Ramanathan, B., & Smerdon, M. J. (1989) *J. Biol. Chem. 264*, 11026-11034.
Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984) *Nature 311*, 532-537.
Sen, D., & Crothers, D. M. (1986) *Biochemistry 25*, 1495-1503.
Smit-McBride, Z., Schnier, J., Kaufman, R. J., & Hershey, J. W. (1989) *J. Biol. Chem. 264*, 18527-18530.
Staros, J. V., Bayley, H., Standring, D. N., & Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun. 80*, 568-572.
Tabor, C. W., & Tabor, H. (1976) *Annu. Rev. Biochem. 45*, 285-306.
Tabor, C. W., & Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749-790.
Thomas, T. J., & Messner, R. P. (1986) *Nucleic Acids Res. 14*, 6721-6733.
Thomas, T. J., & Messner, R. P. (1988) *J. Mol. Biol. 201*, 463-467.
Thomas, T. J., Bloomfield, V. A., & Canellakis, Z. N. (1985) *Biopolymers 24*, 725-729.
Waterborg, J. H., & Matthews, H. R. (1983) *Biochemistry 22*, 1489-1496.
Wells, D. E. (1986) *Nucleic Acids Res. 14*, r119-r149.

Relationship between 2'-Hydroxyls and Magnesium Binding in the Hammerhead RNA Domain: A Model for Ribozyme Catalysis†

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Received November 26, 1990; Revised Manuscript Received January 16, 1991

ABSTRACT: The use of deoxyribonucleotide substitution in RNA (mixed RNA/DNA polymers) permits an evaluation of the role of 2'-hydroxyl groups in ribozyme catalysis. Specific deoxyribonucleotide substitution at G^9 and A^{13} of the ribozyme decreases the catalytic activity (k_{cat}) of the ribozyme by factors of 14 and 20, respectively. The reduction of the reaction rate concomitant with the absence of these 2'-OHs or the 2'-OH of the substrate U^7 position can be partially compensated by increasing the Mg^{2+} concentration above 10 mM. The K_{Mg} of the all-RNA ribozyme is 5.3 mM, and the lack of either of the three influential 2'-OHs increases this value by a factor of approximately 3. These and other reaction constants for the ribozyme and the deoxy-substituted analogues have been determined by assuming a three-step mechanism. The data presented here provide the basis for the formulation of a molecular model of ribozyme activity.

The introduction of deoxyribonucleotides in specific positions of RNA molecules is made possible by an RNA synthesis scheme (Usman et al., 1987; Scaringe et al., 1990) which is compatible with the chemical synthesis procedure for DNA (Wu et al., 1989). We expect deoxyribonucleotide incorporation in polyribonucleotide chains to have minimal structural consequences, since unlike site-directed mutagenesis, where one nitrogen base is replaced by another, neither base-pairing nor base-stacking, the principle determinants of nucleic acid structure, should be affected. Deoxyribonucleotide substitution (i.e., modification of the sugar moiety) in RNA thus offers a unique and incisive tool in the study of the functional and structural role of 2'-hydroxyls in RNA. Using these mixed RNA/DNA polymers, we are exploring structure–function relationships in the self-cleaving activity associated with the RNA hammerhead domain (Perreault et al., 1990). Hammerhead catalysis is characterized by the cleavage of a specific phosphodiester bond producing 5'-hydroxyl and 2',3'-cyclic phosphate products (Uhlenbeck, 1987). As shown in Figure 1, this domain can be partitioned into 2 RNA fragments which allows the formalism of defining a ribozyme fragment of 35 nucleotides and a substrate fragment of 14 nucleotides (Gerlach & Haseloff, 1988; Koizumi et al., 1988; Forster et al., 1988; Jeffries & Symons, 1989). We have previously established that the 2'-OH adjacent to the scissile phosphate of the substrate at position C^8 (Figure 1) is the nucleophile in the reaction (Perreault et al., 1990) and that this is the only essential 2'-OH in the substrate fragment, even though absence of the 2'-OH of U^7 has a negative effect on the reaction rate (Yang et al., 1990).

As for the ribozyme fragment itself, no single 2'-OH is essential in the reaction, in spite of the fact that the 2'-OH of G^9 and at least one other 2'-OH of the central core strongly affect catalytic efficiency (Perreault et al., 1990). We now report the identification of a second influential hydroxyl in the ribozyme fragment, that of A^{13}. Furthermore, our data show that the 2-OHs of the ribozyme G^9 and A^{13} and the substrate U^7 are implicated in a low-affinity binding of the Mg^{2+} cofactor to a specific site in the substrate/ribozyme complex. These data allow the formulation of a structural model of ribozyme activity.

Materials and Methods

Synthesis and Purification of DNA/RNA Mixed Polymers. All oligonucleotides were synthesized on an automated oligonucleotide synthesizer (Milligen/Biosearch and Pharmacia) and deprotected as described elsewhere (Perreault et al., 1990;

† This work was supported by a grant from the Medical Research Council of Canada. J.-P.P. was supported by Fonds FCAR (Québec). R.C. is a Fellow of the Canadian Institute of Advanced Research and D.L. of the Fonds de recherches en santé de Québec. N.U. holds a NASA and an NIH Fogarty International Research Fellowship.

