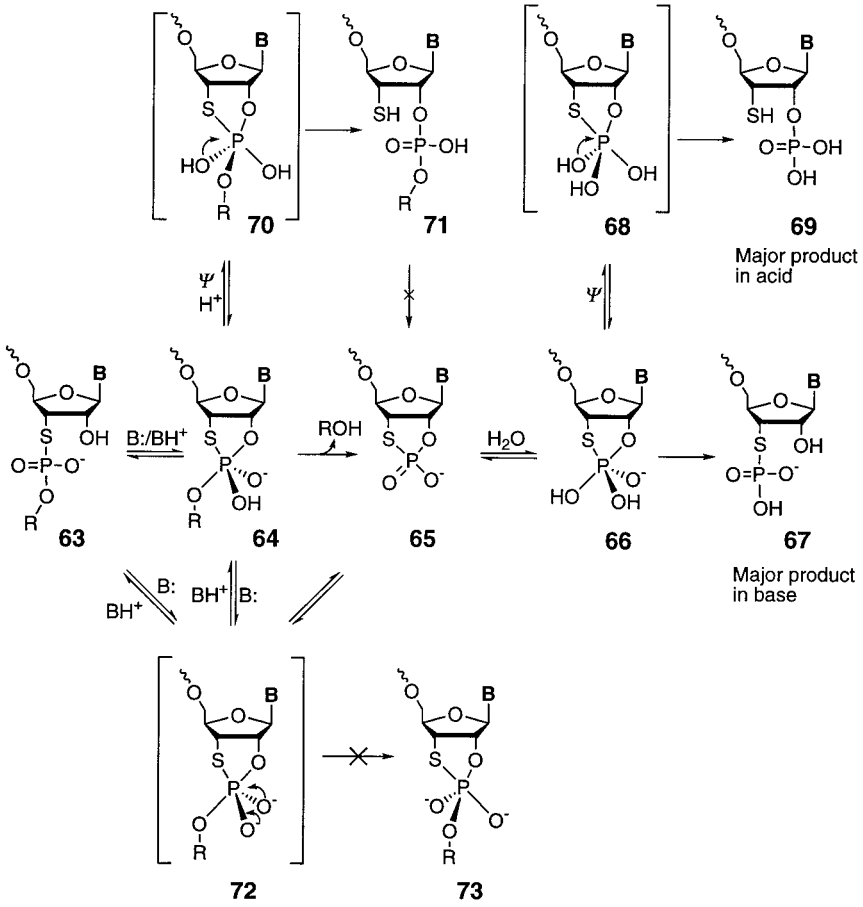
B. Structures of Transition States and Intermediates

As mentioned in the previous section, since the pK_a of a thiol is more than 5 units lower than that of the corresponding alcohol, **TS2** for **61** is significantly stabilized with respect to the corresponding **TS2** for **1** (Scheme 11), with the result that cleavage of **61** is more than 10^5 times faster than that of **1**. As a result, formation of **TS1** becomes the rate-limiting step for the hydrolysis of **61**. As discussed in sections III and IV, all the available data indicate that the

Scheme 11



Scheme 12



monoanionic phosphorane **2** exists as an intermediate (Scheme 11), whereas **62** with an apical sulfur ligand does not exist as an intermediate (recall that, in Scheme 6, no TBP species with an apical sulfur ligand exists as an intermediate; see section III.C). The latter statement is in agreement with the general conclusion made by Anslyn that RNA analogues with efficient leaving groups are hydrolyzed with a single transition state (without formation of a TBP intermediate).[27] It is to be noted that, if intermediate **62** had been produced from **61**, the energy level of **62** would have to be higher than that of **2** (as indicated by the dashed, hypothetical energy curve in Scheme 11) since the latter **2** but not the former **62** can be stabilized by the electronegative 5′-oxygen at the apical position. Then, if formation of **TS1** were the rate-limiting step for native RNAs, such as **1**, and if **62** were to exist as an intermediate, **61** should be hydrolyzed more slowly than **1**. Such a result has not been obtained.[100−108] Therefore, we can safely conclude that formation of **TS2** is the rate-limiting step for native RNAs and **62** is unlikely to exist as an intermediate.

Lastly, we must emphasize that transition-state structures of **TS1** for **1** and **61** are expected to be very similar since they are early transition states with very limited formation of a bond between the phosphorus and the attacking 2′-oxygen. Therefore, the influence of the 5′-thio group on the hydrolysis of **61** is minimal at **TS1** (but maximum at hypothetical **TS2**: since **TS2** is stabilized to a significant extent by the efficent departing group, in fact, **TS2** has disappeared completely). As a result, hydrolysis of **61** is faster than that of **1**. Note again that, if **TS1** for **61** resembled **62**, **TS1** for **61** should have significantly higher energy than **TS1** for **1** (if the latter **TS1** resembled **2**), since **TS1** for **1** but not **TS1** for **61** can be stabilized by the electronegative 5′-oxygen at the apical position

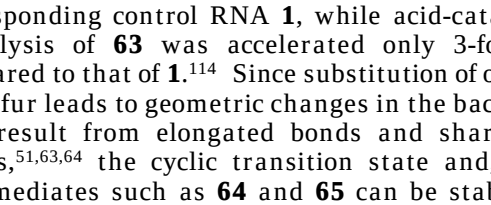
C. Hydrolysis of 3′-Thio-Modified RNA

An interesting RNA analogue, **63**, with a 3′-thio linkage was synthesized and characterized by Cosstick's, Reese's, and Cech's groups.[112−114] Basically, the hydrolytic reaction followed the general pathway for hydrolysis of RNA that is shown in Scheme 1. The details of the hydrolysis of **63** are shown in Scheme 12. Under basic conditions, the major product is **67**, which can be formed from **63** as follows: **63** → **64** → **65** → **66** → **67**. By contrast, under acidic conditions, which allow pseudorotation, the major product is **69**, which is probably formed as follows: **63** → **64** → **65** → **66** → **68** → **69**. Pseudorotation under acidic conditions favors cleavage of the weaker P−S bond to produce **69**, while basic conditions prohibit pseudorotation, limiting the mechanism to direct displacement of the P−O bond, which leads to the production of **67**. Analysis of related compounds led to the same conclusion of acid-catalyzed cleavage of the P−S bond and base-catalyzed cleavage of the P−O bond.[115−117]

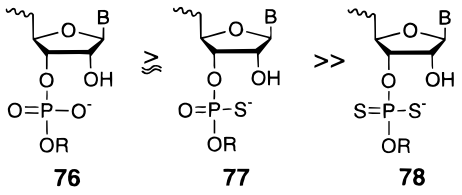
It is of great interest that, over the entire range from pH 10 to pH 14, the 3′-thio-modified RNA **63** was hydrolyzed 2000-fold more rapidly than the

corresponding control RNA **1**, while acid-catalyzed hydrolysis of **63** was accelerated only 3-fold as compared to that of **1**.[114] Since substitution of oxygen by sulfur leads to geometric changes in the backbone that result from elongated bonds and sharpened angles,[51,63,64] the cyclic transition state and, also, intermediates such as **64** and **65** can be stabilized by relief of the strain energy in the five-membered ring.[113,114] However, the relief of ring strain does not explain why the rate was accelerated 2000-fold only under basic conditions. Therefore, it was proposed that the observed acceleration of the rate might reflect stabilization of the anionic transition state by the polarizable sulfur atom. If we consider the phosphorus atom as a center of negative charge, which increases in anionic character on moving to the transition state, the adjacent sulfur in the 3′-bridging position might facilitate distribution of the charge, thereby stabilizing the transition state **TS1**. This type of effect, coupled with geometric factors, would be consistent with the observed enhancement of the rate of base-catalyzed hydrolysis, which utilizes an anionic nucleophile, and it would also be consistent with the modest enhancement seen in the case of acid-catalyzed hydrolysis in which the nucleophile is a neutral hydroxyl group.[114] In this proposal, it was assumed that the attack of the 2′-hydroxyl (**TS1**) is the rate-limiting step in the cleavage of RNA. However, our results (Scheme 11) indicate that the cleavage of the P−O(5′) bond is the overall rate-limiting step in the hydrolysis of natural RNAs, whereas the attack of the 2′-hydroxyl on phosphorus is the rate-limiting step for the 5′-thio RNA (**61** in Scheme 11), if we assume that the leaving sulfur in the 5′-bridging position in **61** does not accelerate the attack of the 2′-hydroxyl (namely, no enhancement by the polarizable sulfur of the nucleophilic attack).

The stabilizing effect of α-sulfur atoms is not necessarily operative in all hydrolytic reactions. For example, carboxylic esters such as **74** and **75** were hydrolyzed at pH 10.5 and with rate constants of 4.6 × 10^{-3} and 2.8 × 10^{-3}, respectively.[109] In the hydrolysis of these esters, the rate-limiting step was formation of tetrahedral intermediates, which was not accelerated by the α-sulfur atom. Other examples include phosphorothioates, the most commonly used phosphate-modified analogues.[118−120] Rates of hydrolysis of phosphorothioates (**77**) are comparable with or somewhat lower than those of the corresponding phosphates (**76**).[121,122] It may be argued that, in the case of phosphorothioate **77**, the sulfur atom in the nonbridging position has a localized full negative charge[123,124] and, thus, it cannot accommodate an extra negative charge. As a consequence, the transition state is not stabilized by the α-sulfur atom. However, phosphorodithioate **78** was much more resistant to hydrolysis than **77** under strongly basic conditions.[125,126] Moreover, a greater accumulation of the α-sulfur-containing cyclic phosphate intermediate (**65** in Scheme 12) than of the corresponding cyclic phosphate intermediate (**3** in Scheme 11) was recognized.[114] If α-sulfur atom were polarizable, we would expect that **65** would be much more reactive than **3**.



74 **75**



76 **77** **78**

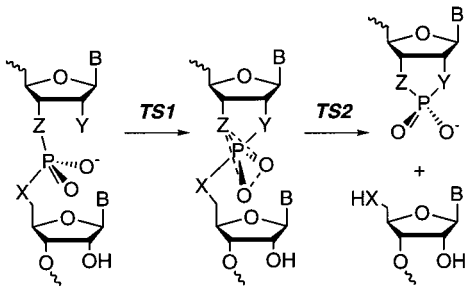
One possible reason for the similar rates for cleavage of **63** and **1**, under acidic conditions, could be that **63** is trapped as **71** (**63** → **64** → **70** → **71**). Then, the slower hydrolysis of **63** in acid might be explained by the dead-end isomer **71**. Since **71** is not expected to undergo the reversible reaction (**71** → **70** → **64**), once **71** has been formed, it would not be hydrolyzed further or it would be hydrolyzed only very slowly (although such an intermediate, **71**, was not reported).[114]

Even if the stabilizing effect of α-sulfur atoms were operative in the hydrolysis of α-sulfur-containing phosphates, enhancement of the rate would be expected to be around 2000-fold.[114] Moreover, the stabilizing effect of an α-sulfur atom at an apical position, as in **62** (Scheme 11), for example, cannot be greater than the stabilizing effect when the sulfur atom is at the equatorial position, as in **64**. Recall that, in Scheme 6, the transition-state structure **39**, with an apical sulfur atom, was higher in energy than the corresponding transition state **31**, with an equatorial sulfur (ab initio calculations, in the gas phase, should be very sensitive to the polarizability of α-sulfur atoms). Therefore, the observed enhancement of more than 10^5-fold in the rate of hydrolysis of **61** relative to that of **1** (Scheme 11) requires that cleavage of the P−O(5′) bond be the rate-limiting step in the hydrolysis of natural RNAs.

D. Reactivities of Other Modified RNA Analogues

The hydrolysis of uridine dinucleotide UpU (**79**) has been studied extensively (Scheme 13).[88,90,127−130] This dinucleotide is extremely stable toward acid/base hydrolysis and at neutral pH, with a hydrolysis rate constant of $k_{obs} = 10^{-8}$.[127] Recently, RNA oligonucleotides containing a 2′-thiol, a 3′-bridging sulfur atom or a 5′-bridging sulfur atom have been synthesized.[100−108,113,114,131] Investigations of such modified oligonucleotides have provided very useful information about the intrinsic reactivity of RNA. In contrast to the significant stability of the UpU dinucleotide **79**, the dinucleotide of uridylyl(3′-5′)-5′-thiouridine, **80**, is very susceptible to hydrolysis under neutral conditions and it is exceptionally susceptible to hydrolysis under mildly basic conditions, forming a uridine 2′,3′-cyclic phosphate and 5′-thiouridine, as discussed in section V.A (Scheme 11). The exceptional base-lability of uridylyl(3′-5′)-5′-thiouridine **80** is attributable to the pK_a of the 5′-thiol (pK_a = 11) which is more than 5 units lower than that of the corresponding 5′-hydroxyl (pK_a = 16)[132] and to the fact that **TS2** is the overall rate-

Scheme 13



79	X=O Y=OH Z=O	Very stable, $k_{obs}=10^{-8}$
80	X=S Y=OH Z=O	Very laible, $k_{obs}=10^{-4}$
81	X=NH Y=OH Z=O	Very labile, $k_{obs}=10^{-4}$
82	X=O Y=SH or NH_2 Z=O	No hydrolysis
83	X=O Y=OH Z=S	Labile, $k_{obs}=10^{-5}$
84	X=S Y=NH_2 Z=O	Very labile, $k_{obs}=10^{-4}$

limiting transition state for the cleavage of unmodified RNA (Scheme 11).[107,108] The strength of the P−S bond (50 kcal/mol) is also lower than that of the P−O bond (ca. 91 kcal/mol).[133] Such a dinucleotide is also a good substrate for ribonuclease A.[100,107]

The lability of oligonucleotides that contain a 5′-amino group (**81**), which are also readily hydrolyzable, can also be attributed to the good leaving ability of the 5′-amino group, upon its protonation, because of its lower pK_a of 6.2.[134,135] The dependence of rates of hydrolysis of RNA model compounds on the pK_a of the leaving group has been investigated extensively.[136,137] For example, an RNA analogue, containing 2,4-dinitrophenol with the low pK_a of 4, was hydrolyzed with a rate constant of $k_{obs} = 10^{-1}$ min^{-1}.[138] Thus, it was almost 7 orders of magnitude more sensitive to hydrolysis than a normal unmodified dinucleotide.

Replacement of the 2′-OH at the cleavage site by an amino group[139] or a thiol group[105,106,131] (**82**) prevents hydrolysis and, as a result, such modifications also protect the RNA against alkaline and RNase A-mediated hydrolysis.[106,140,141] It is unrealistic to assume that nucleophiles such as amino and thiol groups would not attack the phosphorus atom. As discussed above, since the lower pK_a of 2′-amino (pK_a = 6.2) and 2′-thiol (pK_a = 11) moieties make them better leaving group than the 2′-hydroxyl moiety (pK_a = 16) and, since **TS2** is the overall rate-limiting transition state, even if a pentacoordinate intermediate/transition state were formed by attack of either the amino or the thiol group, it is significantly more likely to undergo disruption of the P−N or P−S bond, returning to the original state via **TS1** and never reaching **TS2**. It is to be noted that, in the case of **82**, **TS1** is even lower than the corresponding **TS1** of the normal RNA **2** (Scheme 11). It is important to keep in mind the energy diagrams, such as those shown in Scheme 11. In the case of hydrolysis of **61**, which contains a 5′-thio leaving group, the rate-limiting step changes from **TS2** for the normal RNA **1** to attack of the 2′-oxygen on the phosphorus atom, namely **TS1** (Scheme 11).[100,108] It is noteworthy, in this context that the very stable RNA dinucleotide with a 2′-amino moiety can become labile when the 5′-oxygen atom is replaced by 5′-sulfur, as in **84**,[100] clear evidence in support of the lowering of **TS2** by the 5′-sulfur.

