Evidence for the Role of Solvated Metal Hydroxide in the Hammerhead Cleavage Mechanism†

SueAnn C. Dahm,‡ Wesley B. Derrick, and Olke C. Uhlenbeck*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received June 7, 1993; Revised Manuscript Received September 13, 1993®

ABSTRACT: The log of the rate of the chemical step of hammerhead cleavage in Mg^{2+} increases linearly with pH between pH 5.7 and 8.9. A slope of approximately 1 indicates that a single deprotonation is required for cleavage. Hammerhead pH–rate profiles with Ca^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+} correlate well with the pK_a's of these ions in water. This relationship between the pK_a's and the pH–rate profile suggests that a metal hydroxide bound to the hammerhead RNA acts as the base in the cleavage mechanism.

Monomeric RNA genomes of several plant virusoids and viroids contain a small (approximately 35 nucleotides) autolytic RNA motif termed the hammerhead (Hutchins et al., 1986; Prody et al., 1986; Keese & Symons, 1987). Formation of the hammerhead is thought to occur transiently and elicit cleavage of multimeric genomes in a rolling circle replication cycle (Branch et al., 1981). The transesterification mechanism of hammerhead cleavage includes deprotonation of a 2′-hydroxyl of a unique ribose, nucleophilic attack on the adjacent phosphodiester bond, and protonation of the 5′-oxyanion leaving group, generating 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Prody et al., 1986; Uhlenbeck, 1987). The cleavage mechanism proceeds with inversion of configuration, which is consistent with an in-line attack by the 2′-oxygen group via a trigonal bipyramidal transition state (van Tol et al., 1990; Slim & Gait, 1991). As expected, the 2′-hydroxyl adjacent to the cleavage site is essential for the mechanism (Dahm & Uhlenbeck, 1990; Perreault et al., 1990).

RNA cleavage by the protein ribonucleases A and T_1 occurs by a similar mechanism. The folded polypeptide chain permits substrate binding in such a way that a basic imidazole is positioned to deprotonate the 2′-hydroxyl at the cleavage site, and an acidic imidazolium ion is positioned so that it can protonate the 5′-oxyanion leaving group (Richards & Wycoff, 1971; Nishikawa et al., 1987). The extensive sequence requirements necessary for efficient hammerhead cleavage (Koizumi et al., 1988, 1989; Sheldon & Symons, 1989; Ruffner et al., 1990) suggest that a well-defined folded RNA structure similarly is required to correctly position catalytically essential functional groups. However, the identity of these putative acidic and basic groups is unknown.

A number of experiments have indicated that divalent ions are required for hammerhead cleavage (Prody et al., 1986; Uhlenbeck, 1987). In low ionic strength buffers, hammerhead cleavage rates increase rapidly within a narrow range of divalent ion concentrations (Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991; Perreault et al., 1991). Since the folding of RNA is often promoted by divalent ions in a cooperative fashion (Stein & Crothers, 1976), it was possible that divalent ions were simply required for hammerhead folding. However, when alternate counterions such as spermine or sodium are present to stabilize the folded structure, divalent metal ions are still required for the reaction, suggesting that they may participate directly in the reaction mechanism (Dahm & Uhlenbeck, 1991).

A relevant, but nonphysiological, example of a reaction similar to hammerhead cleavage is the specific cleavage of yeast $tRNA^{Phe}$ between residues D17 and G18 by Pb^{2+}. This reaction, like hammerhead cleavage, also produces 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Dirheimer et al., 1972). In this reaction, crystallographic (Brown et al., 1983; Rubin & Sundaralingam, 1983; Brown et al., 1985) and biochemical (Behlen et al., 1990) data indicate that a lead-bound hydroxyl ion is responsible for the deprotonation of the 2′-hydroxyl at the cleavage site. Several researchers (Brown et al., 1985; Haydock & Allen, 1985; Dahm & Uhlenbeck, 1991; Koizumi & Ohtsuka, 1991) have speculated that a similar mechanism could function in the hammerhead.

We have previously shown that a substantial number of different divalent ions are active in the hammerhead cleavage reaction (Dahm & Uhlenbeck, 1991). Since divalent metal ions differ greatly in their abilities to form metal-bound hydroxides as a function of pH, it was instructive to examine the pH–rate profiles of hammerhead cleavage in the presence of different metal ions.

MATERIALS AND METHODS

RNA Synthesis. The substrate RNA was synthesized chemically using ribonucleoside phosphoramidites with *tert*-butyldimethylsilyl-blocked 2′-hydroxyl groups (Usman et al., 1987) and phenoxyacetyl-blocked amino groups of adenine and guanine bases (Schulhof et al., 1987), which were supplied by American Bionetics. The synthetic oligonucleotide was deprotected with ethanolic ammonia (Usman et al., 1987) at room temperature for 24 h, followed by treatment with tetra-*n*-butylammonium fluoride at room temperature for 24 h. The synthetic RNA was desalted by DEAE–Fractogel chromatography (Supelco) and purified by denaturing gel electrophoresis followed by ethanol precipitation. The ribozyme molecule was synthesized by in vitro transcription with T7 RNA polymerase using a synthetic DNA template (Milligan et al., 1987) and purified by 20% denaturing polyacrylamide gel electrophoresis. Transcription products were located by UV shadowing. The product band was crushed and soaked overnight in two volumes of a buffer containing 0.5 M NaOAc, 0.1 M Tris (pH 8.0), and 1 mM EDTA. The extracted RNA was concentrated by ethanol precipitation

† This research was funded by NIH Grant GM 36944.