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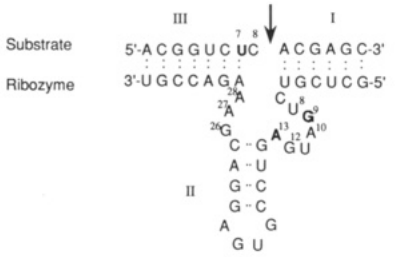

FIGURE 1: RNA structure of ribozyme and substrate produced by chemical synthesis. Note that the G^{6}-C^{30} base pair present in the consensus structure has been changed to C^{6}-G^{30} in the synthetic molecules. The positions of nucleotides replaced with deoxyribonucleotides are numbered, and nucleotides having catalytically influential 2′-OH groups are in boldface.

Scaringe et al., 1990; Wu et al., 1989). The RNA was purified by 20% polyacrylamide gel electrophoresis (PAGE)[1] in 50 mM (0.5× TBE is 45 mM) Tris–boric acid containing 1 mM EDTA and 7 M urea, pH 8.3. Major bands detected by autoradiography were cut out and extracted twice with sterile water at 4 °C or at room temperature overnight. The supernatant was concentrated to 200 μL in a Speed-Vac and then passed through a 10-mL Sephadex G-25 column. Finally, the oligonucleotides were lyophilized, and their concentration was determined by the absorption at 260 nm. Structural characterization was carried out by the rapid gel sequencing technique using RNases (Nicoghosian et al., 1985). The substrate was 5′-^{32}P-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The mixture was purified on 20% PAGE; the 14-mer was extracted with water, ethanol-precipitated, and passed through a Sephadex G-50 spun column.

Standard Cleavage Reaction. Standard reaction conditions were as follows: substrate (1.15 μM) and analogue ribozymes (0.05 μM) were heated to 60 °C for 15 s in 75 mM Tris-HCl buffer, pH 7.5. After snap-cooling, $MgCl_2$ was added to 10 or 50 mM, and the final volume of 10 μL was incubated for 60 min at 37 °C. The reaction was stopped by the addition of formamide dye and analyzed by 20% PAGE.

Kinetic Determination. The reactions were performed as described above. The analogue ribozyme concentrations were 0.025 or 0.05 μM, and four substrate concentrations were used varying from 0.12 to 3.0 μM or from 0.25 to 6.0 μM, respectively. Aliquots were taken, and values up to 10% cleavage were used in the calculations. All reactions were done in duplicate. For [Mg^{2+}] dependence, the reaction conditions were as described above except for the concentrations of substrate (4.0, 1.15, and 0.25 μM) and analogue ribozymes (0.05 μM). The Mg^{2+} concentrations varied between 0 and 50 mM.

RESULTS

Implication of 2′-Hydroxyls in Catalysis. At the inception of this work, the 2′-OHs of the consensus, unpaired nucleotides of the hammerhead domain were considered prime candidates to have an important role in catalysis, since ribonucleotide 2′-OHs in base-paired regions were most likely engaged in interactions within the helices. To evaluate the 2′-OHs of the single-stranded nucleotides of the core, several mixed RNA/DNA polymers, corresponding to both ribozyme and substrate sequences, were prepared, and their activities were determined (Perreault et al., 1990; Yang et al., 1990). It was concluded from the kinetic data of two hexadeoxy analogues of the ribozyme that the 2′-OH of G^{9} was influential in the catalytic process. To confirm this result and to identify a purported second 2′-OH in this region impinging on the catalytic activity, a series of *minimally* deoxyribonucleotide-substituted ribozyme analogues were prepared and analyzed.

[1] Abbreviations: The first letter of the analogues designates the predominant nucleotide used in the synthesis, R for ribonucleotides and D for deoxyribonucleotides. The second letter indicates that the oligomer is R for a ribozyme or S for a substrate analogue. The nature and the position of variant nucleotides are indicated by rA, rC, rG, or rU for ribonucleotide substitution followed by the number of the position from the 5′ terminus and dA, dC, dG, and dT and the position for deoxyribonucleotide substitution. D and R used before the words substrate, analogue, or ribozyme indicate the predominant nucleotide in the molecule. PAGE is polyacrylamide gel electrophoresis. A_{260} is the absorbance of a solution at 260 nm in a 1-cm light path.