It seems impossible to explain the reactivities of modified RNA analogues listed in Scheme 13 unless our conclusion, that the cleavage of the P−O(5′) bond (**TS2**) is rate-limiting in the nonenzymatic hydrolysis of RNAs with native phosphodiester linkages, is valid. Therefore, lowering of the overall energy barrier of the transition state **TS2** is essential for the ribozyme-mediated and proteinacious enzymatic cleavage of a phosphodiester bond in an RNA molecule. In the case of the ribozyme-mediated cleavage of native RNAs, the buildup of charge at the 5′-leaving oxygen occurs in the rate-limiting transition state **TS2** and the metal ion stabilizes the leaving group by coordinating directly to it, as described below.

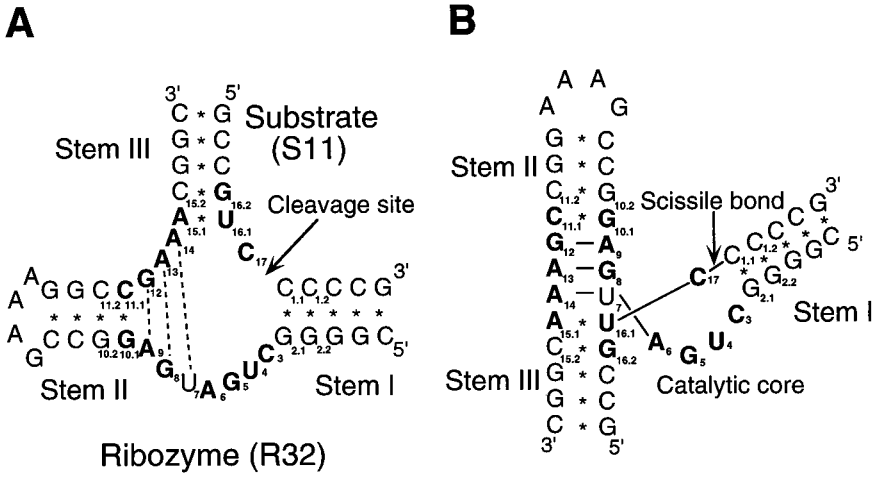
VI. General Features of Hammerhead Ribozymes

We will now move on to the mechanism of ribozyme-mediated cleavage of RNA, bearing in mind the various conclusions derived from the theoretical calculations and model studies described above. First, we shall examine the general features of hammerhead ribozymes, which are small self-cleaving RNAs found in certain viruses and satellite RNAs that replicate via a rolling-circle mechanism.[6−14] For details of studies of structure−function relationships the reader is referred to other articles as well.[3,10,21,142−144]

A. Structure of Hammerhead Ribozymes

The *trans*-acting hammerhead ribozyme consists of an antisense section (stem I and stem III) and a catalytic domain with a flanking stem II−loop section (Scheme 14). Over the past a few years, there have been several attempts to determine both the overall global structure and the detailed atomic structure of the hammerhead ribozyme. These studies have involved, for example, measurements of electrophoretic mobility,[145,146] nuclear magnetic resonance,[147−154] transient electric birefringence,[155,156] fluorescence resonance energy transfer (FRET),[157−159] and X-ray diffraction.[160−165] The X-ray crystal structures determined by McKay's group and Scott and Klug's group are nearly identical in terms of tertiary folding and conformation although the components of the two types of crystals examined were quite different: one type was an RNA−DNA complex that was crystallized in ammonium sulfate, and the crystals were adopted to a high concentration (2.4 M) of Li_2SO_4,[160] and the other type was an all-RNA complex with a 2′-methoxy-2′-deoxyribose at the cleavage site in a solution of lower ionic strength[161]

Scheme 14



or, alternatively, a freeze-trapped intermediate of an unmodified all-RNA complex.[162] The freeze-trapped conformational intermediate was stabilized primarily by a hydrogen bond between the furanose oxygen of C_{17} and the 2′-OH of $U_{16.1}$. All ribozymes were γ-shaped in the crystals, with stems I and II forming the arm of the γ and stem III forming the base (Scheme 14B), with stem I and stem II being adjacent to each other and stems II and III being stacked collinearly to form a pseudo-A-form helix, in agreement with results deduced from fluorescence energy transfer,[157] electrophoretic,[145,146] and chemical cross-linking[140,166] studies.

In the X-ray structures the catalytic core is divided into two regions: domain I consisting of $C_3U_4G_5A_6$ and domain II consisting of nucleotides $G_{12}A_{13}A_{14}$ and $U_7G_8A_9$. The nucleotides of domain II form two reversed-Hoogsteen G-A base pairs between G_8-A_{13} and A_9-G_{12}, and a non-Watson−Crick A_{14}-U_7 base pair that consists of one hydrogen bond. This extended stem II stacks onto the non-Watson−Crick base pair, $A_{15.1}$-$U_{16.1}$, resulting in formation of a pseudo-A-form helix by stems II and III (Scheme 14B).[160−165] The adjacent non-Watson−Crick A-U base pairs (A_{14}-U_7 and $A_{15.1}$-$U_{16.1}$) form the basis of a three-way junction. The four nucleotides ($C_3U_4G_5A_6$) of domain I form a "uridine-turn" motif, allowing the phosphate backbone to turn and connect with stem I. Since the uridine-turn motif conforms to the general sequence requirement UNR (where N is any nucleotide and R represents a purine) and since other sequences have been found to adopt a structure similar to this motif,[167] an attempt at selection in vitro was made to determine whether other sequences might be possible in the hammerhead's catalytic core.[168] Active sequences conformed broadly to the consensus core sequence except at A_9, and no sequences were associated with higher activity than that of the hammerhead with the consensus core, an indication that the consensus sequence derived from viruses and virusoids is probably the optimal sequence. However, chemical modification at U_7 produced a nonnatural ribozyme that had higher activity than the wild-type hammerhead ribozyme.[169] We also found that, in some cases and depending upon the target-recognition sequences, the insertion of G between A_9 and $G_{10.1}$ results in production of a ribozyme that is slightly more active than the wild-type hammerhead ribozyme.[170−172] This finding seems to suggest that it was unnecessary, during evolution, to increase to any further extent the catalytic activity of hammerhead ribozymes for use in rolling-circle mechanisms since, most probably, the present trimming power was adequate with respect to rates of replication. Such speculation indicates that it might be possible to select in vitro or in vivo, or to engineer, hammerhead ribozymes that are better catalysts than wild-type forms.[17,21,168,173−182]

It has been well-established that ribozymes are metalloenzymes.[78,79,183−193] Scott et al. proposed various Mg^{2+} binding sites, two of which are thought to be important for catalysis.[161,162] The first site is thought to involve $Mg(H_2O)_5^{2+}$ bound to the *pro-S* oxygen of the 5′-phosphate of A_9, with further hydrogen bonding associated with G_8, $G_{10.1}$, and G_{12} (alternatively, $Mg(H_2O)_5^{2+}$ might be bound to the *pro-R* oxygen[160]), and this binding is thought to have a structural role. The second, and perhaps a more interesting site, seems to be in the vicinity of the cleavage site. At this site a Mg^{2+} ion is thought to be bound directly to the *pro-R* oxygen of the scissile phosphate in the freeze-trapped conformational intermediate.[162] The hydrated Mg^{2+} ion might be able to participate directly in catalysis by acting as a base to facilitate the deprotonation of the 2′-OH of C_{17}, prior to nucleophilic attack at the scissile phosphate.[194] Such involvement of C_3 and C_{17} in the binding of a Mg^{2+} ion has also been postulated on the basis of replacement of the cytosine by a 2-pyrimidone nucleoside.[195] However, crystal structures generally represent energy minima and do not provide direct and detailed structural information about transition states unless the structural data represent a deliberately designed analogue of a transition state. Because the conformation revealed by all the available crystallographic structures would not allow in-line attack by the 2′-OH on the scissile phosphorus−oxygen bond that is absolutely required for activity, none of them represents the exact catalytic conformation.[160−165,196] It is apparent that substantial twisting at the cleavage site would be required for in-line attack and a specific proposal for rearrange-

ment at the cleavage site has been presented, on the basis of the X-ray structures of hammerhead ribozymes.[154,162−164]

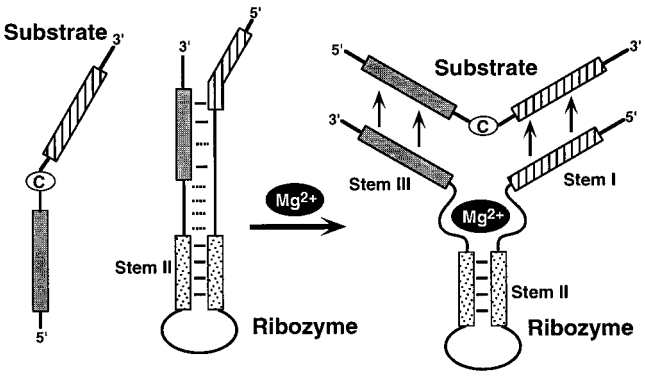
Further analysis by Scott indicates the probable invalidity of the earlier postulate[162] that the Mg^{2+} ion bound to the *pro-R* oxygen of the scissile phosphate in the ground state might move, together with the phosphate, into a conformation more suitable for in-line attack: Scott recently trapped an intermediate with an advanced conformational change, where the phosphate had moved considerably.[197] However, the metal ion (this time a Co^{2+} ion) remained associated with N7 of $A_{1.1}$ and did not move with the *pro-R* oxygen.[197] The exact number and location of catalytic metal ion(s) in the transition state remain to be determined (see section VII.E).

B. Dual Roles of Metal Ions

In striking contrast to proteinaceous enzymes, which do not always require metal ions for their activity, ribozymes in general have a requirement for metal ions for their activity. Metal ions in ribozymes have two distinct functions: in one case they aid in the structural stabilization of the folded RNA and in the second case they act as the catalytic cofactor that allows the RNA to act as a metalloenzyme.[145,183−200] In hammerhead ribozymes, metal ions such as Mg^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+} can participate both in RNA folding and in catalysis. Similarly, Sr^{2+} and Ba^{2+} ions can perform both roles but to a much lesser extent. Other metal ions, such as Cd^{2+} and Zn^{2+}, can participate in catalysis only in the presence of RNA-folding agents such as polyamines and spermine, an observation that suggests that Cd^{2+} and Zn^{2+} ions can only play a catalytic role and the structure of the ribozyme is dependent on polyamines or spermine.[198]

Recently, Lilley's group[145,146] systematically examined the ion-dependent conformational changes in the hammerhead ribozyme by monitoring shifts in electrophoretic mobility. Three discrete conformations were observed: in the absence of Mg^{2+} ions, the hammerhead ribozyme existed in an extended form in which the catalytic core appeared to be unstructured; at a low concentration of Mg^{2+} ions, stems II and III were aligned coaxially, forming a pseudo-continuous helix with stem I being adjacent to stem III; at high concentrations of Mg^{2+} ions, stem I was reoriented and was adjacent to stem II, as in the crystal structure (Scheme 14B).[145,146] Such reorientation of the helical arms by divalent metal ions could also be achieved, to a lesser extent, using singly charged cations such as Na^+, although the Na^+-mediated folded structure was different from the Mg^{2+}-mediated structure. Using NMR spectroscopy, we investigated the effects of metal ions on the formation of an active complex between the hammerhead ribozyme and its substrate by adding Mg^{2+} ions.[152] In our case, in the absence of Mg^{2+} ions, a complex between ribozyme (R32 in Scheme 14) and substrate (S11 with deoxy-C_{17}) could not be formed because the substrate-recognition regions of the ribozyme formed intramolecular base pairs. In other words, the ribozyme remained in an inactive conformation in the absence of metal ions (Scheme 15).

Scheme 15



Upon addition of Mg^{2+} ions to the mixture of ribozyme and substrate, the substrate-recognition regions of the ribozyme opened up and the ribozyme−substrate complex was formed. It is important to note that monovalent Na^+ ions could not replace the Mg^{2+} ions: the ribozyme remained in its inactive conformation in the absence of Mg^{2+} ions and in the presence of Na^+ ions.

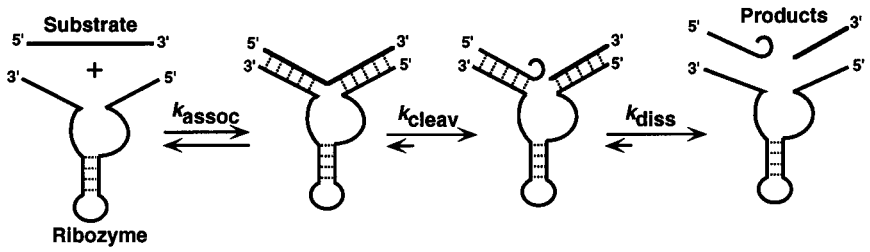
To quantify the binding of Mg^{2+} ions, Eckstein's group incorporated nucleoside 2-aminopurine, which is known to respond to changes in stacking interactions by changes in its fluorescence, at several positions in the hammerhead ribozyme.[201] By monitoring the intensity of fluorescence as a function of the concentration of Mg^{2+} ions, they determined dissociation constants that ranged from 100 μM to 2 mM. These values represent the concentrations of Mg^{2+} ions required to induce the structural rearrangements of the hammerhead that yield the three-dimensional structure, but they do not necessarily represent the dissociation constants of the catalytic metal ions.

C. Cleavage Sites and the NUX Rule

In attempts to define the sequence requirements for an active structure of a hammerhead ribozyme that help the ribozyme to select appropriate target sequences, extensive mutagenesis studies of the conserved region have been performed.[202−207] Several such studies were carried out to examine the importance of the conserved trinucleotide GUC at the cleavage site. Early results revealed that G at the third position in the triplet, which might extend stem I by forming a G_{17}:C_3 pair, inhibited the cleavage reaction in all but one case and, moreover, that U at the central position was required for efficient cleavage.[202,204,205] More detailed analysis by Uhlenbeck's group showed that the ground state of the G_{17} substrate was stabilized by about 2 kcal/mol upon the proposed formation of a G_{17}:C_3 pair.[208] However, the cleavage rate of the G_{17} substrate was much too slow to be explained simply by the need to disrupt this base pair before the transition state could be reached. Therefore, they concluded that G_{17} is unable to make all the contacts necessary to stabilize the transition state or that it is more difficult to reach the transition-state structure because of steric constraint.