* Corresponding author.

‡ Current address: Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, CO 80301.

® Abstract published in *Advance ACS Abstracts,* November 1, 1993.

0006-2960/93/0432-13040$04.00/0

and resuspended in water. To verify that the ribozyme transcript was the correct length, the transcript was 3′-end-labeled with [5′-^{32}P]pCp ([5′-^{32}P]cytidine 3′,5′-bisphosphate) and RNA ligase and subjected to total digestion with ribonuclease T_2 (England & Uhlenbeck, 1978). The digestion product nucleosides were separated and identified by two-dimensional (2D) thin-layer chromatography (Kuchino et al., 1980).

Cleavage Reaction. Cleavage reactions contained 50 mM buffer, 0.5 mM spermine, 1 μM substrate, a trace of 5′-^{32}P-labeled substrate, 5–50 nM ribozyme, and varying concentrations of divalent ions. The sulfonate buffers used to maintain the desired pH were Mes (pH 5.7–6.5), Pipes (pH 6.2–7.0), Mops (pH 7.2–7.9), Taps (pH 8.2–8.9), and Ches (pH 9.2–9.8). The reaction protocol involved heating ribozyme and substrate separately in sulfonic acid buffer to 90 °C for 1 min and slowly cooling to 25 °C. Immediately before a reaction was initiated, spermine followed by metal ions was added to both the substrate and ribozyme solutions. Reactions (40 μL) were initiated by the addition of 20 μL of ribozyme to 20 μL of substrate. At least eight 3–4-μL aliquots were taken at appropriate intervals, and reactions were terminated by the addition of an equal volume of 50 mM EDTA, 7 M urea, 0.02% bromophenol blue, and 0.02% xylene cyanol. The radiolabeled substrate and product were separated by denaturing 20% polyacrylamide gel electrophoresis. The gels were scanned and quantitated using an Applied Biosystems Phosphorimager with the corresponding software. Cleavage rates were determined over the first 10–15% of each reaction. Each rate was determined at least twice and varied by less than 20%.

The three transition metal ions used (Mn^{2+}, Co^{2+}, and Cd^{2+}) form insoluble hydroxides more readily than Mg^{2+} and Ca^{2+} at pH's above 7.0, potentially reducing the free metal ion concentration as well as precipitating RNA. To minimize this problem, the weak chelator, Bis-Tris [2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol], was added at a concentration equivalent to the metal ion concentration to aid in solubilizing the transition metal ions. The reaction protocol was followed as previously described, except that Bis-Tris was added along with the other buffer used in each reaction. Additionally, Mn^{2+} and Co^{2+} are easily oxidized with increasing pH, so that it was necessary to perform cleavage assays that contained these metal ions in a glove bag inflated with argon gas. The previously described reaction conditions and protocol were followed except that after the heating and cooling steps, tubes were introduced into the glove bag and individually flushed with argon to remove dissolved oxygen. After the addition of spermine and metal ions, substrate was immediately added to ribozyme to minimize the time that the metal ions were incubated at the elevated pH.

RESULTS

Previous experiments suggested that the rate of hammerhead cleavage increased only slightly with increasing pH (Uhlenbeck, 1987; S. Dahm, unpublished observations). However, ribozyme and substrate oligonucleotides of the hammerheads used in those studies possessed alternate conformations (Ruffner et al., 1989; Fedor & Uhlenbeck, 1990), and because the reactions were carried out under subsaturating conditions, the cleavage rates reflected substrate binding and not cleavage chemistry. The hammerhead used in this work (Figure 1) was chosen because a detailed kinetic analysis of a very similar hammerhead (Fedor & Uhlenbeck, 1990, 1992) indicated a low K_m and no observable alternate conformations in either the substrate or ribozyme molecule. The k_{cat} and K_m values

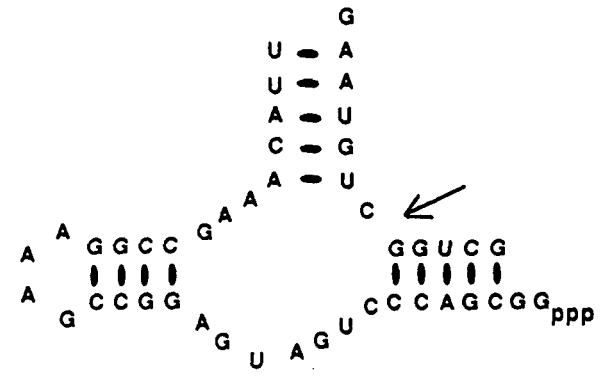

FIGURE 1: Hammerhead formed by 34 nucleotide enzymes and 12 nucleotide substrates. Cleavage occurs at the internucleotide linkage indicated by the arrow.