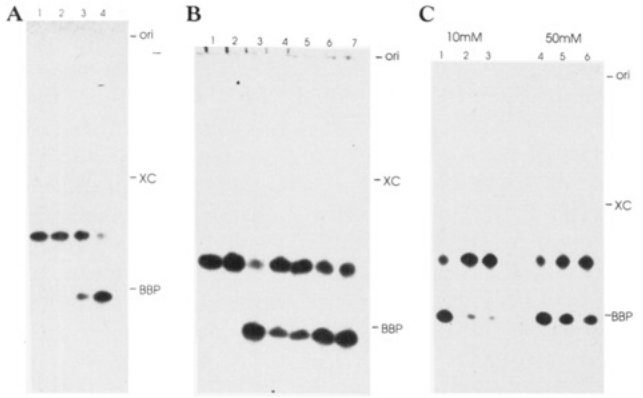

FIGURE 2: Activity gels of analogue ribozyme catalysis using 5′-^{32}P-labeled all-RNA substrate. (A) Controls lacking ribozyme (lane 1 at 0 min and lane 2 at 60 min) are compared with 60-min reactions using the RRdG9 analogue ribozyme (lane 3) and the all-RNA ribozyme (lane 4). (B) Lanes 1 and 2 are controls without ribozyme incubated for 0 and 120 min, respectively. Other lanes are the reaction mixture after 60-min incubation with the all-RNA (lane 3), RRdG12dA13 (lane 4), RRdA13 (lane 5), RRdG12 (lane 6), and RRdG26dA27dA28 (lane 7). (C) Effect of Mg^{2+} concentration on the cleavage activity. Lanes 1 and 4, all-RNA ribozyme; lanes 2 and 5, RRdG9 analogue ribozyme; lanes 3 and 6, RRdA13. The reaction conditions were the same as in panels A and B, except that the [Mg^{2+}] was 10 mM (lanes 1–3) and 50 mM (lanes 4–6).

Table 1: K_M and k_{cat} Values for Various Ribozymes and Substrates[a]

Ribozyme Analogs	Single strand Sequence ^{8}UGAUGA^{13} ^{26}GAA^{28}	k_{cat} min^{-1}	K_M uM	k_{cat}/K_M min^{-1} · uM^{-1}
all-RNA*	UGAUGA GAA	2,6	0,92	2,8
octadeoxy*	(D)(D)(D)(D)(D)(D) (D)(D)(D)	0,014	0,96	0,015
pentadeoxy*	UGAU(D)(D) (D)(D)(D)	0,087	0,95	0,09
RRdG9	U(D)AUGA GAA	0,18	1,0	0,18
RRdG12dA13	UGAU(D)(D) GAA	0,09	0,91	0,10
RRdG12	UGAU(D)A GAA	1,2	0,89	1,4
RRdA13	UGAUG(D) GAA	0,13	0,95	0,14
RRdG26dA27dA28	UGAUGA (D)(D)(D)	1,3	0,84	1,5
Substrate				
RSdT7**	ACGGUC(T)CACGAGC	0,12	7,4	0,016
DSrU7rC8**	(D)(D)(D)(D)UCACGAGC	0,31	13	0,024

[a] Deoxyribonucleotides are noted by circling the nucleotide. A single asterisk indicates values calculated from Perreault et al. (1990) while a double asterisk denotes values calculated from Yang et al. (1990).

Confirmation of our previous result is shown in Figure 2A (also Table I), where an analogue having only the G^9 substituted with a deoxyribonucleotide (RRdG9) has an activity level considerably lower than the all-RNA ribozyme; the k_{cat} of this analogue is approximately 14 times less than the all-RNA ribozyme, although the K_M is virtually identical with that of the parent ribozyme. The strategy for identifying a second 2′-OH is revealed by comparison of the activities of a dideoxy (RRdG12dA13) and trideoxy analogue (RRdG26dA27dA28) in Table I. RRdG12dA13 has a lower activity than the all-RNA ribozyme, whereas the trideoxy analogue's activity is roughly the same as that of the parent ribozyme (Figure 2B). On the basis of the reduced k_{cat} (factor of 29) of the dideoxy analogue (Table I), we concluded that the remaining, unidentified 2′-OH(s) must be at one or both of the substituted positions (dG^{12}, dA^{13}). The monodeoxy analogues RRdG12 and RRdA13 were then made, and their activity level was determined (Figure 2B). The kinetic parameters show that the lack of a 2′-OH at A^{13} strongly reduces the activity (factor of 20 on the k_{cat}), whereas the absence of a 2′-OH at G^{12} has only a minor effect (Table I). Although these experiments were carried out in Mg^{2+} (10 mM), a cofactor in the catalysis, the cation requirement can be satisfied with decreasing efficiency by the following: $Mn^{2+} > Mg^{2+} > Ca^{2+}$ (Uhlenbeck, 1987). Reactions carried out with RRdG9 and the pentadeoxy analogue lacking the 2′-OH group in G^9 and A^{13}, respectively, confirmed a reduced rate with these cations as well (data not shown).

We have previously postulated that the 2′-OH groups affecting catalytic activity could participate in the Mg^{2+} binding required for cleavage (Perreault et al., 1990). In this scenario, absence of an influential 2′-OH would reduce Mg^{2+} binding and consequently the activity of the ribozyme. This model leads to the prediction that a higher concentration of Mg^{2+} could stimulate the activity of the analogue ribozymes. To test this possibility, cleavage rates were compared in 10 and 50 mM $MgCl_2$. In Figure 2C, it is seen that although the activity of the all-RNA ribozyme remains constant at higher Mg^{2+} concentration (lanes 1 and 4), the activities of analogue ribozymes (RRdG9, lanes 2 and 5; RRdA13, lanes 3 and 6) increase dramatically. Interestingly, whereas cleavage of the predominantly ribonucleotide substrate analogue (RSdT7) is stimulated at higher Mg^{2+} concentrations, cleavage of the predominantly deoxyribonucleotide (with T replacing U) substrate analogue (DSrU7rC8) is actually inhibited at higher $[Mg^{2+}]$ (inset of Figure 3). The postulated conformational modification of the catalytic core when predominantly deoxyribonucleotide substrates are used is consistent with this result (Yang et al., 1990).