The accumulated data from mutagenesis studies led to the generally accepted NUX rule (where N can

Scheme 16



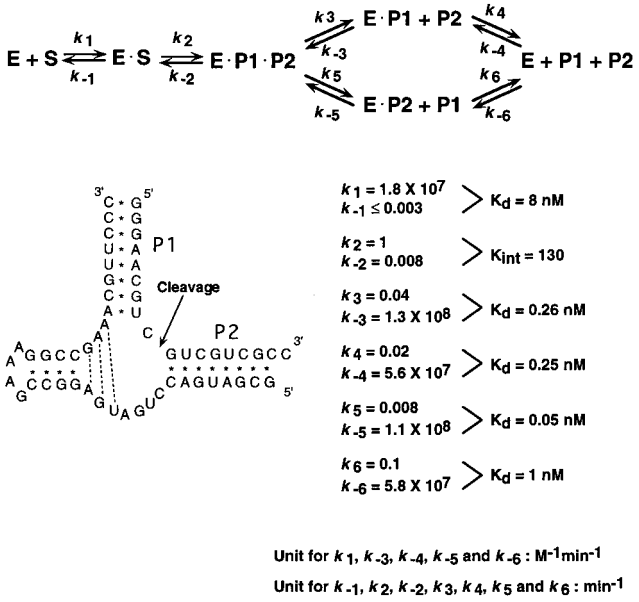
be A, U, G, or C; X can be A, U, or C), which states that any oligonucleotide with a NUX triplex can be cleaved by hammerhead ribozymes. Our detailed kinetic analysis,[207] in which we measured both k_{cat} and K_m, indicated that GUC was cleaved most efficiently in a manner that depended both on k_{cat} and k_{cat}/K_m, with CUC and UUC coming next. Therefore, when a target site in a *trans*-acting system (an intermolecular reaction) is chosen, GUC or CUC may be preferred. However, in *cis*-acting systems (intramolecular reactions), in which K_m values are irrelevant, other triplets, such as AUC, GUA, and AUA, may be chosen since these triplets are associated with high values of k_{cat}. In fact, the minus strand of the virusoid of Lucerne transient streak virus, (−)vLTSV, and the plus strand of the satellite RNA of barley yellow dwarf virus, (+)sBYDV, use the GUA triplet and the AUA triplet, respectively, for hammerhead-catalyzed cleavage during their replication.[207]

D. Kinetic Framework

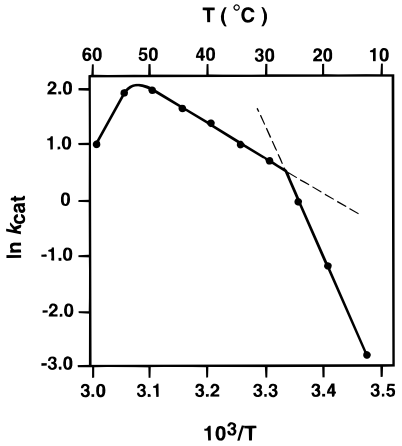
The minimum reaction scheme, consisting at least three steps, is shown in Scheme 16.[190,209] First, the substrate binds to the ribozyme to form a Michaelis−Menten complex via formation of base pairs at stems I and III (k_{assoc}). Then, a specific phosphodiester bond (on the 3′-side of the NUX triplet) in the bound substrate is cleaved by the action of metal ions (k_{cleav}) to produce a 2′,3′-cyclic phosphate and a 5′-hydroxyl group.[209−211] Finally, the cleavage fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events (k_{diss}). More complete analysis, by Uhlenbeck's group with measurements of individual rate constants, yielded a complete kinetic scheme (Scheme 17).[212,213]

The dependence on temperature of the rate-limiting step was detected by analysis of an Arrhenius plot.[214,215] Distinct changes in the slope of the plot (Scheme 18) provided evidence for three different rate-limiting steps in the hydrolysis of an 11-mer substrate (S11 in Scheme 14) by the 32-mer ribozyme (R32). At midrange temperatures of 25−50 °C, the chemical cleavage step (k_{cleav}) is the rate-limiting step, indicating that the cleaved fragments dissociate from the ribozyme at a higher rate than the rate of the chemical reaction ($k_{cleav} < k_{diss}$). At temperatures below 25 °C, the cleaved fragments adhere to the ribozyme more tightly and the product-dissociation step becomes the rate-limiting step ($k_{diss} < k_{cleav}$). Above 50 °C, the rate of the reaction decreases because, at such high temperatures, the formation

Scheme 17



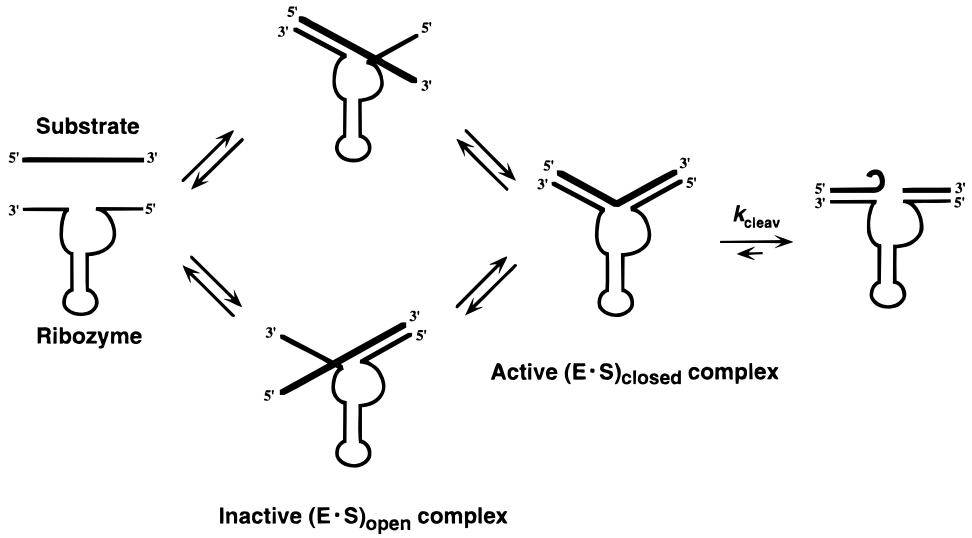
Scheme 18



of the Michaelis−Menten complex (formation of a duplex) is hampered by thermal melting (the melting temperature of stem II of R32 is above 80 °C and, therefore, the stem II and loop region is unaffected between about 50 and 60 °C).

In our kinetic analysis of ribozyme-catalyzed reactions, which will be described below, we consistently used the combination of R32 and S11 because, as the analysis described in this section indicates, so long as the kinetics of the reaction catalyzed by R32 are examined at temperatures of 25−50 °C, the turnover rate constant (k_{cat}) represents the chemical cleavage step (k_{cleav}).

Scheme 19



E. Stem Length and Activity

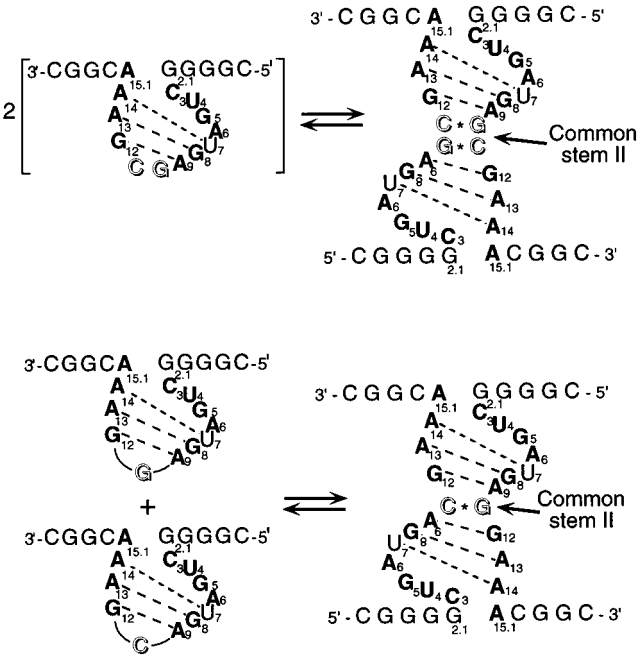
The efficient binding of a hammerhead ribozyme to its cleavage site in an intracellular target RNA is an obvious requirement for the eventual use of the ribozyme as a therapeutic agent. The binding is influenced by the length of the ribozyme antisense arms (stems I and III). Kinetic models of the action of hammerhead ribozymes and analyses of thermodynamic parameters predict that ribozymes with short antisense arms have a high turnover rate when compared to their counterparts with long arms.[212,216−219] However, in some cases, hammerhead ribozymes with long antisense arms have been found to be more active in the cell than short-arm derivatives.[220,221] In the cellular environment, the rate-limiting step is not always the chemical cleavage step and, therefore, a ribozyme's activity in vitro does not necessarily reflect its activity in vivo.[171,172] The stability of ribozymes in vivo appears to be more important in the intracellular efficacy of ribozymes.[222−224] It is, however, also important to use ribozymes with short arms in functional analysis of kinetics in vitro, to ensure measurement of the chemical step.[108,225,226]

Recently, the contribution of several individual ribozyme·substrate base pairs to binding and catalysis was investigated using hammerhead ribozyme substrates that were truncated at their 5′- or 3′-ends.[227] Addition of residues close to the cleavage site contributed in the chemical step of the hammerhead reaction, but not in the substrate binding step, whereas base pairs distal to the cleavage site contributed solely to binding but had no effect on the chemical step. These results led to a "fraying model" in which each ribozyme·substrate helix can exist in either an unpaired [inactive "$(E·S)_{open}$" complex in Scheme 19] state or a helical state [active "$(E·S)_{closed}$" complex], with the closed state required for catalysis. According to this model, the cleavage rate depends on the concentration of the active $(E·S)_{closed}$ complex relative to that of the inactive $(E·S)_{open}$ complex since both helices at the binding arms must be formed for the ribozyme to cleave its substrate. To explain the higher activities of DNA-armed ribozymes, we proposed that the hybrid helices of the DNA-armed ribozyme·substrate complex create a slightly different $(E·S)_{closed}$ structure, resulting in a significantly higher activity of the DNA-armed ribozyme·substrate complex compared with that of the natural ribozyme·substrate complex.[215]

F. Minizymes

Helix II is the only helix in the hammerhead ribozyme that is not directly involved in binding of the substrate. In a systematic study, in which the length and the base composition of helix II were varied, the minimal length of the helix II was found to be two base pairs, with a requirement for a $G_{10.1}$−$C_{11.1}$ base pair for maximal activity.[228,229] So-called minizymes are smaller versions of a hammerhead ribozyme in which helix II and loop II are replaced by a short linker that joins A_9 and G_{12}. The linker can consist of a few nucleotides that cannot form Watson−Crick base pairs among themselves.[230] Several groups have replaced the nucleotide-loop of helix II with poly(ethylene glycol)[231−234] or abasic nucleotide,[235] without complete loss of activity when at least two base pairs remains in helix II. However, the activities of most minizymes are very low.[174,229−236] We found that minizymes with short oligonucleotide linkers instead of the stem−loop II region can form homo- or heterodimers that are very active (Scheme 20).[237−239] These minizymes form dimeric structures with two catalytic centers, two binding sites and a single, common stem II. The activity of one homodimeric minizyme was found to be similar to that of the full-sized ribozyme.[237] Such dimeric hammerhead structures have a number of additional advantages. In particular, it is a very compact divalent structure that (in the case of a heterodimer) can simultaneously cleave at two sites in mRNA[238] and, moreover, it can be designed in such a way that it is only in the presence of a specific sequence that the heterodimeric minizyme can form an active catalytic core.[240]

Scheme 20



We explored the use of dimeric minizymes as gene-inactivating agents by placing minizymes under the control of an appropriate promoter.[241] The promoter we chose was the promoter for a human gene for $tRNA^{Val}$. Although we feared initially that the $tRNA^{Val}$ portion of the transcript might hinder the dimerization of $tRNA^{Val}$-driven minizymes, the novel tRNA-embedded minizyme had strong activity. To our surprise, the cleavage activity of this minizyme that had been expressed either in vitro or in HeLa cells was almost 1 order of magnitude higher than that of the $tRNA^{Val}$-embedded conventional hammerhead ribozyme. Results of NMR and molecular modeling studies supported the putative dimerization of the minizymes. These results indicate that tRNA-embedded minizymes should be considered as potential candidates for gene-inactivating agents. Since minizymes are able to cleave their RNA targets in vitro and in vivo,[241] it is clear that the stem/loop II is not essential for cleavage as long as the $G_{10.1}-C_{11.1}$ base pair is maintained in the dimeric structure, most probably to allow capture of the structurally important Mg^{2+} ion.

G. Chemically Modified Ribozymes

Trans-acting ribozymes exert their activity in a highly specific manner and they are, therefore, not expected to have negative effects on nontargeted cellular functions.[171,172] Because of this specificity, the concept of exploiting ribozymes for cleavage of a specific target mRNA is now emerging as a therapeutic strategy in human diseases such as cancer and AIDS.[14,242−247] For ribozymes to function as therapeutic agents, they must be introduced exogenously or produced endogenously in the target cells.[248,249] In the former case, the chemically modified ribozyme must maintain its catalytic activity while also being resistant to nucleases. A major advantage of chemically synthesized ribozymes is that site-specific modifications can be introduced at any position in the molecule.[250−253] This approach provides flexibility in the design of ribozymes that have wild-type catalytic activity and are not cleaved by nucleases.

A variety of selective and uniform structural modifications have been applied to oligonucleotides to enhance their resistance to nucleases.[254−256] Improvements in the chemical synthesis of RNA[257−262] have led to the ability to modify similarly ribozymes that contain the core motif of hammerhead ribozyme.[263−265] Cedergren's group demonstrated that 2′-O-Me modification of a ribozyme at all positions except G_5, G_8, A_9, $A_{15.1}$, and $G_{15.2}$ yielded a catalytically active molecule with a greatly decreased value of k_{cat} in vitro but with a 1000-fold increase in resistance to nucleases in a yeast extract compared to that of the all-RNA ribozyme.[265] Lamond's group found that a per-substituted 2′-O-allyl-containing ribozyme with ribose residues at positions U_4, G_5, A_6, G_8, G_{12}, and $A_{15.1}$ showed a 5-fold decrease in catalytic activity compared to the all-RNA ribozyme, while the stability of this ribozyme in bovine serum was increased substantially, with 30% remaining intact after 2 h, as compared to the half-life of less than one minute of the all-RNA ribozyme.[266] Simple substitution of the phosphate linkage at positions C_3 and U_4 by phosphorothioate plus replacement of U_7 by A or G in a phosphorothioate and substitution of the RNA arms with DNA arms resulted in a 100-fold increase in stability but a 15-fold ($U_7 \rightarrow A_7$) or a 42-fold ($U_7 \rightarrow G_7$) reduction of catalytic activity as compared to the wild-type ribozyme.[267] Eckstein's group demonstrated that substitution at all pyrimidine nucleotides in a hammerhead ribozyme by 2′-amino or 2′-fluoro resulted in a 25- to 50-fold decrease in activity and a 1200-fold increase in stability in rabbit serum as compared to the unmodified ribozyme.[268] Enhancement of k_{cat} was also recognized with chimeric RNA-DNA hammerhead ribozymes in which the binding arms (stems I and III) were made of DNA (as long as $C_{15.2}A_{15.1}$, that recognize the $G_{16.2}U_{16.1}C_{17}$ triplet, are maintained as RNA).[215,267,269−271] The increase in k_{cat} by DNA arms was demonstrated to be due either to the acceleration of the dissociation of the products from the reaction complex[270] because of the lower stability of the duplex between RNA and DNA than between RNA and RNA, or to the enhancement of the chemical cleavage.[215,269,271] 2′-Deoxy modification at positions G_{12} and U_7 in the active center of the hammerhead was reported to yield a more active enzyme than the unmodified parent as a result of an increase in k_{cat} and even dU_4 was tolerated with a mere 2-fold decrease in activity.[272]

VII. Mechanism of Action of Hammerhead Ribozymes

In section VI, we reviewed the general features of hammerhead ribozymes. We shall now examine the mechanism of action of ribozymes [hereafter, the term ribozymes refers exclusively to hammerhead ribozymes unless otherwise noted; the specific sequences of the ribozyme (R32) and substrate (S11) that we used in our own experiments are shown in Scheme 14].