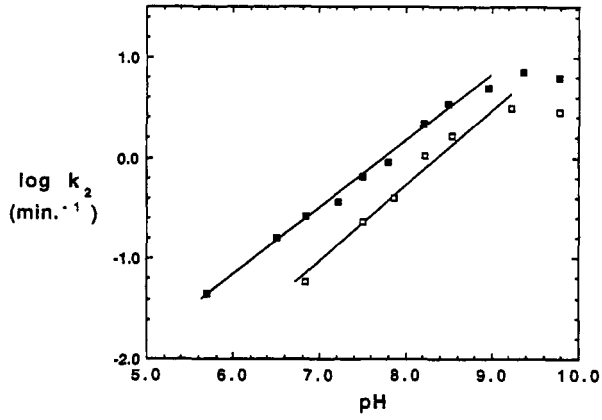

FIGURE 2: Hammerhead cleavage rates as a function of pH in the presence of 2 (□) and 10 mM (■) Mg^{2+}. Both lines have a slope of 0.7.

were found to be 1 min^{-1} and 50 nM, respectively, in 10 mM $MgCl_2$ and 50 mM Tris (pH 7.5). Additionally, the release of products from this ribozyme was faster than cleavage chemistry, so that k_{cat} reflects the rate of cleavage chemistry and can be determined in a multiple-turnover reaction at saturating substrate concentrations. Thus, this hammerhead appeared well-suited for the determination of the rate constant of the chemical step as a function of pH.

Since the substrate is shorter by one unpaired nucleotide, and the reaction conditions used in the current study are slightly different from those used previously, the steady-state kinetic parameters, K_m and k_{cat}, were redetermined. In 50 mM Mops (pH 7.5), 10 mM $MgCl_2$, and 0.5 mM spermine, a K_m of 30 nM and a k_{cat} of 0.7 min^{-1} were obtained, which are very similar to the values determined previously. Clearly, the different type of buffer and the presence of spermine did not greatly alter the kinetic properties of the hammerhead. Spermine was included in the reactions to allow assembly of the hammerhead, independent of the type and concentration of metal ion used (Dahm & Uhlenbeck, 1991).

The cleavage rate was measured as a function of pH at 1 μM substrate and 5–50 nM ribozyme (Figure 2). The log of the cleavage rate increases linearly with pH from pH 5.7 to 8.9 in 10 mM $MgCl_2$. Experiments at higher substrate concentrations confirm that the substrate was saturating throughout this pH range, so that the cleavage rate reflects the chemical step. Experiments also confirmed that the change in rate is due to the pH and not to either the concentration or type of buffer used. For example, in the presence of 10 mM $MgCl_2$ and 0.5 mM spermine, the cleavage rate was unchanged between 20 and 150 mM Mops (pH 7.5). Cleavage

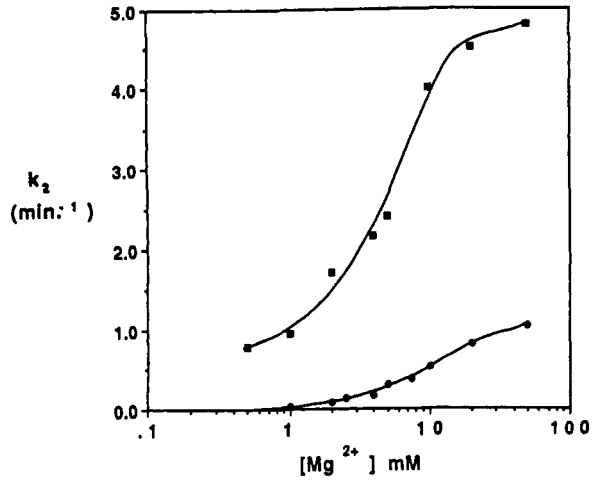

FIGURE 3: Hammerhead cleavage rates as a function of Mg^{2+} concentration at pH 7.2 (●) and 8.9 (■).

rates at a given pH were also the same when different types of buffers were used.

Above pH 9.0, the cleavage rates at both Mg^{2+} concentrations were slower than those predicted from extrapolation of rates at lower pH values (Figure 2). Above pH 9.8, the cleavage rate at 10 mM Mg^{2+} decreases with pH, presumably reflecting the progressive inactivation of the hammerhead resulting from the deprotonation of certain G or U residues. It was important to determine whether the "leveling off" of the cleavage rate between pH 9.0 and 9.8 was the result of the completion of the titration of a basic group with a pK_a of about 8.5 or whether it reflected a change in the rate-limiting step in the reaction. It was possible, for example, that as the rate of the chemical step increased with pH, product release could become rate-limiting for cleavage. However, no "burst" of product formation corresponding to a fast first turnover and subsequent slower product release was observed under a variety of high substrate and enzyme concentrations at pH 9.8. While it was difficult to obtain a reliable K_m value at pH 9.8, the cleavage rate at 5 μM substrate was 14.4 min^{-1}, which is considerably higher than the 4.3-min^{-1} value obtained at 1 μM. This suggests that saturation was not achieved at the higher pH values and that substrate binding rather than chemistry is rate-limiting. Since the 14.4-min^{-1} value is close to the 17 min^{-1} that is predicted by extrapolating the data from lower pH values, it probably reflects the rate of the chemical step. It, therefore, appears that the "leveling off" in Figure 2 simply reflects weaker binding of substrate at higher pH values.