Description of the Reaction Pathway. On the basis of the number of essential components in catalysis, the ribozyme (Rz), substrate (S), and magnesium ion (Mg^{2+}), the reaction scheme leading to the ternary complex ($RzSMg^{2+}$), catalytic cleavage, and products (P_1 and P_2) can be described by the following minimal number of steps [cf. Fehrst (1985)]:

$$Rz + S \overset{K_S}{\rightleftharpoons} RzS + Mg^{2+} \overset{K_{Mg}}{\rightleftharpoons} RzSMg^{2+} \overset{k_3}{\longrightarrow} Rz + P_1 + P_2 + Mg^{2+} \quad (1)$$

where $K_S = [S][Rz]/[RzS]$ and $K_{Mg} = [RzSMg^{2+}]/[RzS][Mg^{2+}]$ are the dissociation constants of the RzS and ternary complex, respectively. k_3 denotes the rate constant of the chemical step and $[Rz_0]$ the total ribozyme concentration. Consequently, the Michaelis–Menten constant (K_M) and catalytic rate (k_{cat}) determined at constant $[Mg^{2+}]$ can be expressed by $K_M = K_S K_{Mg}/(K_{Mg} + [Mg^{2+}])$ and $k_{cat} = k_3[Mg^{2+}]/(K_{Mg} + [Mg^{2+}])$. Plotting the initial velocity of cleavage measured at constant [S] as a function of $[Mg^{2+}]$ by eq 2 permits the evaluation of K_{Mg} and k_3.

$$1/v = 1/k_3[Rz_0] + K_{Mg}(1 + K_S/[S])/k_3[Rz_0][Mg^{2+}] \quad (2)$$

In this way, the dissociation constant K_{Mg} was determined for the all-RNA ribozyme and several analogues (Figure 3 and Table II) using different constant [S]. The K_{Mg} value of 5.3 mM for the all-RNA ribozyme is indicative of a relatively low affinity between the Mg^{2+} and the ribozyme–substrate complex. The Mg^{2+} binding in the case of the D analogues, RRdG9, and a pentadeoxy-substituted ribozyme (which lacks the 2′-OH of A^{13}) demonstrates even lower affinities since each has a K_{Mg} value 3 times higher than in the all-RNA ribozyme (Table II). This effect is enhanced in the octadeoxy analogue which lacks both influential 2′-OHs; K_{Mg} is about 8 times

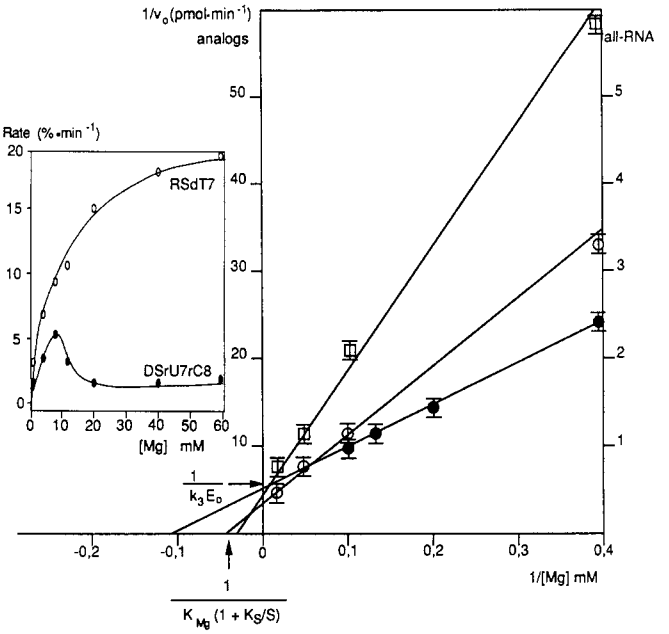

FIGURE 3: Plot of $1/v$ versus $1/[Mg^{2+}]$ as represented in eq 2 for different [S]. The intercept of the abscissa is $1/K_{Mg}(1 + K_S/[S])$. Only the results for 4.0 μM substrate are shown in the plot. The left scale is for the analogue ribozymes, pentadeoxy ribozyme (□) and RRdG9 (○), while the right scale is for the all-RNA ribozyme (●). The insert at the left is the time course (v versus $[Mg^{2+}]$) for the reaction of the substrate analogues RSdT7 (○) and DSrU7rC8 (●) with the all-RNA ribozyme.