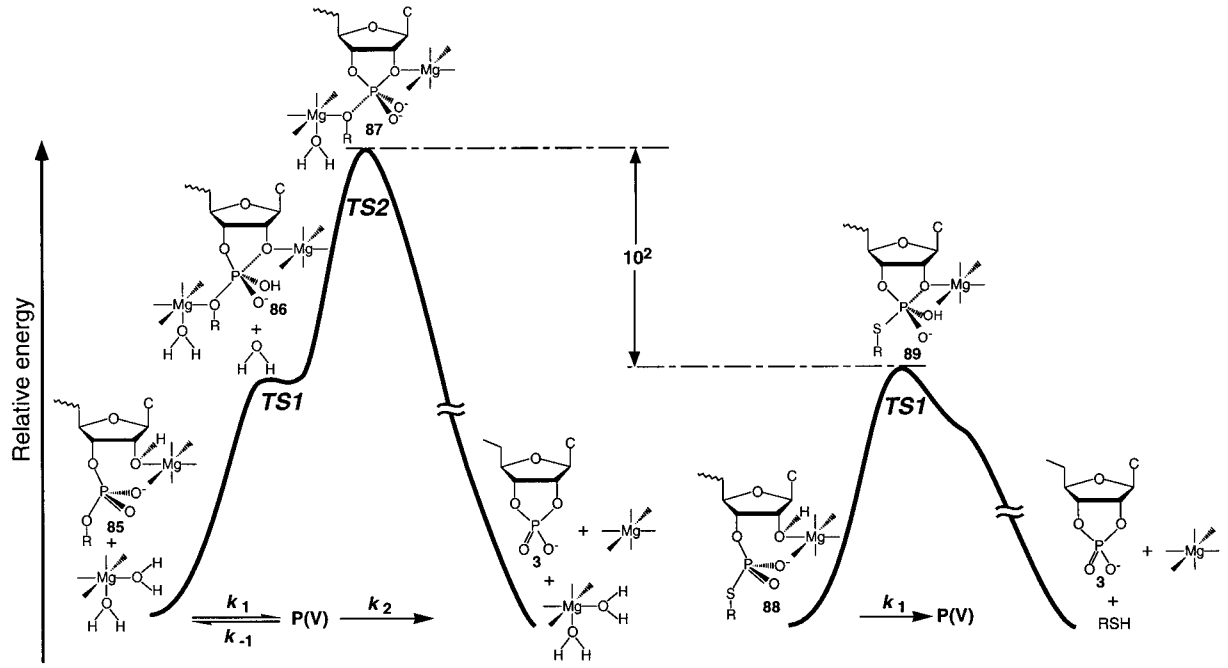


Figure 4. Reaction profiles for the hammerhead ribozyme-mediated cleavage of normal (**85**) and 5′-thio (**88**) RNAs. The direct coordination of a metal ion with 2′-oxygen polarizes and weakens the 2′-OH bond. As a result, higher concentrations of the active nucleophile, the metal-bound 2′-alkoxide of the ribose, will be produced. Therefore, there is an inverse correlation between the pK_a of the metal-bound ribose 2′-OH and the ribozyme activity, i.e., the lower the pK_a of the metal ion, the higher the cleavage rate at a given concentration of metal ions at a fixed pH. Similarly, the direct coordination of the metal ion with the 5′-oxygen of the leaving nucleotide residue weakens the P−(5′-O) bond. Metal ions with lower pK_a values will weaken the P−(5′-O) bond to a greater extent, thereby, activating the ribozyme-mediated cleavage to a greater extent. The rate-limiting step of the reaction for the 5′-thio substrate **88** has shifted from the cleavage of the P−(5′-O) bond (in the normal substrate **85**) to the formation of the P−(2′-O) bond (in **88**). In **88**, the buildup of charge at the 5′-leaving group occurs after the rate-limiting step and, therefore, any metal ion bound at that position is kinetically insignificant. Thus, for the cleavage of the weak P−(5′-S) bond in **88**, a second metal ion at the 5′ position is not required.

A. Evidence for the Rate-Limiting Departure of the 5′-Oxygen in Hammerhead Ribozyme-Catalyzed Reactions

In the case of the nonenzymatic hydrolysis of RNA, the cleavage of the P−O(5′) bond is the overall rate-limiting step in the hydrolysis of natural RNAs, whereas the attack of the 2′-OH on phosphorus is the rate-limiting step for 5′-thio RNA (**61**), since **61** is hydrolyzed much more rapidly than the natural RNA, **1** (Scheme 11).[100−108] The same technique as that used to draw these conclusions was used to determine the rate-limiting step in reactions catalyzed by ribozymes.[78,101,103,108]

Two kinds of substrates were synthesized, both containing a ribonucleotide with a 5′-thio leaving group at the site of cleavage by the ribozyme. In the first case, the substrate was made of DNA that contained a single mandatory ribonucleotide with a 5′-thio leaving group at the cleavage site.[101−104] In the second case, the substrate consisted entirely of RNA with a 5′-thio leaving group at the cleavage site.[78,108] In the first case, the 5′-thio DNA substrate (corresponding to **61**) was cleaved by a ribozyme at a rate similar to that of cleavage of the parental 5′-oxy DNA substrate (corresponding to **1**), at pH 7.5 in the presence of 10 mM Mg^{2+} ions. However, in the second case, the 5′-thio RNA substrate (**88** in Figure 4) was cleaved by the R32 ribozyme almost 2 orders of magnitude more rapidly than the parental 5′-oxy RNA substrate S11 (**85** in Figure 4), at pH 6.0 in the presence of 0.3 mM Mg^{2+} ions [since our 5′-thio RNA substrate was very labile, we had to identify reaction conditions (low pH and low concentrations of metal ions) under which nonribozyme-mediated hydrolysis could be minimized]. The discrepancy between these two results could potentially originate, at least in part, from the differences in substrates used (almost all-DNA vs all-RNA; we realized that, unless GUC triplet is maintained as RNA, the cleavage efficiency drops significantly).[215,267] It is also to be mentioned that since the activity of ribozymes increases linearly with pH from pH 6.0 to above 8.0 with a slope of unity, it should be possible to characterize the nature of the transition state for the chemical cleavage step in the entire pH region from pH 6 to 8, as long as the slope is unity. In fact, kinetics performed at pH 6.5 identified a specific metal ion in the transition state of a hammerhead ribozyme in its active conformation.[200] The discrepancy between the two results (with almost all-DNA vs all-RNA substrate) may be explained as follows: in the case of the ribozyme-mediated cleavage of the 5′-thio DNA−substrate, the slope of the pH−rate profiles was 0.24 or 0.36, respectively, in the presence of either Mn^{2+} or Mg^{2+} ions,[104] an indication that the chemical step is hidden by other step(s) at higher pH regions [an indication that the chemical step is *not* the (sole) rate-limiting step]. Then, since the corresponding slope for the natural substrate was unity (since the chemical step *is* the sole rate-limiting step), it is not valid to make a comparison of the activities

of unmodified and 5′-thio-modified DNA substrates at higher pH regions.[104] At any rate, since our almost all-RNA substrate with a 5′-thio leaving group was hydrolyzed by the ribozyme more than 3 orders of magnitude more rapidly than their corresponding almost all-DNA substrate with a single mandatory ribonucleotide with a 5′-thio leaving group (upon correction of pH and the concentration of metal ions to the same respective values) and since we confirmed by the use of two types of ribozyme that we followed the chemical cleavage step,[78] we conclude that the result obtained with our almost all-RNA substrate should reflect true ribozyme-mediated catalysis.

Following the same argument as used in section V.A, we can see that the attack by the 2′-oxygen at C_{17} on the phosphorus (**TS1**) must be the rate-limiting step for the 5′-thio RNA substrate **88** and the departure of the 5′-leaving group (**TS2** with the transition state structure **87**) must be the rate-limiting step for the parental 5′-oxy RNA substrate **85**, for the cleavage reactions catalyzed by a hammerhead ribozyme (as indicated by the energy diagrams in Figure 4). Note that, if the rate-limiting step were the attack by the 2′-oxygen at C_{17} on the phosphorus (**TS1**) in both cases, both **85** and **88** should have been hydrolyzed at the same rate since sulfur is expected to enhance both specifically and significantly the cleavage of the P−(5′-S) bond because the pK_a of thiol is more than 5 units lower than that of the corresponding alcohol.[110,111]

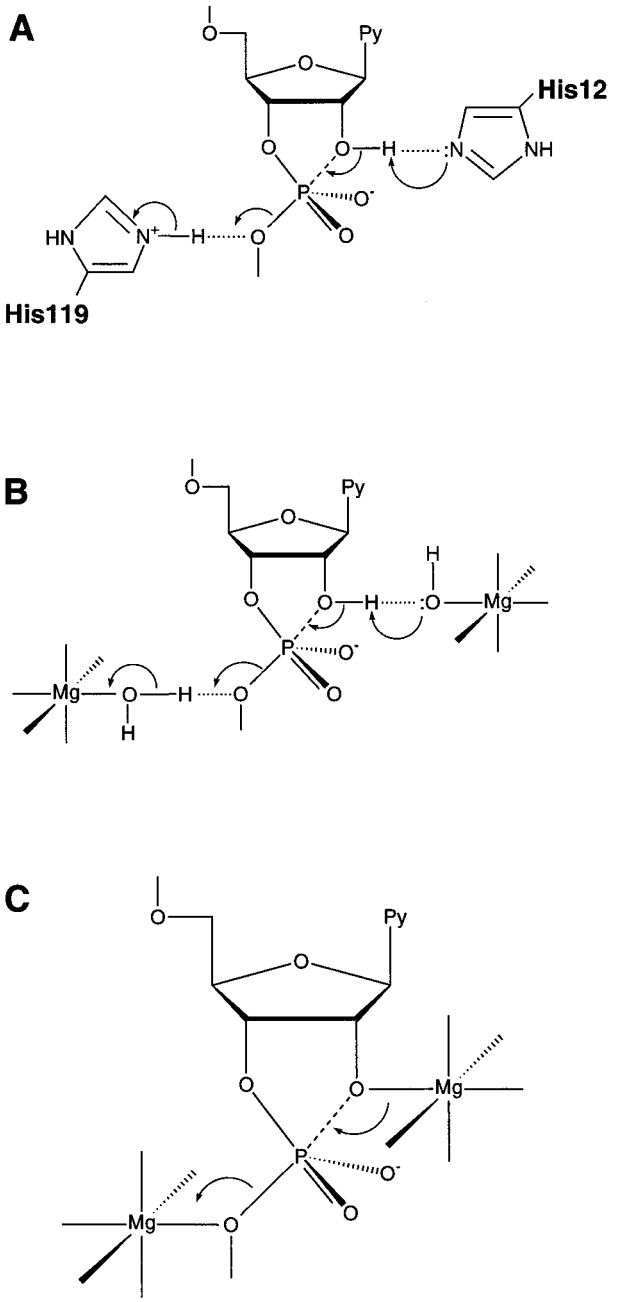
As argued in the case of Scheme 11, a pentacoordinate intermediate should not exist during the ribozyme-catalyzed hydrolysis of **88**. It is uncertain whether a pentacoordinate intermediate [P(V), corresponding to **2** in Scheme 11] is a kinetically significant intermediate, if it exists at all, in the ribozyme-catalyzed hydrolysis of **85**. However, we can conclude the following: the departure of the 5′-leaving oxygen is the rate-limiting step not only in the nonenzymatic hydrolysis of RNA (Scheme 11) but also in the hammerhead ribozyme-catalyzed reaction with the natural RNA substrate (Figure 4). This conclusion does not depend on the lifetime of the pentacoordinate intermediate P(V). If the lifetime of P(V) were kinetically insignificant (if the reaction were concerted), the actual, single transition state for **85** would be **TS2**-like.

The detailed mechanism involving the postulated "double-metal-ion mechanism" of catalysis, shown in Figure 4, will be discussed below in sections VII.D and VII.E.

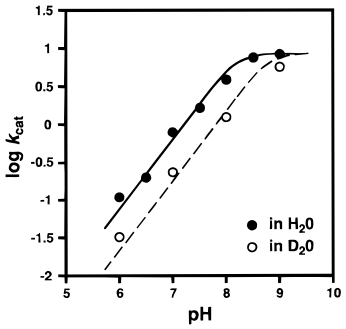
B. Absence of the Transfer of a Proton in the Transition State of a Ribozyme-Catalyzed Reaction

In the hydrolysis of RNA, both acid and base catalysts are required for the efficient cleavage of phosphodiester bonds.[27] In the case of the proteinaceous enzyme RNase A, which does not require metal cofactors, the reaction is initiated by a histidine residue at position 12 (His12), which acts as a base catalyst by abstracting a proton from 2′-OH (Scheme 21A). The resulting, more nucleophilic 2′-oxygen then attacks phosphorus to generate a pentacoordinate intermediate/transition state. Finally, His119 acts as an acid catalyst by supplying a proton to the leaving 5′-oxygen, which stabilizes the overall transition state **TS2** in Scheme 11, with the resultant cleavage of the exocyclic P−(5′-O) bond. The acid/base system provided by the two histidine residues in RNase A can, in principle, be replaced by Mg^{2+}-bound water moieties (Scheme 21B).[7] Alternatively, according to our molecular orbital calculations,[33,53,59,61] direct coordination of Mg^{2+} ions with the attacking or the leaving oxygen can promote formation or cleavage of the P−O bond (Scheme 21C). The latter two mechanisms should be distinguishable because the former (Scheme 21B) involves a proton-transfer process in the transition state, whereas the latter (Scheme 21C) does not.[190] To examine whether a proton-transfer process occurs in the transition state in reactions catalyzed by hammerhead ribozymes, we