The pH–rate profile of the same hammerhead in 2 mM $MgCl_2$ closely resembles the 10 mM data, except that the rate was 3–5-fold slower at all pH values tested (Figure 2). The substrate concentration was confirmed to be saturating between pH 7 and 8.5. This indicates that Mg^{2+} was not saturating at 2 mM, but the proportion of $Mg(OH)^+$ to total Mg^{2+} concentration remains the same, as expected. The cleavage rate as a function of Mg^{2+} concentration at pH 7.2 and 8.9 is shown in Figure 3. While Mg^{2+} does not reach saturation until above 50 mM, the two curves are approximately proportional. This suggests that while both pH and Mg^{2+} concentration affect the chemical step, they do so independently.

The hammerhead cleavage rate as a function of pH in 10 mM Ca^{2+} was determined. We have shown previously that while the hammerhead is active in Ca^{2+} and requires similar concentrations for optimal cleavage, the overall rate of cleavage

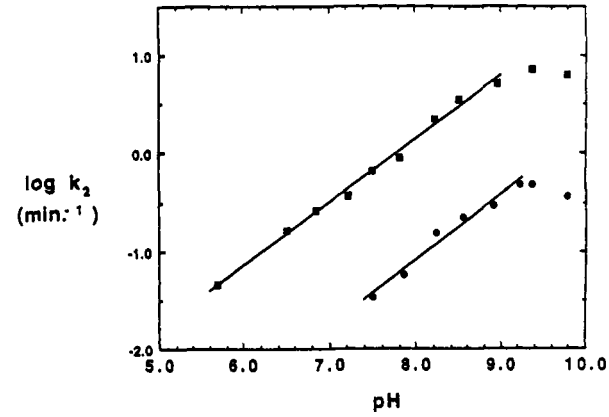

FIGURE 4: Hammerhead cleavage rates as a function of pH in 10 mM Mg^{2+} (■) and 10 mM Ca^{2+} (●). The lines have slopes of 0.65 and 0.63, respectively.

is slower (Dahm & Uhlenbeck, 1991). As shown in Figure 4, the cleavage in 10 mM Ca^{2+} is also linear with log pH, but is approximately 16-fold slower than with 10 mM Mg^{2+} at each pH.

In order to determine the cleavage rates in Mn^{2+}, Co^{2+}, and Cd^{2+} as a function of pH, the experimental protocol had to be changed. When the protocol used for Mg^{2+} and Ca^{2+} was attempted with these three ions, cleavage rates were irreproducible, and virtually no cleavage was observed at pH values above 8.0. This was the result of two properties of these "soft" divalent ions. First, unlike Mg^{2+} and Ca^{2+}, both Mn^{2+} and Co^{2+} react rapidly with dissolved oxygen at higher pH values to form metal oxides. Secondly, all divalent ions can form insoluble metal hydroxides at higher pH values. At 10 mM ion concentration, $Ca(OH)_2$ and $Mg(OH)_2$ begin to form above pH 9.6, $Cd(OH)_2$ and $Mn(OH)_2$ form above pH 8, and $Co(OH)_2$ forms above pH 7.2 (Kragen, 1978; Westermann et al., 1986). In order to avoid these problems, three changes in the protocol were introduced. First, reactions with Mn^{2+} and Co^{2+} were carried out in an argon atmosphere. Second, all cleavage reactions with Mn^{2+}, Co^{2+}, or Cd^{2+} contained an equal concentration of Bis-Tris which, in its unprotonated form (above pH 7.0), is a weak chelator of metal ions. Bis-Tris was found to greatly improve the reproducibility of cleavage data for Mn^{2+}, Co^{2+}, or Cd^{2+} at higher pH values, presumably by reducing the formation of metal hydroxides. In control experiments, Bis-Tris had no effect on the rate of cleavage with Mg^{2+} or Ca^{2+}. Finally, since metal hydroxide formation is both concentration- and time-dependent, 1 mM Mn^{2+} or 2 mM Co^{2+} or Cd^{2+} was used for the experiments, and the amount of time the metal ions were present at the high pH was minimized.

Using the precautions outlined above, the cleavage rates were determined as a function of pH using 1 mM Mn^{2+} and 2 mM Co^{2+} or Cd^{2+}. Although these concentrations are likely to be subsaturating, higher concentrations were not possible. As shown in Figure 5, the log of the cleavage rate increases linearly with pH in a limited range for all three ions. Cleavage rates as a function of pH were fit to a binomial expression reflecting both a pH-dependent increase in cleavage and a pH-dependent inactivation at higher pH values. The absolute cleavage rates in the linear range were about 10-fold faster than those for Mg^{2+}. These rates are faster than those previously reported under the same conditions (Dahm & Uhlenbeck, 1991) because the precautions against metal oxides and hydroxides had not previously been taken.