Table II: Kinetic Parameters for Analogue Ribozyme Catalysis and for Analogue Substrates[a]

Ribozyme Analogs	Single strand Sequence ^{8}UGAUGA^{13}	^{26}GAA^{28}	K_S uM	K_{Mg} mM	k_3 min^{-1}	k_3/K_{Mg} $min^{-1}\cdot mM^{-1}$
all-RNA	UGAUGA	GAA	2,70	5,3	3,99	0,76
octadeoxy	dGdAdGdA	dGdAdA	1,19	42	0,07	0,0017
pentadeoxy	UGAdGdA	dGdAdA	1,50	15,7	0,25	0,016
RRdG9	UGAUGA	GAA	1,71	15,1	0,45	0,03
Substrate						
RSdT7	ACGGUCUCACGAGC		11,6	17,5	0,33	0,019
DSrU7rC8	dAdCdGdGdUCUdCdAdCdGdAdGdC		55	3,1	0,32	0,011

[a] k_3, K_{Mg}, and K_s were determined according to eq 2 from the dependency of reaction rate on $[Mg^{2+}]$ and [S].

higher than in the all-RNA ribozyme. A 3-fold increase in K_{Mg} is also observed with the analogue substrate having a deoxyribonucleotide at position 7 (RSdT7) compared with the all-RNA substrate. Thus, the absence of any of the three identified 2′-OHs results in an increase in the K_{Mg} by a remarkably similar factor of 3, and the K_{Mg} increases with the number of influential 2′-OHs removed from the ribozyme–substrate complex by deoxyribonucleotide substitution. We conclude that the formation of the ternary complex between ribozyme–substrate complex and Mg^{2+} is inhibited when the ribozyme lacks 2′-OHs at G^9 or A^{13} and the substrate at U^7.

Previously, it was argued that a modified catalytic core conformation was responsible for the reaction characteristics of a predominantly deoxyribonucleotide substrate analogue, DSrU7rC8. Here, the K_{Mg} was evaluated at 3.1 mM, approximately half the value of the RNA substrate (Table II). The fact that this higher affinity for Mg^{2+} in the ternary complex is not accompanied by an increase in catalytic efficiency further supports the hypothesis that the conformation of the catalytic complex is somehow altered. Moreover, this conformational modification could be responsible for the observed inhibition of the reaction at higher [Mg^{2+}] (see inset of Figure 3), the possible result of interference from the binding of a second Mg^{2+}. A similar inhibitory effect of a low-affinity Mg^{2+} was observed in the Pb^{2+}-catalyzed cleavage of tRNA (Labuda et al., 1985).

The values of K_S, K_{Mg}, and k_3 are also shown in Table II. The values of K_S for the all-RNA ribozyme and the different analogues might be expected to be very similar because no deoxyribonucleotides have been introduced in the base-paired regions created during the interaction of the ribozyme with the substrate. This expectation is borne out to a certain degree since the K_S values for different ribozyme analogues range from 1.5 to 1.7 μM, and the K_S for the all-RNA ribozyme has a higher value at 2.7 μM. Although the differences between K_S values of the analogues are within experimental error, comparison of these values with that of the all-RNA ribozyme reveals a trend: K_S is inversely proportional to the number of deoxyribonucleotides in the analogue. If an equilibrium between active and inactive forms of the ribozyme fragment exists prior to the addition of substrate (Heus et al., 1990), it is conceivable that deoxyribonucleotide substitution could displace this equilibrium toward the active conformer, thereby promoting duplex formation between the ribozyme and the substrate. Alternatively, the absence of 2′-OHs could reduce steric hindrance to the formation of the substrate–ribozyme duplex. In either case, the similarity of K_S values results in a very high dependency of the formation of the catalytically active ternary complex on the K_{Mg} or on Mg^{2+} binding.

Evaluation of the k_3 for the ribozyme and analogues is shown in Table II. From these values, it is clear that an approximate 10-fold decrease in k_3 accompanies the absence of either of the three influential 2′-OHs, i.e., G^9 in RRdG9, A^{13} in the pentadeoxy analogue, and U^7 in RSdT7. The k_3 value for the all-RNA ribozyme (Table II) is only slightly higher than the corresponding k_{cat} determined at 10 mM Mg^{2+} (Table I), since this [Mg^{2+}] at double the K_{Mg} is almost saturating. The differences between the values of k_3 for the ribozyme analogues and the corresponding values of k_{cat} are more pronounced, since the values of K_{Mg} for these ternary complexes are higher. This explains the stimulation of catalytic activity by increasing [Mg^{2+}] observed with the analogues (Figure 2). The value of k_3, independent of [Mg^{2+}], corresponds to the rate of decomposition of the ternary complex within the framework of the proposed reaction scheme (eq 1). The differences among values of k_3 in Table II can be correlated with the observed differences in the binding of Mg^{2+} to the ribozyme–substrate complex as measured by the increasing K_{Mg} values. We note, however, that higher [Mg^{2+}] cannot completely compensate for lower reaction efficiency, suggesting that a lower k_3 is related to an increased lability of bound Mg^{2+}.

The results presented here show the simple three-step reaction pathway to be at least *sufficient* to analyze the kinetics of the hammerhead-catalyzed reaction described in this paper. We realize, however, that this model may not be sufficient to account for all microscopic events leading to catalysis, such as indicated by the data contained in the inset of Figure 3 which demonstrate that higher [Mg^{2+}] may under certain circumstances be inhibitory to the reaction. Inhibition is not observed in any other system below 50 mM Mg^{2+}. In addition, it should be noted that in some circumstances the three-step scheme may not apply. For example, by extending the recognition sequences between ribozyme and substrate or lowering the temperature, it is possible to render the rate of the reaction dependent on product release (Koizumi et al., 1989; Fedor & Uhlenbeck, 1990). To avoid these types of complications, the ribozyme and substrate sequences have remained unchanged in our experiments, an option made possible by the use of structural modifications in the ribose moiety.