Scheme 21



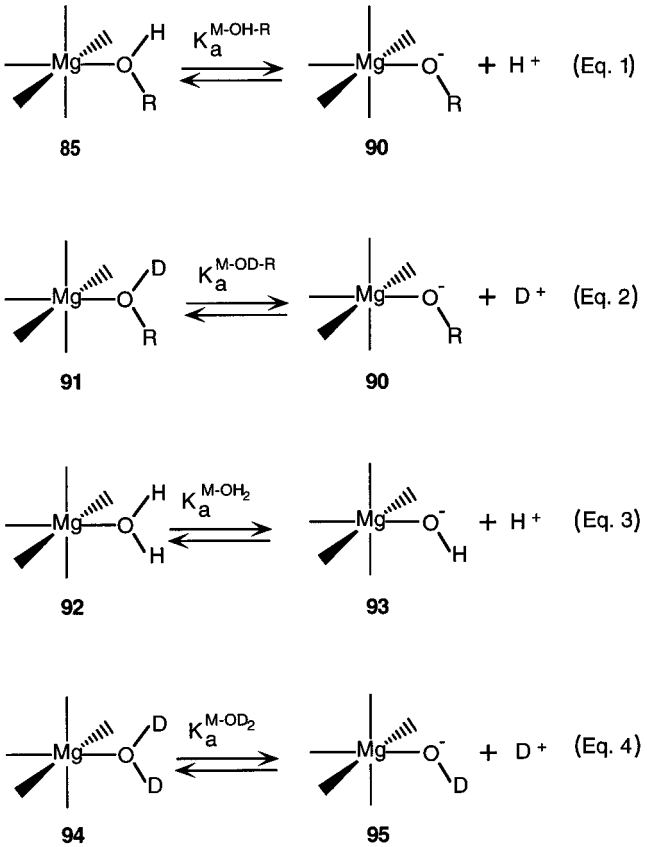
Scheme 22



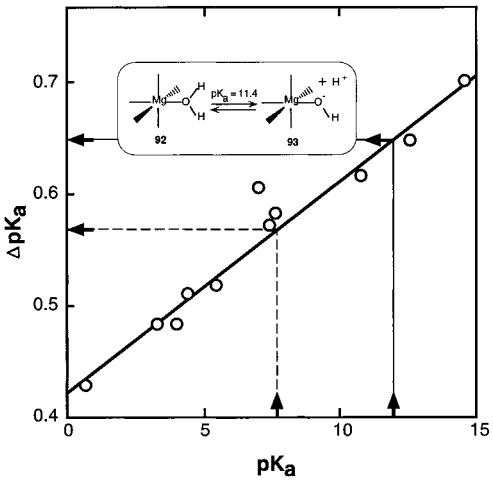
measured solvent isotope effects for the ribozyme R32. We chose R32 because, in this case, the chemical cleavage step has been proven unambiguously to be the sole rate-limiting step in the system; the rate constants (k_{cat}) measured in H_2O or D_2O represent the pure chemical cleavage step (k_{cleav} in Scheme 16).[190,273]

Solvent isotope effects for R32-catalyzed cleavage of S11 were measured under single-turnover conditions at 25 °C at pH 6. The cleavage rate constant in H_2O [$k_{cleav(H_2O)}$, 0.086 min^{-1}] was 4.4 times larger than the corresponding value in D_2O ($k_{cleav(D_2O)}$, 0.020 min^{-1}).[190,273] This apparent isotope effect of 4.4 could be taken as evidence to support the involvement of transfer of a proton in the transition state.[190,191] However, since the measurements were made at pH 6 where the slope is unity in the pH−log(k_{cat}) profile (Scheme 22) and, moreover, since the concentration of the active species (M−O$^-$−R; **90** in eq 1 in Scheme 23) in H_2O was severalfold higher than that (M−O$^-$−R; **90** in eq 2) in D_2O at a fixed pH,[274,275] the reduction in the level of the active species **90** in D_2O could have been the sole cause of the lower rate of the reaction in D_2O. As we shall discuss below, the active species represented by M−O$^-$−R (**90** in Scheme 23) is the Mg^{2+}-bound 2′-alkoxide and the specific structure of its protonated form (M−OH−R) is **85**, shown in Figure 4.

Scheme 23



Scheme 24



Since the concentration of **90** in H_2O (eq 1 in Scheme 23) is higher than that in D_2O (eq 2) at a fixed pH, we must take this difference in pK_a, ΔpK_a, that is log($K_a^{H_2O}/K_a^{D_2O}$), into account when we analyze the data. It is possible to estimate the relative concentrations of the active species (M−O$^-$−R) in H_2O vs D_2O, since a linear relationship exists (Scheme 24) between the magnitude of the isotope effect on the acidities of various organic acids (ΔpK_a) and their values of pK_a,[274,275] if we can assume that (i) hydrated metal ions have the same relative ratios of $K_a^{H_2O}/K_a^{D_2O}$ as organic acids and (ii) the ratio of the equilibrium constants ($K_a^{M-OH-R}/K_a^{M-OD-R}$) in eq 1 and eq 2 is the same as that ($K_a^{M-OH_2}/K_a^{M-OD_2}$) in eqs 3 and 4. That is, a change in the equilibrium concentration of metal-bound 2′-alkoxide at the catalytic center ($K_a^{M-OH-R}/K_a^{M-OD-R}$) is nearly identical to a change in the equilibrium concentration of the metal hydroxide ($K_a^{M-OH_2}/K_a^{M-OD_2}$). The solid arrow in Scheme 24 indicates the estimate of the ΔpK_a for a water molecule coordinated to a Mg^{2+} ion with a $pK_a^{M-OH_2}$ of 11.4 (eq 3).[273−275] The estimated ΔpK_a turns out to be 0.65, as indicated by the solid arrow. Since log($K_a^{H_2O}/K_a^{D_2O}$), ΔpK_a, is 0.65, the equilibrium ratio ($K_a^{M-OH_2}/K_a^{M-OD_2}$) in eq 3 and eq 4 is about 4.5. The ΔpK_a estimated from the linear plot matched the experimentally obtained value in the case of Cu^{2+}−OH_2 (dashed arrow),[273] suggesting that the linear plot shown in Scheme 24, which is based on data for various organic acids, might also be useful for estimating ΔpK_a of hydrated metals, which in turn suggests the validity of the above assumptions i and ii.

The estimation indicated that the concentration of the active species (M−O$^-$−R, **90**) is 4.5 times higher in H_2O than that in D_2O at 25 °C. Therefore, the 4.4-fold lower rate of the reaction in D_2O than in H_2O should be interpreted as the result of the perturba-

tion of the pK_a. We can, therefore, conclude that the reduction in the level of the active species **90** in D_2O was the sole cause of the lower rate of the ribozyme-catalyzed reaction in D_2O at a given pH.[190,273] Thus, the absence of an actual kinetic isotope effect in the step that leads to cleavage of a phosphodiester bond by a ribozyme can be interpreted only in terms of a mechanism in which transfer of a proton does not occur in the transition state. This observation is consistent with the double-metal-ion mechanism of catalysis, proposed by Steitz and Steitz,[187] in which Mg^{2+} ions are directly coordinated with the attacking and the leaving oxygens (Scheme 21C).[190,191,273] Such a direct coordination was also proposed on the basis of our molecular orbital calculations.[53,59,61]

As pointed out by von Hippel's group,[191] the direct coordination of the metal ion with the 2′-oxygen of the attacking nucleotide residue, as shown in Scheme 21C, polarizes and weakens the 2′-OH bond. As a result, the equilibrium in eq 1 shifts to the right, yielding higher concentrations of the active nucleophile, the metal-bound 2′-alkoxide of the ribose (M−O$^-$−R, **90**). Therefore, an inverse correlation between the pK_a^{M-OH-R} of the metal-bound ribose 2′-OH and the ribozyme activity holds. In other words, the lower is the pK_a of the metal ion, the higher is the cleavage rate at a given concentration of the metal ion at a fixed pH.[185,191] von Hippel and his colleagues point out that this kind of inverse correlation between the pK_a of the metal ion and the ribozyme activity would not have been obtained if the metal hydroxide acted as a Brønsted base, as shown in Scheme 21B,[191] because the conjugate base of a strong acid is a weak base. Metal ions with lower values of pK_a (stronger acids) are expected to be present at higher concentrations at the active site in the form of a metal hydroxide **93** (at a given pH), but such ions should be correspondingly weaker bases and, therefore, should be less able to remove the 2′-OH proton, since the 2′-OH group has a relatively high pK_a. As a result, there should be virtually no correlation between the pK_a of the metal ion and the cleavage activity of ribozymes because these two effects (greater basicity and reduced occupancy of the metal hydroxide species **93**) should cancel each other out. Therefore, the metal hydroxide mechanism of Scheme 21B seems not be the true mechanism of the reaction.

C. Profiles of pH vs Rate

The direct coordination of the metal ion with the 5′-oxygen of the leaving nucleotide residue, as shown in Scheme 21C, weakens the P−(5′-O) bond.[61] Metal ions with lower values of pK_a will weaken the P−(5′-O) bond to a greater extent and, thereby, activate the ribozyme-mediated cleavage to a greater extent.[61,191] In this case, the active Lewis acid should be the completely protonated metal-bound water molecules, as depicted in the transition-state structure **87** in Figure 4 [left species in eq 5 below, where (P)R′O represents the phosphorus-bound 5′-oxygen].

$$(P)R'O-M-OH_2 \rightleftharpoons (P)R'O-M-O^-H + H^+ \quad (5)$$

Then, in the pH−rate profile of a ribozyme-catalyzed reaction, we would expect a slope of unity from eq 1, since deprotonated **90** has an active nucleophile, and a slope of −1 from eq 5, since protonated **87** at the 5′-oxygen moieties has the active Lewis acid [(P)−R′(5′-O)−M−OH_2]. The result is a bell-shaped pH−rate profile. Such bell-shaped pH−rate profiles are common for the imidazole-catalyzed hydrolysis of RNA[276,277] and the RNase A-catalyzed hydrolysis of RNA,[278,279] in that maximum activity is found around pH 7, reflecting the pK_a of the catalytic molecule (imidazole or histidine). In the case of ribozyme-catalyzed reactions, as shown in Scheme 22, the activity increases linearly with pH from pH 6 to as high as pH 9,[61,185,215] in accord with eq 1. However, because of the high pK_a of the metal-bound water molecules (>10), the decrease in activity (according to eq 5) at an even higher pH is not discernible experimentally.

The decline in the slope above pH 8.5 in the pH−rate profile in Scheme 22 reflects a change in the rate-limiting step, from a rate-limiting chemical cleavage step (k_{cleav} in Scheme 16) to a rate-limiting association step (k_{assoc}), probably as a result of the deprotonation of uridine and guanosine moieties under more alkaline conditions.[61,185,215] Therefore, in reality, the rate constants obtained above pH 8.5 do not represent k_{cat}; strictly speaking, they represent k_{cat}/K_m.

The absence of a proton-transfer process in reactions catalyzed by a hammerhead ribozyme can be rationalized in terms of the double-metal-ion mechanism of catalysis, as supported by ab initio calculations.[53,61] In the mechanism depicted in Scheme 21C, the metal ion in the 2′ position is expected to be the one that is correlated with the pK_a of the hydrated metal ion. This effect is thermodynamic and not kinetic. Since the transfer of a proton is expected to be rapid,[280] the concentration of the active species, specifically, the concentration of the metal-bound 2′-alkoxide (M−O$^-$−R, **90**, in Scheme 23, that yields **86** in Figure 4), is at a steady state, determined by the pK_a of the metal ion. This phenomenon gives rise to the apparent kinetic effect, as shown by the slope of unity in the pH−rate profile below pH 8 (Scheme 22). The second metal ion at the 5′ position is a true catalyst because it stabilizes the developing charge of the 5′-leaving oxygen in the true transition state **TS2**.[53,61] This configuration should lower the activation energy barrier and accelerate the reaction in a true kinetic fashion (in other words, the effect here is *not* thermodynamic effect that influences the concentration of active species).

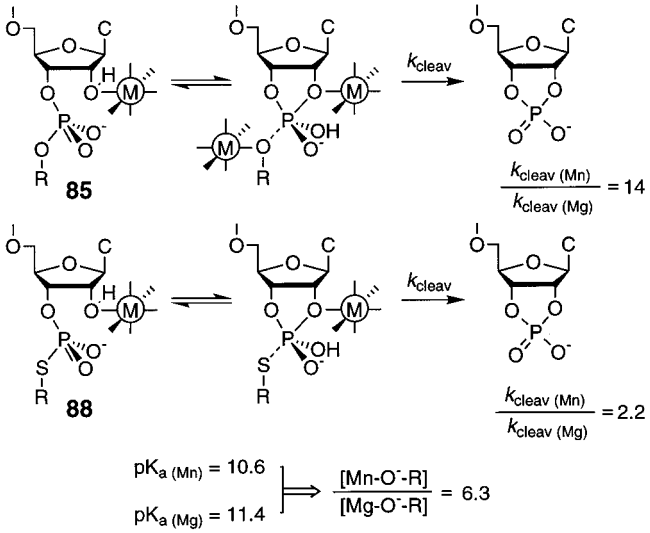
D. Differential Effects of Metal Ions on the Cleavage of 5′-Oxy and 5′-Thio Substrates

As discussed in the previous section, the absence of kinetic isotope effects, which indicates that a proton-transfer process is not involved in the reactions catalyzed by a hammerhead ribozyme, is consistent with the double-metal-ion mechanism of catalysis.[190] However, we cannot completely exclude the possibility that there exists a small isotope effect

"buried" within the estimation of ΔpK_a (Scheme 24). In this section we shall discuss additional direct experimental support for the double-metal-ion mechanism of catalysis.

Two kinds of substrate, the parental 5′-oxy substrate S11 (**85** in Figure 4) and the corresponding 5′-thio substrate **88**, were subjected to R32 ribozyme-mediated cleavage at pH 6.0 in the presence of either 0.3 mM Mg^{2+} ions or 0.3 mM Mn^{2+} ions.[78] The results are summarized in Scheme 25. As expected

Scheme 25



from eq 1, both substrates were cleaved more rapidly in the presence of Mn^{2+} ions than in the presence of Mg^{2+} ions, since metal ions with a lower pK_a produce higher concentrations of active species (M−O$^-$−R, **90**). The pK_a of the Mn^{2+}-bound water molecule ($pK_a^{M-OH_2}$ in eq 3) is 10.6 and that of the Mg^{2+}-bound water molecule is 11.4.[281] Thus, at a given pH, the concentration of the active species (Mn^{2+}−O$^-$−R) is roughly 6.3 times higher in Mn^{2+}-containing solutions than that of the corresponding active species (Mg^{2+}−O$^-$−R) in Mg^{2+}-containing solutions. Therefore, 6.3-fold is the theoretical maximum value of the difference in the steady-state concentrations of the two active species, specifically, the metal-bound 2′-alkoxides (M−O$^-$−R, **90**), which is determined by the pK_a of each metal ion.

However, as can be seen in Scheme 25, R32-mediated cleavage of S11 is 14 times more efficient in the presence of Mn^{2+} ions than in the presence of Mg^{2+} ions, a result that cannot be explained only by the difference in concentrations of active species (M−O$^-$−R) since the difference should be 6.3-fold at most. This discrepancy suggests the involvement of more than one catalytic metal ion. From the energy diagram in Figure 4, we can rationalize the different effects of metal ions as follows.