The deviations from linearity in the pH–rate profiles in Figure 5 are probably related to remaining problems with

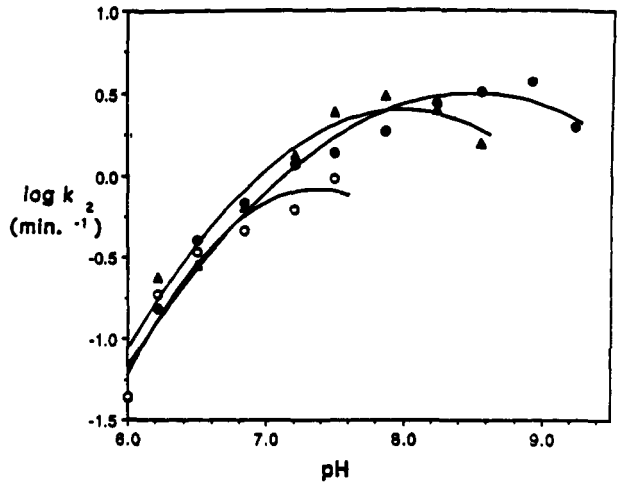

FIGURE 5: Hammerhead cleavage rates as a function of pH in 2 mM Co (▲), Cd (○), and 1 mM Mn (●). Slopes are approximately 1.0 below pH 7.0.

metal hydroxide formation. Presumably, at higher pH values the precautions taken to avoid metal hydroxide are inadequate. Indeed, for all three metals, attempts to obtain data at pH values higher than those reported in Figure 5 failed to show any cleavage at all, due to the precipitation of the metal hydroxide.

DISCUSSION

The rate of hammerhead cleavage was determined as a function of pH under conditions where the chemical step is rate-limiting. In Mg^{2+} and Ca^{2+}, the log of the cleavage rate was found to increase linearly with pH over a broad pH range. A linear plot with a slope of 1 generally suggests that a single deprotonation occurs (Fersht, 1985). Although the observed slope of 0.7 is somewhat less than that expected, the data clearly indicate that a deprotonation occurs. The discrepancy may be due to experimental error or there may be small differences in the ability of the hammerhead to bind Mg^{2+} as a function of pH. Discrepancies between observed and theoretical slopes are also encountered in the determination of pH–rate profiles for other metalloenzymes (Vallee & Galdes, 1984; Derbyshire et al., 1991).

At pH values above 9.0 and at 1 μM substrate, the observed reaction rates deviate from linearity, and the cleavage rate ultimately drops to very low values. At pH 9.8, the reaction rate with 10 mM Mg^{2+} was much faster than that at 5 μM substrate, suggesting that the K_m value had risen sufficiently such that the rate of substrate binding may have become rate-limiting. Since K_m is equal to K_d for this hammerhead (Fedor & Uhlenbeck, 1992), the simplest interpretation of the data is that, at higher pH values, one or more of the guanosine (pK_a = 9.2) or uridine (pK_a = 9.2) residues involved in helix formation between substrate and ribozyme become deprotonated. Since the rate at pH 9.8 and 5 μM is close to that extrapolated from lower pH values, k_{cat} is unimpaired at pH 9.8, suggesting that essential G and U residues in the hammerhead core are not yet titrated or can function as charged species.

The hammerhead cleavage rate in 10 mM Ca^{2+} is approximately 15-fold slower than that in 10 mM Mg^{2+} at every pH tested. Since we have previously shown that both of these divalent ions bind the hammerhead with similar affinities (Dahm & Uhlenbeck, 1991), some other property of the two ions must be responsible for the difference in cleavage rate. While Ca^{2+} has a slightly larger hydrated ionic radius than Mg^{2+} (Weast, 1987) and can adopt different coordination geometries (Snyder et al., 1990), it resembles Mg^{2+} in its preference for coordinating "hard" atoms. Ca^{2+} has been shown to promote the tertiary folding of large RNAs in a manner quite similar to Mg^{2+} (Celander & Cech, 1991). One important difference between Ca^{2+} and Mg^{2+} is that the pK_a of a water molecule coordinated to Ca^{2+} is 12.8 and to Mg^{2+} is 11.4 (Kragten, 1978; Westermann et al., 1986). Thus, at a given pH, the concentration of $[Ca(OH)]^+$ is 25-fold lower than that of $[Mg(OH)]^+$ in aqueous solution. Since this difference correlates closely with the approximately 16-fold difference in hammerhead cleavage rates at each pH, one can propose that Mg^{2+} and Ca^{2+} interact with the hammerhead in a very similar way and, like the cleavage of $tRNA^{Phe}$ by Pb^{2+} (Brown et al., 1985), a metal hydroxide ion is the active species. Presumably this $[M(OH)]^+$ is located near the ribose at the cleavage site and, therefore, can act as a general base and either directly or indirectly deprotonate the 2'-hydroxyl to accelerate chain cleavage.

If this model is correct, only a small fraction of the hammerheads is likely to contain a metal-bound hydroxide ion at low pH, and the fraction increases with increasing pH. If we assume that the pK_a values of Mg^{2+} and Ca^{2+} in water are also correct in the hammerhead, a hammerhead cleavage rate of 0.5 min^{-1} at pH 7 is the result of 0.000 04 of the molecules having a $[Mg(OH)]^+$ in its active site. This would correspond to a rate of 13 000 min^{-1} if all of the molecules contained a $[Mg(OH)]^+$. Similarly, the rate with $[Ca(OH)]^+$ in the active site can be calculated to be 10 000 min^{-1}. While these rates are in reasonable agreement with one another, their order is opposite what would be expected since Ca^{2+} has a higher pK_a and therefore should be a stronger base when fully titrated. It is possible that the pK_a of the metal hydroxide ion in the hammerhead structure may be quite different since it is coordinated to other ligands. Indeed, the pK_a of a Zn^{2+}-coordinated water molecule in the active site of alkaline phosphatase is reduced by nearly 2 units due to its coordination with histidine residues (Coleman & Chlebowski, 1979; Jones et al., 1983). Alternatively, it is possible that the hammerhead folded in Ca^{2+} does not position the metal hydroxide in precisely the same location as the hammerhead folded in Mg^{2+}.