Discussion

The role of the Mg^{2+}, the influence of the 2′-OHs, and the possible interplay between them are critical issues in hammerhead ribozyme catalysis. Although the essential nature of Mg^{2+} binding was known, we have now shown that this binding is characterized by a constant (K_{Mg}) indicating a relatively low affinity. The question of fundamental importance is whether this Mg^{2+} is catalytic, i.e., does the Mg^{2+} play a direct role in cleavage. At first glance, it would seem obvious that the Mg^{2+} is indeed catalytic, since there are no examples of RNA catalysis in the absence of a bivalent metal cation; however, conformational effects cannot be ruled out a priori. The conformational interpretation of these data requires a postulate that the critical Mg^{2+} binding that we have measured either provokes a conformational change or stabilizes an active conformer which leads to catalysis. However, melting experiments of mixtures of ribozyme and substrate in the presence and absence of Mg^{2+} show little difference in T_m or hyperchromicity; a significant difference could be ascribed to a conformational change upon binding of Mg^{2+}. Furthermore, only broadening of imine proton resonances has been observed in NMR studies upon the addition of Mg^{2+} (Heus et al., 1990; Odai et al., 1990; Heus & Pardi, 1990). The lack of significant spectral differences has prompted the authors to conclude that Mg^{2+} enters a preexisting cavity in the ribozyme–substrate complex (Heus & Pardi, 1990); i.e., Mg^{2+} binding neither induces major conformational changes nor dramatically shifts preexisting conformational equilibria. It should be noted that even this negative evidence is not fully conclusive, since it is still possible that a minor conformational change upon Mg^{2+} binding would be sufficient to promote catalysis, but not substantial enough to be detected by these physical methods. Even with this methodological limitation, major conformational change seems highly unlikely.

On the other hand, there is ample precedent for the proposal of a catalytic Mg^{2+}. Many proteoenzymes promote catalysis by the use of cation cofactors including Mg^{2+}, which is at the catalytic site and actively involved in the chemical step of catalysis. One such example, the Klenow fragment, uses either Mg^{2+}, Mn^{2+}, or Zn^{2+} directly in phosphate transesterification

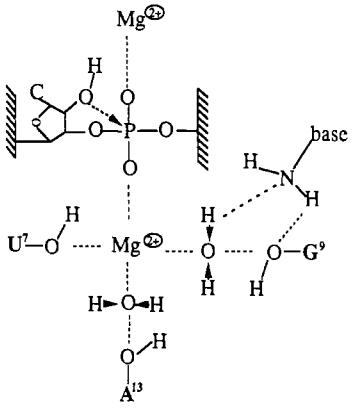

FIGURE 4: Model of the putative active site of the hammerhead ribozyme. The scissile phosphate is shown complexed to two Mg^{2+} ions, which delocalizes its negative charge. Influential 2′-OHs of substrate U^7 and ribozyme G^9 and A^{13} are shown in various configurations representing different ways in which they could interact in the formation of the catalytic site. The two remaining valences of Mg^{2+} could be filled by other functional groups on the ribozyme or by solvent molecules.

as in the present case (Freemont et al., 1988). Herschlag and Jencks (1987) have proposed that divalent metal ions act by stabilizing the leaving group in a displacement reaction at phosphorus, thereby reducing the requirements on the attacking nucleophile. This may be a crucial key factor in hammerhead ribozyme catalysis which depends on the relatively poor nucleophilicity of the adjacent 2′-OH for transesterification.

In considering the role of the 2′-OHs and their effect on K_{Mg}, we must again confront the possibility of a conformational effect, i.e., that the deoxy substitution changes the conformation of the ribozyme in such a way that Mg^{2+} binding is affected. Here again, available evidence argues against such an interpretation, since the K_M's of all ribozymes are virtually identical even though the K_{Mg} varies by a factor of 3. As mentioned previously, the K_S and thus the K_M will be sensitive to conformational changes as demonstrated with the D substrates (Yang et al., 1990). Again, it is obvious that small conformational changes cannot be completely ruled out by these data; in fact, small changes are to be expected, since the preferred conformation of deoxyribonucleotides is 2′-endo in contrast to 3′-endo for ribonucleotides. However surprising as it might be, these small structural alterations of the catalytic domain do not seem to play a significant role in the very consistent kinetic data set presented here and previously (Perreault et al., 1990; Yang et al., 1990).

On the basis of the above arguments and the analysis of the kinetic data, it can be concluded that large conformational changes probably do not take place either in Mg^{2+} binding or in the use of deoxyribonucleotide substitution in the ribozyme. Significantly, even small conformational effects, though present, are not necessary to explain the kinetic data on the formation of the ternary complex presented here. We have thus devised a simple model illustrated in Figure 4 which is sufficient to describe and fully consistent with out data. The critical factor of this proposal involves the binding of a catalytic Mg^{2+} to a low-affinity site which is at least partially defined by the influential 2′-OHs. The 2′-OHs could interact with the Mg^{2+} in a variety of ways: directly, in an inner-sphere complex [cf. Labuda and Pörschke (1982)], or via solvent-mediated bridges either alone or in combination with functional groups on the nitrogen bases.