From the energy diagram for the R32-mediated cleavage of **88**, when the 5′-oxygen at the scissile bond has been replaced by sulfur to yield a much better leaving group, nucleophilic attack by the 2′-oxygen becomes the rate-limiting step, with an early transition state (**TS1**) and little bond formation. The rate of cleavage of **88** is then governed by k_1, namely:

$$k_{cat} = k_{cleav} = k_1 \quad (6)$$

Since the rate of cleavage of **88** by R32 was increased 2.2-fold (Scheme 25) when Mg^{2+} ions were replaced by Mn^{2+} ions, the ratio of the rate constants can be expressed by eq 7, where the subscripts (Mg) and (Mn) identify the rate constants obtained in the presence of Mg^{2+} ions and Mn^{2+} ions, respectively:

$$k_{1(Mn)}/k_{1(Mg)} = 2.2 \quad (7)$$

The fact that value of $k_{1(Mn)}/k_{1(Mg)}$ is 2.2 for the R32-mediated cleavage of **88** means that, despite the 6.3-fold higher concentration of the Mn^{2+}-bound nucleophile, the nucleophilicity of the Mn^{2+}-bound 2′-O$^-$ moiety is lower than that of the Mg^{2+}-bound 2′-O$^-$ moiety (Brønsted rule). Note that a nucleophile with a higher pK_a (Mg^{2+}-bound 2′-O$^-$) is expected to be less stable, more reactive, and a better nucleophile than a nucleophile with a lower pK_a (Mn^{2+}-bound 2′-O$^-$). Thus, according to the well-established correlation, $\log k = \beta \cdot pK_a + \text{constant}$, the Brønsted β value turns out to be 0.57 for the R32-catalyzed reactions, a reasonable value for this kind of nucleophilic reaction.

For the normal substrate **85**, the chemical cleavage is determined by both **TS1** and **TS2**, if the reaction proceeds in a stepwise manner. Then, the rate of the chemical cleavage can be expressed by

$$k_{cat} = k_{cleav} = k_1 k_2/(k_{-1} + k_2) \quad (8)$$

The overall value of $k_{cleav(Mn)}/k_{cleav(Mg)}$ was found to be 14 (Scheme 25) for the R32-mediated cleavage of **85** (eq 9):

$$[k_{1(Mn)}k_{2(Mn)}/(k_{-1(Mn)} + k_{2(Mn)})]/[k_{1(Mg)}k_{2(Mg)}/(k_{-1(Mg)} + k_{2(Mg)})] = 14 \quad (9)$$

Rearrangement of eq 9 and substitution of $k_{1(Mn)}/k_{1(Mg)}$ by 2.2 for the R32-mediated cleavage of **85** leads to eq 10, as follows:

$$k_{2(Mn)}/k_{2(Mg)} = [14(k_{-1(Mn)} + k_{2(Mn)})]/[2.2(k_{-1(Mg)} + k_{2(Mg)})] = 6.4[(k_{-1(Mn)} + k_{2(Mn)})]/[(k_{-1(Mg)} + k_{2(Mg)})] \quad (10)$$

In eq 10, since $k_{-1(Mn)} > k_{2(Mn)}$, $k_{-1(Mg)} > k_{2(Mg)}$ (see left energy diagram in Figure 4), and since the value of $k_{-1(Mn)}/k_{-1(Mg)}$ is expected to be larger than unity {because of the principle of microscopic reversibility for the reverse direction [disruption of P(V) by P−(2′-O) bond cleavage] of the forward process [formation of P(V)] that resulted in eq 7}, the value of $k_{2(Mn)}/k_{2(Mg)}$ for **TS2** can be estimated to be greater than 6.4.

If there were no involvement of metal ions in **TS2** for the cleavage of **85**, the overall value of $k_{cleav(Mn)}/k_{cleav(Mg)}$ should be lower than the theoretical maximum value of 6.3, as in the case of **88** (eq 7). However, the fact that the value of $k_{2(Mn)}/k_{2(Mg)}$ for the **TS2** state appears to be greater than 6 suggests the involvement of metal ions not only at **TS1** but also at **TS2**, reflecting the superior catalytic power of the

Mn^{2+} ion as a Lewis acid at **TS2** because of its lower pK_a value compared to that of the Mg^{2+} ion.

E. Double-Metal-Ion Mechanism

The argument laid out in the previous section VII.D can be rephrased as follows. Scheme 25 provides two sets of data that compare the ribozyme-mediated cleavage of a substrate in the presence of either Mg^{2+} or Mn^{2+} ions. The first set of data uses the normal substrate **85** and the second set uses the 5′-thio analogue **88**. In the first case, we observed that the kinetic ratio $[k_{cleav(Mn)}/k_{cleav(Mg)}]$ for **85** is 14; the magnitude of this ratio can be taken as an indication of the involvement of two required metal ions because it is greater than the theoretical maximum of 6.3, the maximum difference in the concentrations of active species calculated from a $pK_a^{Mn-OH_2}$ of 10.6 and a $pK_a^{Mg-OH_2}$ of 11.4. Thus, the value of 14 represents the additive effects of two essential metal ions.

In the second case, the same experiment was performed with **88**. For this substrate, the corresponding ratio was 2.2 which is significantly lower than the theoretical maximum of 6.3 and it is also very much lower than the experimental value for **85** of 14. This difference can best be interpreted, on the basis of the energy diagram in Figure 4, as indicating that the rate-limiting step of the reaction with **88** has shifted from the cleavage of the P−(5′-O) bond (in **85**) to the formation of the P−(2′-O) bond (in **88**). In **88**, the buildup of charge at the 5′-leaving group occurs after the rate-limiting step and, therefore, any metal ion bound at that position is kinetically insignificant. The value of $k_{cleav(Mn)}/k_{cleav(Mg)}$, of 2.2 for the R32-mediated cleavage of **88** indicates that the nucleophilicity of the Mn^{2+}-bound 2′-O^- is lower than that of the Mg^{2+}-bound 2′-O^-, in view of the fact that the concentration of the Mn^{2+}-bound nucleophile is 6.3 times higher than that of the Mg^{2+}-bound nucleophile.

In short, in the case of the normal substrate **85**, two metal ions are required because the kinetic ratio $[k_{cleav(Mn)}/k_{cleav(Mg)}]$ is too large to be accounted for by a single-metal model, and the same ratio for **88** is small enough to represent involvement of a single metal ion. Note that the theoretical maximum value of 6.3 is the calculated ratio of catalytically active species and is based upon the difference in values of pK_a of the two aqueous metal ions, Mg^{2+} and Mn^{2+}. Anything over this value *must* then be due to involvement of a second metal ion. If there were no involvement of metal ions in **TS2** for the cleavage of **85**, the overall value of $k_{(Mn)}/k_{(Mg)}$ should be lower than 6.3, as in the case of **88** with the extremely weak P−S bond that does not require an acid catalyst for cleavage. Note also that the Mn^{2+} ion with a lower pK_a will weaken the P−(5′-O) bond to a greater extent than does the Mg^{2+} ion, just as the former weakens a metal-bound O−H bond to a greater extent than does the latter, with the resultant large, overall kinetic ratio $[k_{cleav(Mn)}/k_{cleav(Mg)}]$ for **85** of 14.

The above argument is also valid for concerted reactions with a single transition state, which is **TS2**-like and **TS1**-like for **85** and **88**, respectively. The metal ion at the 2′ position is the one that is correlated with the pK_a of the hydrated metal ion, reflecting the slope of unity in the pH−rate profile.[61,185,215] Unless a metal ion is coordinated directly with the 5′-oxygen, the kinetic ratio $[k_{cleav(Mn)}/k_{cleav(Mg)}]$ for **85** should not exceed the theoretical maximum of 6.3 because only at the 5′ position can a Mn^{2+} ion be a better catalyst than a Mg^{2+} ion because of its lower pK_a value and capacity to weaken the P−(5′−O) bond to a greater extent. At the 2′ position, the nucleophilicity of the Mn^{2+}-bound 2′-O^- should be lower than that of the Mg^{2+}-bound 2′-O^-, as observed in the case of **88**, where the kinetic ratio $[k_{cleav(Mn)}/k_{cleav(Mg)}]$ is smaller than the theoretical maximum of 6.3: the observed kinetic ratio for **88** was 2.2. Therefore, the present kinetic evidence demands that we invoke the double-metal-ion mechanism of catalysis for reactions catalyzed by hammerhead ribozymes,[78] regardless of the presence or absence of a pentacoordinate intermediate P(V). Such double-metal-ion catalysis has also been proven to be an efficient mechanism for the cleavage of phosphodiester bonds in nonenzymatic reactions.[282−286]

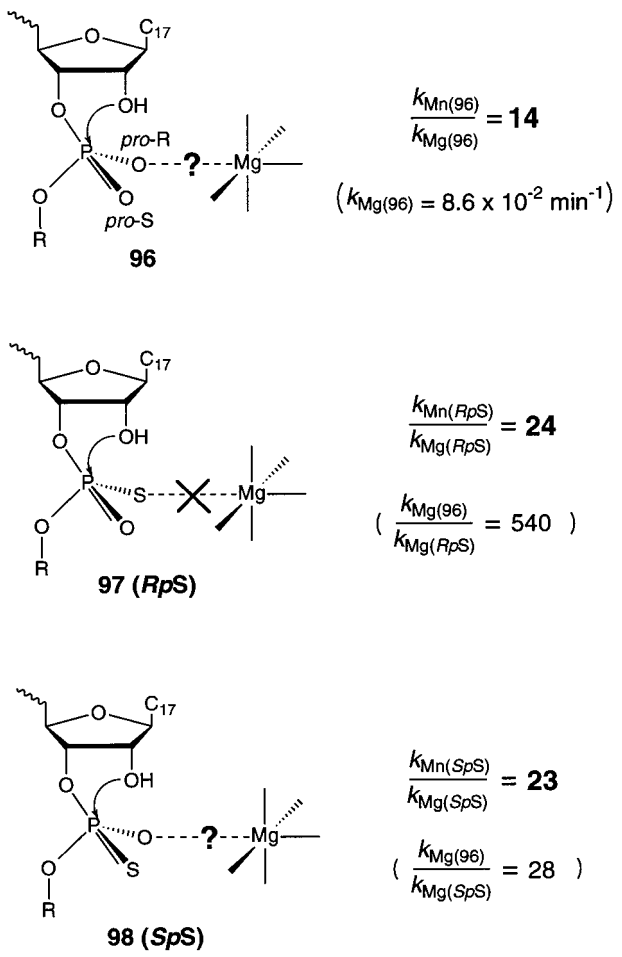
Thus, on the basis of the relative energies of reactions catalyzed by a hammerhead ribozyme depicted in Figure 4, for both a natural substrate and a modified substrate with a 5′-bridging thiophosphate linkage, we have been able quantitatively to analyze the differential effects of metal ions. We can conclude that (i) the departure of the 5′-leaving group is the rate-limiting step in the hammerhead ribozyme-catalyzed reaction with the natural substrate;[53,78,108] (ii) resembling the cofactor that is involved in reactions catalyzed by the *Tetrahymena* ribozyme (see below, section VIII.A),[186,193,287] a second metal cofactor appears to interact with the leaving group at the transition state (**TS2**) for the natural substrate, although no switch in metal ion specificity was observed for the 5′-thio substrate (because of a change in the rate-limiting step);[78] and (iii) it seems likely that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis.[61,187,190,191] An independent investigation by von Hippel's group led to the identical reaction mechanism, for the hammerhead ribozyme catalyzed reactions, as ours shown in the left diagram of Figure 4 (see Figure 5 in ref 79). The direct coordination of the metal ion with the 2′-oxygen (as shown in Scheme 21C)[61,78,79,190,191] was also supported by Piccirilli's group on the basis of their investigation using 2′-thio-substituted RNA substrate.[288]

VIII. Catalytic Roles of Metal Ions

The cleavage of RNA can be accelerated by a number of catalytic factors, which include (i) an acidic or a basic group to aid in deprotonation of the 2′-OH which, in effect, enhances the nucleophilicity of the 2′-oxygen, (ii) an acidic group that can neutralize and stabilize the leaving 5′-oxyanion group, and (iii) any environment that can stabilize the pentavalent species P(V), which is either a transition state or a short-lived intermediate.[275,281,289] In general, metal ions or protons can act as such factors in the cleavage of RNA, irrespective of whether cleavage is catalyzed by enzymes or by nonenzymatic systems. In some

cases, proton inventory studies support the involvement of acid/base catalysis.[290,291] In the case of ribonuclease A, proton inventory data revealed a single transition state (the concerted mechanism shown in Scheme 21A) in which each of two protons makes a normal contribution of $k_{H_2O}/k_{D_2O} = 1.75$, with a resultant overall isotope effect of 3.07.[290] Similarly, in a model of a bifunctional ribonuclease, in which two histidine residues act as an acid catalyst and as a base catalyst, respectively, isotope effects (k_{H_2O}/k_{D_2O}) of 2.12 and 1.90 were observed for each proton with a resultant overall isotope effect of 4.03.[291] In these cases, to avoid complexities due to ΔpK_a, proton inventory studies were carried out in the plateau regions of pH−rate profiles. We must emphasize that, if a similar proton-transfer reaction with an isotope effect of similar magnitude were to occur in the ribozyme-catalyzed reaction, even with involvement of a single proton, the apparent isotope effect (without a correction based on ΔpK_a) would be greater than 7, rather than the observed apparent value of $k_{H_2O}/k_{D_2O} = 4.4$ (a similar value was also obtained by McLaughlin's group[103]). If two protons were involved in such a transition state, a much higher value would have been observed (section VII.B). Therefore, in the case of ribozymes, which belong to an important new class of metalloenzymes insofar as divalent metal ions are essential for the cleavage of RNA, metal ions appear to have adopted the roles of histidine residues, as shown in Scheme 21C. In reality, during evolution, histidine residues of proteinacious enzymes (Scheme 21A) must have adopted the roles of catalytic metal ions (Scheme 21C) of the RNA world.

Scheme 26



A. Metal Ions in Catalysis by Hammerhead Ribozymes

The possible roles of metal ions in ribozyme-catalyzed reactions are as follows. (i) A metal-coordinated hydroxyl group might act as a general base, abstracting the proton from the 2′-OH (Scheme 21B) or, alternatively, a free metal ion might act as a Lewis acid to coordinate directly with the 2′-oxygen to accelerate its deprotonation (Scheme 21C). (ii) The developing negative charge on the 5′-oxygen leaving group might be stabilized by direct coordination with a metal ion (Scheme 21C) or by a proton, provided by a metal-bound water molecule (Scheme 21B). (iii) Direct coordination of a metal ion to the nonbridging oxygen or a hydrogen bond between metal-bound water and the nonbridging oxygen might stabilize the trigonal-bipyramidal transition state or intermediate and might render the phosphorus center more susceptible to nucleophilic attack (electrophilic catalysis; Scheme 26).

The first possibility (i) is supported by the finding that the cleavage activity of ribozymes increases linearly with pH from pH 5.5 to pH 9.0 with a slope of about 1 (Scheme 22).[61,185,215,236] From this observation, it is now generally concluded that a metal hydroxide is bound by the ribozyme to act as a base that abstracts the proton from the 2′-OH (Scheme 21B).[3,10,185] However, this argument was recently challenged by von Hippel's group because such a pH−rate profile is also consistent with a model (Scheme 21C) in which direct coordination between a divalent metal ion and the attacking 2′-oxygen takes place.[191] An increase in the concentration of OH^- ions stimulates formation of both possible active species. A higher concentration of OH^- ions increases the concentration of metal hydroxide and facilitates catalysis by deprotonating the nucleophilic 2′-OH, which leads to an increased concentration of the attacking 2′-O^- species. Similarly, a higher concentration of OH^- ions, which can deprotonate the 2′-OH that is bound directly to the metal ion, generates more of the attacking 2′-O^- species. Thus, the pH−rate profile cannot be used to distinguish between these two possible mechanisms.