In order to obtain support for the notion that a bound metal hydroxide is needed for hammerhead cleavage, rates of cleavage were measured with three other metal ions which had lower pK_a values. While the experiments were complicated by the fact that subsaturating metal concentrations had to be used to avoid the formation of metal oxides and metal hydroxides, pH–rate profiles were roughly linear in a limited pH range, and as expected, the hammerhead cleavage rates with these ions were faster than those with Mg^{2+}. For example, at pH 6.5, the cleavage rate with Co^{2+} is approximately 10-fold faster than that with Mg^{2+}, which correlates well with the 16-fold higher $[Co(OH)]^+$ concentration expected from the pK_a of 10.2 for Co^{2+} in water. The Mn^{2+} hammerhead had a 2–5-fold faster rate than the Mg^{2+} hammerhead, which agrees reasonably well with the 6-fold higher $[Mn(OH)]^+$ concentration predicted by a pK_a of 10.6 for Mn^{2+} in water. However, although the Cd^{2+} hammerhead cleaved 6–10-fold faster than Mg^{2+}, it did not show cleavage rates as fast as would be expected by a pK_a of 9.0 for Cd^{2+} in water.

In general, the faster hammerhead cleavage rates observed for the three divalent ions with lower pK_a values support our proposal that a metal-bound hydroxide ion acts as a base in the hammerhead cleavage reaction. Our previous survey (Dahm & Uhlenbeck, 1991) of hammerhead cleavage at pH

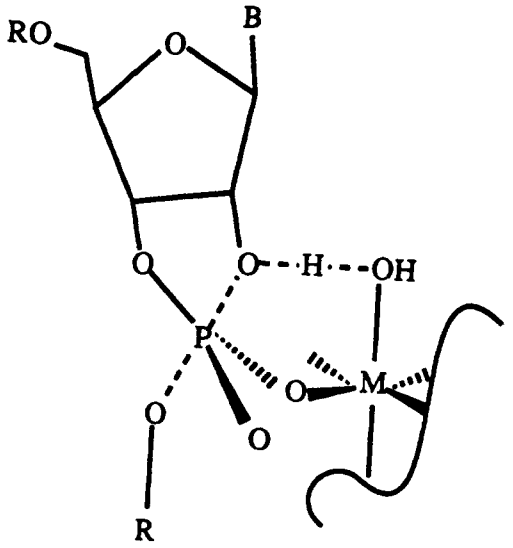

FIGURE 6: A possible transition state for a minimal cleavage mechanism showing a single divalent ion directly coordinated to the *pro-R*P oxygen and providing a hydroxide ion near the attacking 2′-oxygen.

7.5 in 0.5 mM spermine and different divalent ions is also consistent with this conclusion. While the formation of metal oxides and hydroxides was not minimized in those experiments, it is clear that, among the divalent ions not tested here, Zn^{2+} (pK_a = 9.0) cleaved quite well whereas Sr^{2+} (pK_a = 13.3) and Ba^{2+} (pK_a = 13.5) did not (Kragten, 1978; Westermann et al., 1986). However, since Pb^{2+} (pK_a = 7.7) was not active in hammerhead cleavage, it is clear that other factors are involved as well. As discussed above, there are many possible reasons for the discrepancies between the pK_a of a metal ion in water and the hammerhead cleavage rate. A particularly likely possibility for Co^{2+}, Mn^{2+}, Cd^{2+}, and other "soft" transition metals is that they have very different preferred coordination ligands than Mg^{2+} and Ca^{2+} when binding to RNA (Jack et al., 1977; Teeter et al., 1981). This could result in quite different positioning of the metal with respect to the cleavage site and, thus, could modify reaction rates.

Any model for the role of divalent metal ions in the hammerhead cleavage mechanism must incorporate several experimental facts. First, on the bases of the metal ion specificity of a phosphorothioate-substituted hammerhead, we propose that the *pro-R*P oxygen of the phosphate at the cleavage site must be directly coordinated to a metal ion for hammerhead cleavage to occur (Dahm & Uhlenbeck, 1991). The absence of any large change in cleavage rate upon substitution of the corresponding *pro-S*P oxygen by sulfur makes it unlikely that a metal ion (or anything else) directly coordinates that oxygen (Koizumi & Ohtsuka, 1991; Slim & Gait, 1991). Second, we show here that, for a number of metal ions active in cleavage, the pH–rate profile correlates with the pK_a of the divalent ion in water, therefore suggesting that a metal hydroxide ion bound close to the cleavage site acts as the base in the reaction.