Interactions between 2′-OHs and solvent or Mg^{2+} have been found in the tRNA crystal structure (Holbrook et al., 1977; Hingerty et al., 1978) and have been proposed for the Mg^{2+} implicated in group I intron activity (Sugimoto et al., 1988, 1989). Inner-sphere complexes of Mg^{2+} with the hydroxyl groups of threonine and serine are well-documented in the crystal structure of the Ras protein (Schlichting et al., 1990). The relatively low affinity of the site for Mg^{2+}, which decreases further in the absence of the 2′-OHs, suggests that the putative metal ion binding pocket is located in an area of rather low electrostatic potential. However, as indicated above, the positively charged Mg^{2+} should be located near the negatively charged phosphate, the catalytic target. This apparent contradiction can be resolved by the consideration of the effect of previously bound Mg^{2+}'s, which serve the role of phosphate counterions. This higher affinity (lower specificity) binding would shield or delocalize negative charges associated with the phosphodiester bond, thereby reducing the electrostatic potential of the catalytic pocket. Although shielding could result from a "cloud" of positively charged counterions, it may be pertinent to consider a specific counterion. For instance, two metal ions have been found at the catalytic center of the Klenow fragment exhibiting an endonucleolytic activity similar to that of the ribozyme (Freemont et al., 1988). It has not escaped our attention that a phosphodiester interacting with two cations would be considerably more electrophilic than others and as a result a prime target for a nucleophilic attack.

Both the 2′-OH of G^9 and A^{13} of the ribozyme are implicated in some aspect of Mg^{2+} binding necessary for catalysis, and on the basis of kinetic parameters determined previously for an extensively substituted analogue (octadeoxy analogue), these two are the only hydroxyls of the single-stranded region so implicated. Also, it seems that the effect of influential ribozyme 2′-OHs is not cooperative, for if we assume that only these hydroxyls are influential and that their effects are independent, the product of their k_{cat}'s ($0.18 \times 0.13 = 0.023$) is very close to the k_{cat} observed in the case of the octadeoxy analogue (0.014). Clearly these results dispel fears that the introduction of deoxyribonucleotides in single-stranded regions of the ribozyme leads to a major alteration of the catalytic properties or the conformation of the ribozyme. Our results rather imply that these 2′-OHs have a role to play in the binding of Mg^{2+} in the catalytic pocket. The fact that other examples of RNA catalysis have metal ion requirements in the range of 10 mM certainly raises the issue of whether all of these RNA-catalyzed reactions are related to the molecular mechanism proposed herein (Takada et al., 1986; Sugimoto et al., 1988; Grosshans & Cech, 1989).

Finally, the reaction pathway is summarized as an energy profile in Figure 5, where Gibbs free energy differences were calculated from the appropriate kinetic parameters (Fersht, 1985). The first step, formation of the ribozyme–substrate complex, is of course dependent on the initial conformation of both the ribozyme and the substrate, which in turn is related to the ability of the substrate and/or the ribozyme to fold into a stable intramolecular structure, as recently demonstrated by Heus et al. (1990). In the case of the ribozyme used in our study, an inactive conformation resulting from helix formation between ^{5}GUCU^8 and ^{29}AGAC^{32} (Figure 1) is possible as suggested above to rationalize the effect of deoxyribonucleotide substitution on K_S. The stability of this structure would be approximately -3 kcal mol^{-1} from the thermodynamic parameters derived by Freier et al. (1986). The reaction scheme could therefore include the melting of the intramolecular structure before the formation of the ribozyme–substrate complex. On the basis of the K_S for the all-RNA ribozyme and substrate, the free energy related to substrate