In reactions catalyzed by ribozymes, an inverse correlation has been noted between the pK_a of metal ions and the ability to promote cleavage of RNA.[185,198] The lower the pK_a is, the higher is the cleavage rate at a given concentration of metal ions. This dependence on pK_a has been taken as evidence that a metal hydroxide participates in the cleavage reaction by abstracting a proton from the attacking 2′-OH,[185] by analogy to the role of the Pb^{2+} ion in the Pb^{2+}-dependent cleavage of phosphodiester bonds in tRNA.[292] However, as pointed out by von Hippel[191] and as discussed in section VII.D, the 2′-OH that is directly coordinated with a metal ion (Scheme 21C) is more likely to be the active species since the dependence on pK_a cannot be explained by the

solvated metal hydroxide acting as a base. Deprotonation of 2′-OH can be greatly accelerated by the direct binding to it of a metal ion because the pK_a of a metal-bound 2′-OH can be reduced by 3−8 units.[281]

Evidence for the second role of a metal ion (ii), namely, as a Lewis acid that stabilizes the leaving group, was first obtained with the *Tetrahymena* ribozyme.[186] In this case a Mg^{2+} ion coordinates to the leaving 3′-oxygen, with stabilization of the developing negative charge on the leaving 3′-oxygen. This mechanism became apparent when a switch in metal ion specificity was observed with a 3′-thio substrate.[186] The replacement of 3′-leaving oxygen by sulfur reduced the activity of the *Tetrahymena* ribozyme more than 1000-fold in the presence of Mg^{2+} ions. However, a change in metal ions from Mg^{2+} to Mn^{2+} ions reversed the effect, and the P−(3′-S) bond was cleaved nearly as rapidly as the P−(3′-O) bond. In view of the fact that Mn^{2+} ions coordinate with oxygen and sulfur more or less equally but Mg^{2+} ions prefer to coordinate with oxygen rather than sulfur,[293,294] these results indicate that the metal ion contributes directly to catalysis by coordination to the 3′-leaving oxygen in the transition state, stabilizing the developing negative charge on the leaving group.[186] Similar analysis identified the second metal ion in reactions catalyzed by the *Tetrahymena* ribozyme.[193] In the case of hammerhead ribozymes, the direct coordination of the second metal ion with the 5′-oxygen leaving group (Scheme 21C) was supported by an analysis based on Figure 4 and Scheme 25.[78]

The third role (iii) of a metal ion, namely, as an electrophilic catalyst that coordinates directly with the *pro*-R_p oxygen of the scissile phosphate was proposed, in the case of hammerhead ribozymes (Scheme 26), on the basis of results of a similar "rescue" experiment with Mn^{2+} ions.[198,295,296] Thio substitution at the *pro*-R_p oxygen at the cleavage site of a substrate (**R_pS**) for a hammerhead ribozyme resulted in a large thio effect that was relieved by replacement of Mg^{2+} by Mn^{2+} ions, which have higher affinity for sulfur than do Mg^{2+} ions.[198,295,296] This observation led to the general conclusion that a Mg^{2+} ion is directly coordinated with the *pro*-R_p oxygen. In this arrangement, the bound metal ion can act as an electrophilic catalyst and, thus, the proposed mechanism is very attractive as an explanation for the catalytic activity of metalloenzymes.[199] However, our reexamination of the thio effects argues against the generally accepted mechanism of electrophilic catalysis, namely, the direct coordination of a metal ion with the *pro*-R_p oxygen,[297] as described in the next section.

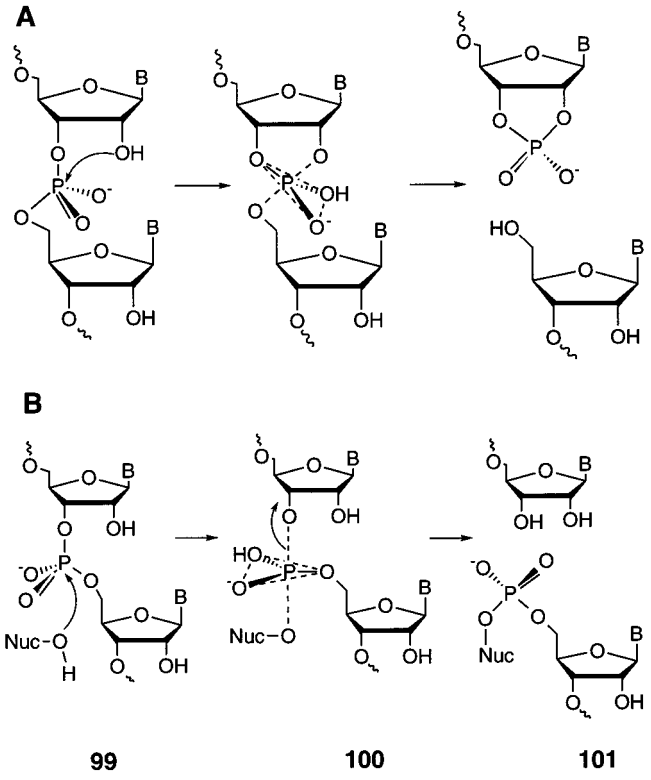
B. Possibility of Electrophilic Catalysis and Thio Effects

A double-metal-ion model has been proposed not only for hammerhead ribozymes[61,78,79,187,190,191] but also for leadzyme,[192] ribonuclease P,[189,298] and the *Tetrahymena* ribozyme,[193,287] as well as for alkaline phosphatase and DNA polymerases.[299−303] In the case of hammerhead ribozymes, it seems likely that the first metal ion interacts directly with the 2′-attacking oxygen, while the second metal ion interacts with the 5′-leaving oxygen.[61,78,79,187,190,191] In addition, the proposal of electrophilic catalysis is supported by the cleavage rate in the presence of Mg^{2+} ions, which is greatly reduced in the case of the phosphorothioate substrate **97** (**R_pS**), wherein the *pro*-R_p oxygen at the scissile phosphate is replaced by sulfur, while the cleavage rate is reduced to a much lesser extent for the **S_pS** isomer **98**, wherein the *pro*-S_p oxygen at the scissile phosphate is replaced by sulfur (Scheme 26). The observation[198,295,296,304] that thio substitution at the *pro*-R_p oxygen resulted in a large "thio effect" (the thio effect = $k_{phosphate}/k_{phosphorothioate}$),[305] which was relieved by replacement of Mg^{2+} ions by Mn^{2+} ions, with their higher affinity for the sulfur atom than that of Mg^{2+} ions[293,294] led to the general conclusion that a Mg^{2+} ion is directly coordinated with the *pro*-R_p oxygen of the scissile phosphate in the transition state of hammerhead ribozyme-catalyzed reactions.[198,199,295,296]

In an attempt to quantitate the rescue ability of Mn^{2+} ions (the rescue effect = $k_{Mn^{2+}}/k_{Mg^{2+}}$), we reexamined the thio effects for two epimeric thio substrates, **R_pS** and **S_pS**. Our results are summarized in Scheme 26. The thio effects of 540 and 28, respectively, for **R_pS** and **S_pS** were obtained experimentally.[297] However, careful examination of the rescue ability of Mn^{2+} ions with these isomers demonstrated that Mn^{2+} ions could rescue the reaction not only with the **R_pS** isomer but also with the **S_pS** isomer, and to a similar extent ($k_{Mn^{2+}}/k_{Mg^{2+}}$ = ∼23). Moreover, the rate of the ribozyme-mediated hydrolysis of the unmodified substrate **96** was about 14-fold higher in the presence of Mn^{2+} ions than in the presence of Mg^{2+} ions, under identical reaction conditions with **R_pS** and **S_pS** in 0.3 mM $MgCl_2$ or 0.3 mM $MnCl_2$ in 50 mM MES buffer (pH 6.0) at 25 °C. Since Mn^{2+}-mediated cleavage occurs about 20-fold more rapidly than Mg^{2+}-mediated cleavage with **R_pS**, **S_pS** and, also, with the natural substrate **96**, it seems unlikely that the previously observed "rescue" effects[198,295,296] support the proposed direct and specific coordination of a metal ion to the *pro*-R_p oxygen in the transition state of the hammerhead ribozyme catalyzed reaction. The previously observed rescue effect might, rather, have originated, at least in part, from the intrinsic properties of the metal ions, which include the specific values of pK_a of the different metal ions as discussed in earlier sections (see, for example, sections VII.D and VII.E).

The observed rescue effect of Mn^{2+} ions [$k_{cleav(Mn)}/k_{cleav(Mg)}$] of about 20 for these substrates is too large to be accounted for by a single-metal model since the theoretical maximum value of 6.3 is the calculated ratio of catalytically active species that is based upon the difference in pK_a of the two aqueous metal ions, Mg^{2+} and Mn^{2+} (see section VII.E). Since anything above this value must be due to involvement of a second metal ion, the ribozyme-mediated cleavage of **R_pS** and **S_pS** appears likely to proceed via the double-metal-ion mechanism of catalysis (Scheme 21C), as does the cleavage of the natural substrate **96**, without the specific interaction of a metal ion with the *pro*-R_p oxygen of the scissile phosphate in the transition state.

Scheme 27



IX. Stereochemistry of the Ribozyme-Mediated Cleavage of RNA

All the ribozymes shown in Figure 1 specifically cleave a specific phosphodiester bond of RNA through one of two mechanisms, which are dependent on either internal (Scheme 27A) or external (Scheme 27B) nucleophilic attack on phosphorus.[199,306−310] The catalytic RNA molecules that activate an internal nucleophile are small ribozymes that include the hammerhead (as discussed above), hepatitis delta virus, and hairpin ribozymes.[199,281,311,312] The well-characterized hammerhead ribozyme catalyzes a hydrolytic reaction that yields two fragments, one containing a 5′-hydroxyl and the other containing a 2′,3′-cyclic phosphate (**3**).[8,210,211] Analysis of the configuration of the reaction products, based on substrates that contained either an S_p or a R_p phosphorothioate linkage at the cleavage site,[295,296,304] indicated the inversion of the configuration at the phosphorus center, which resulted from in-line S_N2 attack with the 2′-hydroxyl group adjacent to the cleavage site as the nucleophile. These features are those of the reactions catalyzed by common ribonucleases, such as RNase A, for the hydrolysis of RNA,[313,314] as well as those of the random and nonrandom cleavage of RNA by metal ions.[315−319]

In the case also of large ribozymes, such as group I and group II ribozymes and the RNA subunit of ribonuclease P, an external nucleophile, such as the 3′-hydroxyl of guanosine (group I), the 2′-hydroxyl of adenosine that is provided intramolecularly (group II; Scheme 28) or water (RNase P), attacks phosphorus also through an in-line S_N2 nucleophilic displacement mechanism, releasing a 3′-hydroxyl leaving group (Scheme 27B, see also Table 1).[163,199,320−322] Because these large ribozymes do not use the adjacent 2′-OH as a nucleophile, they must adopt complicated structures that assist in the attack by the exogenous nucleophile, thus with large size and the novel ability to cleave DNA.[323,324] These features are also involved in reactions catalyzed by DNases and other exonucleases for the cleavage of DNA.[311]

A. Asymmetry of the Two Nonbridging Oxygens in the Ribozyme-Mediated Cleavage of RNA

The phosphate diester in RNA has tetrahedral geometry and it generally exists as a monoanion above pH 2. The negative charge is shared equally between the two nonbridging oxygens. The phosphorus atom is a prochiral center and the two nonbridging oxygens are diastereotopic and nonequivalent. Despite the different mechanisms (internal vs external) adopted by these two types of ribozyme when they catalyze cleavage of RNA, catalysis proceeds in each case via an in-line, S_N2 transesterification with inversion of the configuration at the scissile site. Substitution of sulfur for one of

Scheme 28

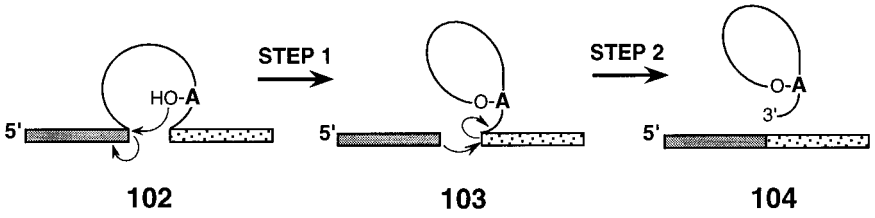


Table 1. Some Characteristics of Ribozyme-Dependent RNA Hydrolysis

ribozyme species	nucleophile	reaction	products
small			
hammerthead	2′-OH (adjacent)	transesterification	5′-OH and 2′,3′ cyclic phosphatase
hairpin	2′-OH (adjacent)	transesterification	5′-OH and 2′,3′ cyclic phosphatase
hepatitis delta virus	2′-OH (adjacent)	transesterification	5′-OH and 2′,3′ cyclic phosphatase
$tRNA^{Phe}$	2′-OH (adjacent)	transesterification	5′-OH and 2′,3′ cyclic phosphatase
large			
Group I	3′-OH of G/H_2O	transesterification/hydrolysis	3′-5′ linkage, 3′-OH/5′-phosphate, 3′-OH
Group II	2′-OH of A/H_2O	transesterification/hydrolysis	2′-5′ linkage, 3′-OH/5′-phosphate, 2′-OH
RNase P	H_2O	hydrolysis	5′-phosphate and 3′-OH

the nonbridging phosphoryl oxygens at the scissile site generates the epimer R_p or S_p phosphorothioate, which differs in the configuration at the phosphorus atom.[198,295,296,322,325] The difference in bond length, bond order, charge location, and stereospecificity between epimer pairs R_p and S_p leads to varying degrees of stereoselectivity in their interactions with enzymes.[118,119,124] Bond lengths and bond angles differ by the following values: S−P = 1.95 Å; O−P = 1.57 Å; C−S = 1.82 Å; C−O = 1.43 Å;[326] ∠P−O−H = 112°; and ∠P−S−H = 99.5°.[327]

In the case of the *Tetrahymena* ribozyme, one of the group I introns in which the external nucleophile group is the 3′-hydroxyl of guanosine, substitution by sulfur for the *pro*-S_p nonbridging phosphoryl oxygen atom reduces its activity more than 1000-fold in the presence of Mg^{2+} ions, but replacement of the *pro*-R_p oxygen with sulfur has only a modest effect on the rate of chemical step.[186] These findings have usually been taken as evidence that supports direct coordination of a metal ion with the S_p oxygen but not with the R_p oxygen in the transition state of the *Tetrahymena* ribozyme-mediated transesterification,[328−331] although the significant thio effect for the S_p-thio epimer cannot be relieved by Mn^{2+} ions.[186]

The self-splicing of the group II intron, another ribozyme with more complicated external nucleophilic attack, involves the folding of intronic RNA into a catalytically active structure, with two subsequent and sequential reactions that result in release of the intron and the ligation of flanking exons (Scheme 28).[332−334] The nucleophilic attack on the phosphorus also occurs via an in-line S_N2-type displacement mechanism, releasing a 3′-OH leaving group (Scheme 27B).[321,322] The first transesterification step in this splicing reaction has been shown to be the rate-limiting step and it is followed by a rapid second ligation step that leads to removal of the intron and ligation of exons.[335] During the rate-limiting transesterification step, the nucleophile can be either the 2′-OH of adenosine[332−334] or a water molecule.[336,337] Padgett found that *cis*-splicing is inhibited by substitution with an S_p phosphorothioate linkage at the 5′-splice site, with a reduction of about 30-fold in the rate as compared to the corresponding wild-type ribozyme.[322,325] The R_p substitution seemed to have a more complicated effect because complete inhibition of splicing was obsvered.[322] However, Pyle found recently that, with respect to the chemical step, this ribozyme could cleave an R_p phosphorothioate linkage only 3-fold more slowly than a phosphate at the cleavage site and she argued that the previously obtained kinetic parameters[322] did not reflect the rate-limiting chemical step.[338] Moreover, she found that a mixture of equal amounts of R_p and S_p isomers could only be cleaved to 50%,[338] an indication that the R_p isomer was completely cleaved but the S_p isomer was not. Such results together clearly demonstrate that, just as in the case of the *Tetrahymena* ribozyme and because of a similar mechanism, the replacement of the S_p oxygen with sulfur at the scissile site of group II introns leads to a significant thio effect at the chemical cleavage step. By contrast, substitution of the R_p oxygen with sulfur has only a marginal effect on the chemical cleavage.