It is possible that these two independent functions for metal ions in the hammerhead reaction may be fulfilled by a single metal ion. In such a simple model, the catalytic metal would be positioned to coordinate directly with the *pro-R*P oxygen of the cleavage phosphate, perhaps in such a way as to stabilize the trigonal bipyramidal transition state. A hydroxide ion coordinated to the same metal can easily be oriented to abstract the proton from the 2′-hydroxyl (Figure 6). Alternatively, an additional water molecule can be inserted between the hydroxide ion and the 2′-hydroxyl, as has been proposed for the Pb^{2+} cleavage of $tRNA^{Phe}$ (Sundaralingam et al., 1984).

In analogy to the precise location of metal ions in tRNA, the catalytic metal ion must be positioned correctly by coordination to other functional groups in the hammerhead tertiary structure. These coordination sites currently are unknown, but many atomic or functional group substitutions in the hammerhead core have been shown to reduce cleavage rates. These include the deletion of nucleotide functional groups (i.e., the amino group of guanosine) (Odai et al., 1990), deletion or substitution of a 2′-hydroxyl group (Yang et al., 1990; Perreault et al., 1991; Paolella et al., 1992; Heidenreich et al., 1993), and substitution of phosphates by thiophosphates (Ruffner & Uhlenbeck, 1990). It is difficult to determine whether these changes affect metal ion binding or disrupt RNA tertiary structure. While the effect of the substitution can sometimes be "rescued" by raising the divalent ion concentrations (Perreault et al., 1991), one cannot necessarily conclude that this demonstrates the disruption of a metal ion coordination site. The higher divalent ion concentration could stabilize the hammerhead by reducing ionic repulsion and thereby offset the destabilizing effect of structural perturbation on the stability or tertiary structure. Thus, the identification of metal ion coordination sites in the hammerhead still remains an important goal.

While the one metal ion model can explain all of the available experimental data, we cannot eliminate the possibility that other metal ions participate in the reaction. The interaction of a divalent ion with the leaving group in phosphoryl-transfer reactions has been suggested previously (Herschlag & Jencks, 1987; Chipman & Jencks, 1988). Hammerhead cleavage mechanisms have been proposed involving either direct (Taira et al., 1990) or indirect (Haydock & Allen, 1985) coordination of a metal ion to the 5′-hydroxyl leaving group in order to stabilize the oxyanion leaving group. Two similar metal ion mechanisms are believed to be employed by alkaline phosphatase (Kim & Wyckoff, 1991), phospholipase C (Hough et al., 1989), and the 3′ to 5′ exonuclease activity of *Escherichia coli* DNA polymerase I (Beese & Steitz, 1991). Determination of the number of metal ions participating in the catalytic step of hammerhead cleavage and their location in the active site awaits additional experiments.

ACKNOWLEDGMENT

We thank Tao Pan and Pascale Legault for comments on this manuscript.

REFERENCES

Beese, L. S., & Steitz, T. A. (1991) *EMBO J. 10,* 25–33.

Behlen, L. S., Sampson, J. R., DiRenzo, A. B., & Uhlenbeck, O. C. (1990) *Biochemistry 29,* 2515–2523.

Branch, A. D., Robertson, H. D., & Dickson, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78,* 6381–6385.

Brown, R. S., Hingerty, B. E., Dewan, J. C., & Klug, A. (1983) *Nature 303,* 543–546.

Brown, R. S., Dewan, J. C., & Klug, A. (1985) *Biochemistry 24,* 4785–4801.

Celander, D. W., & Cech, T. R. (1991) *Science 251,* 401–407.

Chipman, D. M., & Jencks, W. P. (1988) *Biochemistry 27,* 5707–5712.

Coleman, J. E., & Chlebowski, J. F. (1979) in *Advances in Inorganic Biochemistry* (Eichhorn, G. L., & Marzilli, L. G., Eds.) Vol. 1, pp 1–66, Elsevier, Amersterdam.

Dahm, S. C., & Uhlenbeck, O. C. (1990) *Biochimie 72,* 819–823.