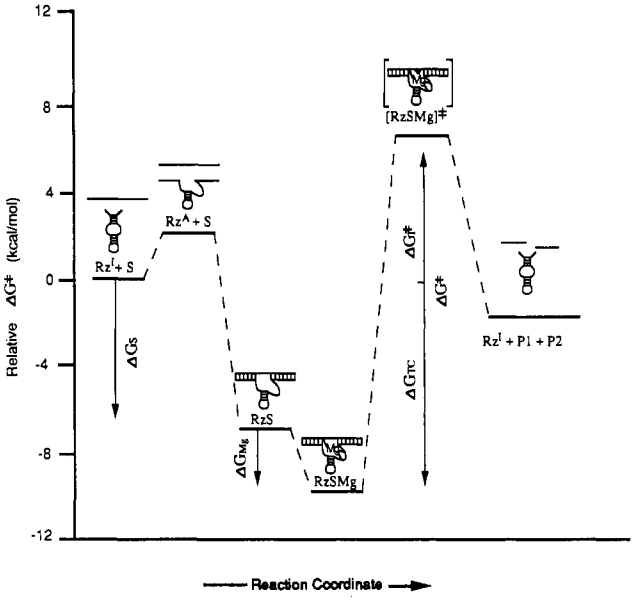

FIGURE 5: Energy profile proposed for hammerhead catalysis. The ΔG_S is calculated from the equation $\Delta G_S = RT \ln K_S$, where R is 1.987 cal mol^{-1} and T the reaction temperature in degrees kelvin. The energy change going from the ribozyme–substrate complex to the ternary complex is $\Delta G_{Mg} = RT \ln K_{Mg}$ (−3.2 kcal mol^{-1}), and ΔG_{TC} the free energy difference in the formation of the ternary complex RzSMg, is thus equal to $\Delta G_S + \Delta G_{Mg}$. Assuming the transition-state theory applied to Michaelis–Menten kinetics, $\ln k_{cat}/K_M = \ln kT/h - \Delta G^{\ddagger} - \Delta G_{TC}$, where k is Boltzmann's constant, h is Planck's constant, and $\Delta G_T^{\ddagger}$ is the transition energy. The activation energy $\Delta G^{\ddagger}$ is +17.6 kcal mol^{-1}, and $\Delta G_T^{\ddagger}$ is −6.5 kcal mol^{-1}. Rz^I and Rz^A refer to putative inactive and active conformations of the ribozyme, respectively.

binding (ΔG_S) is estimated at −7.9 kcal mol^{-1}.

Following the formation of the RzS complex, the Mg^{2+} essential for catalysis can bind to give the ternary complex $RzSMg^{2+}$. The energy involved in Mg^{2+} binding (ΔG_{Mg}) is −3.2 kcal mol^{-1}. From the K_{Mg}'s obtained with different analogue ribozymes, the contribution of each influential 2′-OH is evaluated at about −0.7 kcal mol^{-1} per hydroxyl. Subsequently, the ternary complex decomposes by way of the transition state whose energy level is calculated at +6.5 kcal mol^{-1} (17.6 kcal mol^{-1} higher than the ternary complex, Figure 5). Finally, chemical cleavage proceeding through the transition state and likely a trigonal bipyramid intermediate leads to product (Cedergren et al., 1987). It remains to be seen whether this step involves major conformational changes or other as yet undefined aspects of the structure.

ACKNOWLEDGMENTS

We thank Pascal Chartrand for technical assistance, Milligen/Biosearch for supplying a DNA synthesizer used for some of the mixed polymers, and Professor A. Rich for his encouragement.

REFERENCES

Cedergren, R., Lang, B. F., & Gravel, D. (1987) *FEBS Lett. 226*, 63–66.
Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668–1672.
Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
Forster, A. C., Davies, C., Sheldon, C. C., Jeffries, A. C., & Symons, R. H. (1988) *Nature 334*, 265–267.
Freemont, P. S., Friedman, J. M., Beese, L. S., Sanderson, M. R., & Steitz, T. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8924–8928.
Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9373–9377.
Gerlach, W. L., & Haseloff, J. (1988) *Nature 334*, 585–591.
Grosshans, C. A., & Cech, T. R. (1989) *Biochemistry 28*, 6888–6894.
Guerrier-Takada, C., Haydock, K., Allen, L., & Altman, S. (1986) *Biochemistry 25*, 1509–1515.
Heus, J. A., & Pardi, A. (1990) *J. Mol. Biol.* (in press).
Heus, H. A., Uhlenbeck, O. C., & Pardi, A. (1990) *Nucleic Acids Res. 18*, 1103–1108.
Hingerty, B., Brown, R. S., & Jack, A. (1978) *J. Mol. Biol. 124*, 523–534.
Holbrook, S. R., Sussman, J. L., Warrant, R. W., Church, G. M., & Kim, S.-H. (1977) *Nucleic Acids Res. 8*, 2811–2821.
Jeffries, A. C., & Symons, R. H. (1989) *Nucleic Acids Res. 17*, 1371–1377.
Koizumi, M., Iwai, S., & Ohtsuka, E. (1988) *FEBS Lett. 228*, 228–230.
Koizumi, M., Hayase, Y., Iwair, S., Kamiya, H., Inoue, H., & Ohtsuka, E. (1989) *Nucleic Acids Res. 17*, 7059–7071.
Labuda, D., & Pörschke, D. (1982) *Biochemistry 21*, 49–53.
Labuda, D., Nicoghosian, K., & Cedergren, R. (1985) *J. Biol. Chem. 260*, 1103–1107.
Nicoghosian, K., Gu, X.-R., & Cedergren, R. (1985) *FEBS Lett. 193*, 255–260.
Odai, O., Kodama, H., Hiroaki, H., Sakata, S., Tanaka, T., & Uesugi, S. (1990) *Nucleic Acids Res. 18*, 5955–5960.
Perreault, J.-P., Wu, T.-F., Cousineau, B., Ogilvie, K. K., & Cedergren, R. (1990) *Nature 344*, 565–567.
Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res. 18*, 5433–5441.
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990) *Nature 345*, 309–315.
Sugimoto, N., Kierzek, R., & Turner, D. H. (1988) *Biochemistry 27*, 6384–6392.
Sugimoto, N., Tomka, M., Kierzek, R., Bevilacqua, P. C., & Turner, D. H. (1989) *Nucleic Acids Res. 17*, 355–371.
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*, 7845–7854.
Wu, T., Ogilvie, K. K., Perreault, J.-P., & Cedergren, R. J. (1989) *J. Am. Chem. Soc. 111*, 8531–8533.
Yang, J.-H., Perreault, J.-P., Labuda, D., Usman, N., & Cedergren, R. (1990) *Biochemistry 29*, 11156–11160.