In the case of hammerhead ribozymes, as discussed in section VIII.B, the cleavage rate in the presence of Mg^{2+} ions was greatly reduced with a phosphorothioate substrate in which the R_p oxygen had been replaced by sulfur at the cleavage site, but the cleavage rate was only marginally affected for the S_p isomer. However, careful examination of the rescue effect for R_p and S_p isomers demonstrated that substitution of Mg^{2+} by Mn^{2+} ions not only stimulated cleavage of the R_p-phosphorothioate substrate significantly but also stimulated cleavage of the S_p-phosphorothioate substrate, as well as of the unmodified substrate to the similar extent (Scheme 26).[269,297] These results demonstrated that the rescue effect might have originated from the intrinsic properties of metal ions and, in part, from the different pK_a values of the different metal ions used, without the specific interaction of a metal ion with the *pro*-R_p oxygen of the scissile phosphate in the transition state.

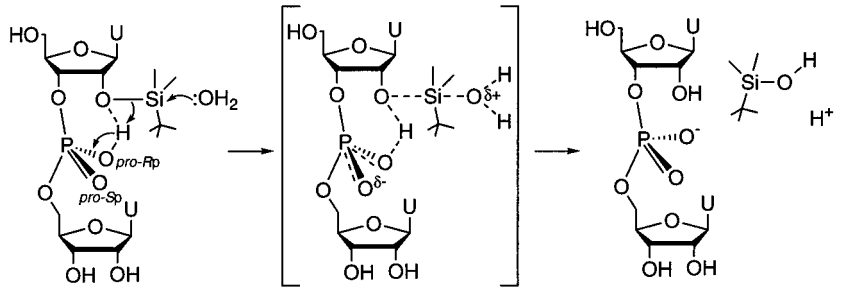
Similarly, unless rescue values can be evaluated quantitatively for group I and group II ribozymes, it is dangerous to conclude that a metal ion is coordinated directly to the *pro*-S_p oxygen in the transition state because such a conclusion can be drawn only when substitution of Mg^{2+} by Mn^{2+} ions significantly and specifically rescues the inhibited activity of the S_p phosphorothioate substrate.[144] Otherwise, since the two prochiral phosphoryl oxygens are not equivalent, minor structural differences could result in different stereospecificities due to changes in interactions with the active site of each enzyme.[338]

B. Asymmetry of the Anchimeric Assistance

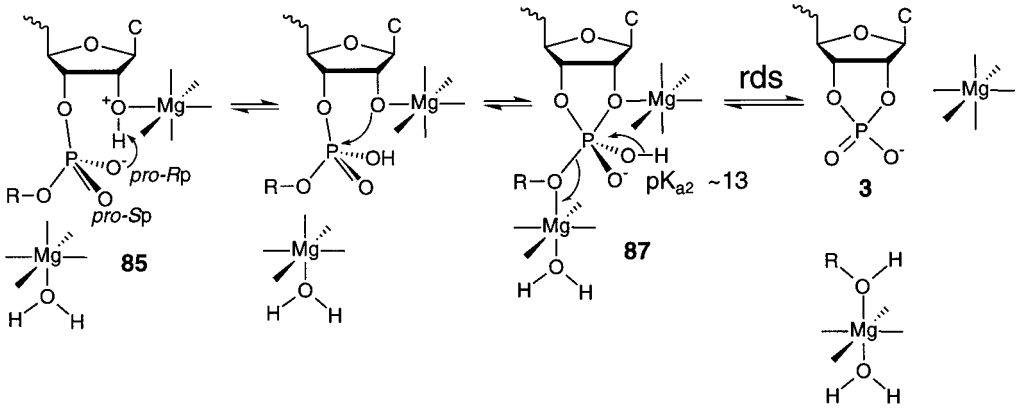
Sekine's group confirmed the asymmetry of the two diastereomeric oxygens during hydrolytic removal of the 2′-*tert*-butyldimethylsilyl (TBDMS) group from a 2′-O-TBDMS-protected UpU dinucleotide, **105** (Scheme 29).[339] In this case, the *pro*-R_p oxygen favors formation of the hydrogen bond with the 2′-oxygen because the distance between the *pro*-R_p oxygen and the 2′-oxygen is short enough for formation of such a hydrogen bond in the energetically favorable conformation. However, such a hydrogen bond between the *pro*-S_p oxygen atom and the 2′-oxygen leads to an energetically unfavorable conformation.[339] Therefore, when the *pro*-R_p oxygen was replaced by sulfur, the participation of the neighboring group in the resultant R_p phosphorothioate became weaker, with slower consequent deprotection of the TBDMS group as compared with the corresponding S_p phosphorothioate isomer.

The favorable hydrogen bond between the 2′-oxygen and the *pro*-R_p oxygen not only stabilizes a transition state such as **106** but it should also assist in the deprotonation of the 2′-OH and in the protonation of the nonbridging phosphoryl *pro*-R_p oxygen, when the internal nucleophile 2′-oxygen attacks the phosphorus (Scheme 27A). Therefore, we cannot exclude the possibility that, in hammerhead ribozyme catalyzed reactions, the proton from the 2′-OH is transferred to the *pro*-R_p oxygen prior to or in concert

Scheme 29



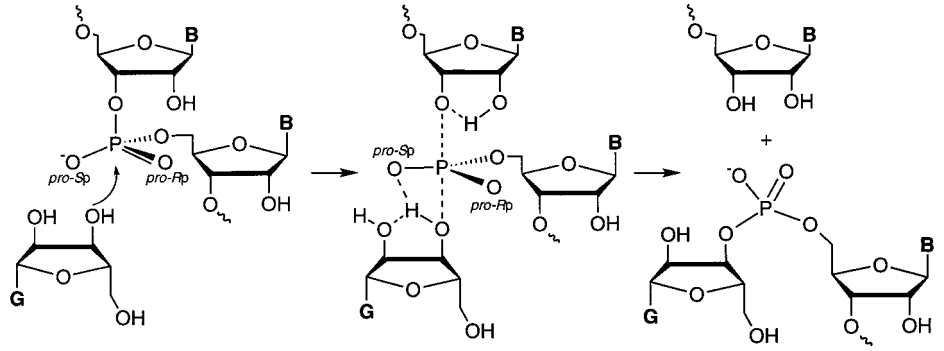
Scheme 30



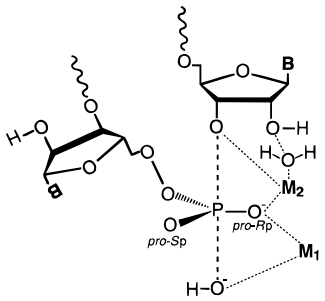
with the attack of the 2′-oxygen on the phosphorus (**85** → **87** in Scheme 30). Such a transfer of a proton to the pentacoordinate species should be energetically feasible because the second pK_a of the phosphorane is estimated to be ~13 (Figure 3).[27,30,35] This kind of transfer of a proton during the formation of a pentacoordinate species (**85** → **87**) and the subsequent deprotonation prior to the rate-limiting cleavage of the P−(5′-O) bond (**rds** step in Scheme 30) is in accord with Breslow's mechanism.[27,277,340−345] Such a mechanism involving the nucleophilic attack of an oxyanion on the neutral phosphate is supported by the recent solution-phase ab initio calculations.[346] The active hammerhead ribozyme complex might then have evolved in such a way that it can further enhance the chemically and energetically favorable transfer of a proton from the 2′-OH to the *pro*-R_p oxygen in concert with the attack of the 2′-oxygen on the phosphorus atom. If this is the case, when the *pro*-R_p oxygen is replaced by sulfur, such transfer of a proton becomes more difficult, with resultant inhibition of hammerhead ribozyme-mediated cleavage. By contrast, when the *pro*-S_p oxygen is replaced by sulfur, the very important hydrogen bond between the *pro*-R_p oxygen and the 2′-OH is maintained, and there is no effect on hammerhead-dependent RNA hydrolysis. This result has been demonstrated experimentally.[297,347] Therefore, without the assumption of the direct coordination of a metal ion with the *pro*-R_p oxygen, the experimental observations can be reconciled with this proton-transfer process. If the dianionic pentacoordinate species (after deprotonation of **87** that leads to the transition state **TS2**) is the active species at steady-state concentrations, all the theoretical calculations and kinetics described in earlier sections for the hammerhead ribozymes can still be reconciled with the experimental data.

As discussed above, substitution of one of the nonbridging oxygens with sulfur at the scissile site perturbs the configuration of the phosphate group and produces an asymmetric reaction center. The chiral R_p or S_p phosporothioate isomers have different stereoconformations when the nucleophilic group attacks the phosphorus atom from different directions. In terms of the geometry, the *pro*-R_p oxygen that is catalytically important for the internal nucleophile 2′-OH of hammerhead ribozymes corresponds to the *pro*-S_p oxygen when the nucleophilic hydroxyl group is supplied externally (when the nucleophilic attack occurs from the opposite direction), either from the 3′-OH of guanosine of the *Tetrahymena* ribozyme or from the 2′-OH of adenosine of group II introns, provided that the proton on the nucleophile is located between the nucleophilic oxygen and either the 2′-oxygen or the 3′-oxygen (Scheme 31). This feature of the reaction might explain why large ribozymes, such as the *Tetrahymena* ribozyme and group II introns, require the *pro*-S_p oxygen for the chemical step of the first transesterification reaction (Scheme 31, and **102** → **103** in Scheme 28), whereas small hammerhead ribozymes require the *pro*-R_p oxygen for the chemical cleavage step. We should note, however, that the RNA of RNase P requires the *pro*-R_p oxygen for the external attack by a water molecule, and the conventional double-metal-ion mechanism (Scheme 32), originally proposed by Steitz and Steitz,[187] appears to operate in this system.[298] It is possible, therefore, that the switch in stereospecificity at nonbridging oxygens between the small and large ribozymes, as discussed

Scheme 31



Scheme 32



above, might just be coincidental, although we should keep in mind that the nucleophile of the RNase P is not a hydroxyl group of a ribose ring.

It is important to note that only one of the phosphoryl oxygens is involved in tertiary contact with the metal ion or with a proton in the transition state, in accord with our finding that a cationic species is unlikely to bifurcate two equatorial oxygens in pentacoordinate species.[61] The particular oxygen that plays the more important role depends on the active site of the ribozyme. In the case of hammerhead ribozymes, the *pro*-R_p oxygen plays a much more important role than the *pro*-S_p oxygen. By contrast, the *pro*-S_p oxygen is more important than the *pro*-R_p oxygen in the *Tetrahymena* ribozyme.

Thio substitution at the scissile phosphate of a substrate had a limited effect on the cleavage−ligation reactions mediated by hairpin ribozymes in the presence of Mg^{2+} ions and, also, in the presence of $Co(NH_3)_6^{3+}$.[348−352] However, in the case of hairpin ribozymes, spermidine can induce catalytic cleavage in the absence of metal ions, albeit at a very low rate, even in the presence of chelating agents such as EDTA and EGTA. Thus, the possibility exists for metal-independent catalysis in the chemical cleavage step of hairpin ribozyme-catalyzed reactions.[312,353] Indeed, three independent groups have provided clear evidence for this hypothesis using cobalt hexaamine, $Co(NH_3)_6^{3+}$, which is an inert transition metal complex [i.e., $Co(NH_3)_6^{3+}$ can play a structural role but cannot play a catalytic role in the chemical cleavage step], in place of divalent metal ions.[348−350] Even in the absence of other metal ions, $Co(NH_3)_6^{3+}$ promotes cleavage reactions mediated by hairpin ribozymes as effectively as Mg^{2+} ions and $Co(NH_3)_6^{3+}$-promoted reactions are not quenched by addition of a chelating agent (EDTA).[348−350] In addition, $Co(NH_3)_5Cl^{2+}$, which has a reduced charge and should have reduced ability to act as an electrostatic catalyst, had the same effect on the rate of hairpin ribozyme-mediated reactions as Mg^{2+} ions and $Co(NH_3)_6^{3+}$.[350] It should also be noted that rates of hairpin ribozyme-mediated cleavage−ligation reactions in Mg^{2+}- or $Co(NH_3)_6^{3+}$-containing buffer are hardly affected by pH between pH 5 and pH 10,[349,354] while the rates of the cleavage reaction catalyzed by hammerhead ribozymes increase linearly with pH (Scheme 22), reflecting the deprotonation of the 2′-hydroxyl group prior to the rate-limiting chemical cleavage step (Scheme 25).[61,185,215] Since unusual independence of pH was observed under conditions of excess hairpin ribozymes, where no product dissociation-step was involved,[349] it seems possible that the rate-limiting step of the hairpin ribozyme-catalyzed reaction might not be the chemical cleavage step and, thus, no thio effects were observed.

X. Conclusion

We have reviewed here the results of recent molecular orbital calculations on phosphate/phosphorane systems, and we have used some of the results to speculate about the mechanisms of action of ribozymes. Calculations are useful for characterizing the structures of transition states that are not easily obtainable in experiments, but mechanistic speculations based on such calculations need to be tested experimentally. Some calculations, such as the reaction pathway shown in Scheme 6, have been confirmed experimentally. With respect to the mechanism of RNA hydrolysis, both calculations and results of experiments with 5′-thio substrates have demonstrated that cleavage of the P−(5′-O) bond is the overall rate-limiting step (Scheme 11). The energy diagram shown in Scheme 11 for normal RNA was used to predict differences in reactivity among variously modified RNA analogues (Scheme 13). The conclusion that the cleavage of the P−(5′-O) bond is the overall rate-limiting step also holds true for hammerhead ribozyme-mediated hydrolysis of RNA (Figure 4). Despite the extensive kinetic and structural analysis of hammerhead ribozymes, there is still significant controversy. In this review, we have presented our preferred model of the "double-metal-ion" mechanism of catalysis, together with our ex-

perimental data that support such a mechanism, as shown in Figure 4 and Scheme 21C. Some mechanisms, such as the one shown in Scheme 30, are purely speculative, and many more experiments are clearly required to test the various hypotheses. Elucidation of the mechanisms of action of ribozymes is clearly important, in the context of the original, putative RNA world and the origins of enzymatic catalysis, in terms of the various catalytic factors, including metal ions, that were available on the primitive earth. As we contemplate the distant past, we may learn enough about ribozymes to utilize them effectively as therapeutic agents in the future.

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