Dahm, S. C., & Uhlenbeck, O. C. (1991) *Biochemistry 30,* 9464–9469.
Derbyshire, V., Grindley, N. D., & Joyce, C. M. (1991) *EMBO J. 10,* 17–24.
Dirheimer, G., Ebel, J. P., Bonnet, J., Gangloff, J., Keith, G., Krebs, B., Kuntzel, B., Roy, A., Weissenbach, J., & Werner, C. (1972) *Biochimie 54,* 127–144.
England, T., & Uhlenbeck, O. C. (1978) *Nature 275,* 560–561.
Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 1668–1672.
Fedor, M. J., & Uhlenbeck, O. C. (1992) *Biochemistry 31,* 12042–12054.
Fersht, A. (1985) *Enzyme Structure and Mechanism,* 2nd ed., pp 155–175, W. H. Freeman & Company, New York.
Haydock, K., & Allen, L. C. (1985) *Prog. Clin. Biol. Res. 172A,* 87–98.
Heidenreich, O., Pieken, W., & Eckstein, F. (1993) *FASEB J. 7,* 90–96.
Herschlag, D., & Jencks, W. P. (1987) *J. Am. Chem. Soc. 109,* 4665–4674.
Hough, E., Hansen, L. K., Birknes, B., Jynge, K., Hansen, S., Hordvik, A., Little, C., Dodson, E., & Derewenda, Z. (1989) *Nature 338,* 357–360.
Hutchins, C. J., Rathjen, P. D., Forster, A. C., & Symons, R. H. (1986) *Nucleic Acids Res. 14,* 3627–3640.
Jack, A., Ladner, J. E., Rhodes, D., Brown, R. S., & Klug, A. (1977) *J. Mol. Biol. 111,* 315–318.
Jones, D. R., Lindoy, L. F., & Sargeson, A. M. (1983) *J. Am. Chem. Soc. 105,* 7327–7336.
Keese, P., & Symons, R. H. (1987) in *Viroids and Viroid-like Pathogens* (Semancik, J. S., Ed.) pp 1–47, CRC Press, Inc., Boca Raton, FL.
Kim, E. E., & Wyckoff, H. W. (1991) *J. Mol. Biol. 218,* 449–464.
Koizumi, M., & Ohtsuka, E. (1991) *Biochemistry 30,* 5145–5150.
Koizumi, M., Iwai, S., & Ohtsuka, E. (1988) *FEBS Lett. 239,* 285–288.
Koizumi, M., Hayase, Y., Iwal, S., Kamiya, H., Inoue, H., & Ohtsuha, E. (1989) *Nucleic Acids Res. 17,* 7059–7071.
Kragten, J. (1978) *Atlas of metal-ligand equilibria in aqueous solution,* Halsted Press, Chichester, England.
Kuchino, Y., Watanabe, S., Harada, F., & Nishimura, S. (1980) *Biochemistry 19,* 2085–2089.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15,* 8783–8798.
Nishikawa, S., Morioka, H., Kim, H. J., Fuchimura, K., Tanaka, J., Uesugi, S., Hakoshima, T., Tomita, K., Ohtsuka, E., & Ikehara, M. (1987) *Biochemistry 26,* 8620–8624.
Odai, O., Hiroaki, H., Sakata, T., Tanaka, T., & Uesugi, S. (1990) *FEBS Lett. 267,* 150–152.
Paolella, G., Sproat, B. S., & Lamond, A. I. (1992) *EMBO J. 11,* 1913–1919.
Perreault, J. P., Wu, J., Coussineau, B., Ogilvi, K. K., & Cedegren, R. (1990) *Nature 344,* 565–567.
Perreault, J.-P., Labuda, D., Usman, N., Yang, J.-H., & Cedergren, R. (1991) *Biochemistry 30,* 4020–4025.
Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., & Bruening, G. (1986) *Science 231,* 1577–1580.
Richards, F. M., & Wycoff, H. W. (1971) *The Enzymes,* pp 647–806, Academic Press, New York.
Rubin, J. R., & Sundaralingam, M. (1983) *J. Biomol. Struct. Dyn. 1,* 639–646.
Ruffner, D. E., & Uhlenbeck, O. C. (1990) *Nucleic Acids Res. 18,* 6025–6029.
Ruffner, D. E., Dahm, S. C., & Uhlenbeck, O. C. (1989) *Gene 82,* 31–41.
Ruffner, D. E., Stormo, G. D., & Uhlenbeck, O. C. (1990) *Biochemistry 29,* 10695–10702.
Schulhof, J. C., Molko, D., & Teoule, R. (1987) *Nucleic Acids Res. 15,* 397–416.
Sheldon, C. C., & Symons, R. H. (1989) *Nucleic Acids Res. 17,* 5679–5685.
Slim, G., & Gait, M. J. (1991) *Nucleic Acids Res. 19,* 1183–1188.
Snyder, E. E., Buoscio, B. W., & Falke, J. J. (1990) *Biochemistry 29,* 3937–3943.
Stein, A., & Crothers, D. M. (1976) *Biochemistry 15,* 157–160.
Sundaralingam, M., Rubin, J. R., & Cannon, J. F. (1984) *Int. J. Quant. Chem.: Quant. Biol. Symp. II,* 355–366.
Taira, K., Uebayasi, M., Maeda, H., & Furukawa, K. (1990) *Protein Eng. 3,* 691–701.
Teeter, M., Quigley, G., & Rich, A. (1981) in *Metal Ions in Genetic Information Transfer* (Eichhorn, G. L., & Marzilli, L. G., Eds.) pp 233–272, Elsevier, Amersterdam.
Uhlenbeck, O. C. (1987) *Nature 328,* 596–600.
Usman, N., Ogilvie, K. K., Jiang, M.-Y., & Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109,* 7843–7853.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56,* 283–430.
van Tol, H., Buzayan, J. M., Feldstein, P. A., Eckstein, F., & Bruening, G. (1990) *Nucleic Acids Res. 18,* 1971–1975.
Weast, R. C. (1987) *Handbook of Chemistry & Physics,* 68th ed., CRC Press, Boca Raton, FL.
Westermann, K., Naser, K.-H., & Brandes, G. (1986) *Inorganic Chemistry (Anorganische Chemie),* 12th ed., pp 53–55, VEB Deutscher Verlag für Grundstoffindustrie, Leipzig, GDR.
Yang, J.-H., Perreault, J. P., Labuda, D., Usman, N., & Cedergreen, R. (1990) *Biochemistry 29,* 11156–11160.