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Importance of Specific Adenosine N^7-Nitrogens for Efficient Cleavage by a Hammerhead Ribozyme. A Model for Magnesium Binding†,‡

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ABSTRACT: Five modified hammerhead ribozyme/substrate complexes have been prepared in which individual adenosine N^7-nitrogens have been excised. The modified complexes were chemically synthesized with the substitution of a single 7-deazaadenosine (c^7A) base analogue for residues A_{11}, A_{14}, A_{26}, A_{27}, or A_{28}. Two of the base analogues, c^7A_{11} and c^7A_{14}, occur in a 19-mer ribozyme, while the remaining three residues, c^7A_{26}, c^7A_{27}, and c^7A_{28}, are present in a 24-mer substrate. Under stoichiometric conditions, four of the complexes are cleaved with relatively little change in rate when compared with that of the native complex. However, the relative rate for the c^7A_{11} complex is some 35-fold slower than that of the native complex. Steady-state kinetic analyses indicate that the cleavage efficiencies, as measured by k_{cat}/K_M, for the c^7A_{14}, c^7A_{26}, c^7A_{27}, and c^7A_{28} complexes are reduced 18-fold, 10-fold, 34-fold, and 16-fold, respectively. These reductions in cleavage efficiency are primarily a result of lower k_{cat} values. By comparison, the cleavage efficiency of the c^7A_{11} complex is reduced more than 200-fold relative to that of the native complex, again primarily as a result of a lower k_{cat} value. The results suggest that the N^7-nitrogen of A_{11} in the hammerhead ribozyme/substrate complex is critical for efficient cleavage activity. The results of the present work, in combination with those from previous reports, indicate that five critical functional groups are located within the tetrameric sequence $G_{10}A_{11}U_{12}G_{13}$. A preliminary model for the binding of a single magnesium cofactor to this portion of the sequence is proposed. In this model, the five critical functional groups interact with a partially hydrated magnesium cofactor, and the sixth coordination site remains open for complexing an oxygen from the scissile phosphodiester or for a similar interligand interaction involving a coordinated water molecule.

Self-cleavage reactions of RNA have been observed in the genomes of several plant satellite RNAs, where it is believed that these reactions are an essential step in replication [for reviews, see Symons (1989) and Bruening (1989)]. One of the smallest consensus sequences containing the requisite secondary structure necessary for such autolytic transesterifications is that represented by the hammerhead RNAs (Forster & Symons, 1987; Uhlenbeck, 1987). These complexes consist of three helices (one or more of which can terminate as a hairpin loop) and include 11 consensus nucleotides that appear to be responsible for the formation of a catalytically active domain. Cleavage of the RNA occurs as the result of a transesterification reaction and generates two products, one containing a terminal 5′-hydroxyl and a second with a terminal 2′,3′-cyclic phosphodiester (Forster et al., 1987; Uhlenbeck, 1987). In the in vivo examples, these structures result from the folding of a single RNA molecule, but synthetic hammerhead complexes composed of two or even three fragments also exhibit cleavage activities (Haseloff & Gerlach, 1988; Koizumi et al., 1988; Jefferies & Symons, 1989; Koizumi et al., 1989). Divalent metal ions such as Mg^{2+} or Mn^{2+} are required for the cleavage reaction (Uhlenbeck, 1987; Olsen et al., 1991; Dahm & Uhlenbeck, 1991). At least one, but possibly two metal cofactors, is

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necessary for activity (Koizumi & Otsuka, 1989).

Sequence mutations of the 11 conserved nucleotide residues have resulted in dramatic decreases in cleavage activity (Koizumi et al., 1988; Jefferies & Symons, 1989; Ruffner et al., 1989, 1990; Fedor & Uhlenbeck, 1990), suggesting that specific functional groups of the conserved U, C, A, and G nucleotide residues are critical for the formation of the catalytically competent complex. Results of a number of functional group alterations within the ribozyme complex have been reported; such studies permit functional group mutagenesis at the atomic level within the catalytic complex. A series of "deletion substitutions" have been reported in which the 2′-hydroxyls have been excised at specific sites by the introduction of the corresponding 2′-deoxynucleosides (Yang et al., 1990, 1992; Perreault et al., 1990, 1991; Pieken et al., 1991; Williams et al., 1992; Fu & McLaughlin, 1992). The role of the exocyclic amino groups of the conserved purines in the hammerhead domain has been examined by replacement of single adenosine residues by nebularine or single guanosine residues by inosine (Odai et al., 1990; Fu & McLaughlin, 1992; Slim & Gait, 1992). The substitution of 2′-fluoro, 2′-amino, or 2′-*O*-methyl derivatives into ribozyme complexes (Koizumi et al., 1989; Olsen et al., 1991; Williams et al., 1992), has, in some cases, not dramatically altered cleavage activity. These latter derivatives also provide some increase in the stability of the ribozyme to intracellular nucleases (Pieken et al., 1991). Studies employing the (R_p)- and (Sp)-phosphorothioate diastereomers at the cleavage site suggest that the Mg^{2+} (Mn^{2+}) cofactor is bound to the *pro-R* oxygen in the unmodified complex and that transesterification occurs by an in-line mechanism (van Tol et al., 1990; Koizumi & Otsuka, 1991; Slim & Gait, 1991). Three other specific phosphodiesters within the conserved central core sequence appear to be necessary for efficient folding of the complex (Buzayan et al., 1990; Ruffner & Uhlenbeck, 1990).

The present work focuses on the role of the N^7-nitrogens of the five conserved adenosine residues and their importance in the self-cleavage reaction. We have incorporated single residues of 7-deazaadenosine (tubercidin) into each conserved site normally occupied by adenosine. We report here the syntheses of the appropriately protected 7-deazaadenosine (tubercidin) phosphoramidite derivative, the construction of the "deletion-modified" ribozymes and substrates, and the kinetic characterization of these 7-deazaadenosine-substituted ribozyme complexes. We also propose a preliminary model for the binding of a partially hydrated magnesium cofactor to five critical functional groups in the tetrameric sequence $G_{10}A_{11}U_{12}G_{13}$.

EXPERIMENTAL PROCEDURES

Materials

Tubercidin (7-deazaadenosine, c^7A) is a product of Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography (TLC) was performed on 5 × 10 cm silica gel 60 F_{254} glass-backed plates (E. Merck, Darmstadt, Germany). The compounds were visualized by UV light or by spraying with 10% sulfuric acid followed by heating. The following solvents were used to develop the TLC plates: solvent A, dichloromethane/methanol (90:10); solvent B, dichloromethane/methanol (95:5); solvent C, hexane/dichloromethane (50:50); solvent D, hexane/ethyl acetate (50:50); solvent E, dichloromethane/ethyl acetate/triethylamine (49.5:49.5:1); solvent F, dichloromethane/petroleum ether/triethylamine (33:66:1). Silica gel 60 (particle size less than 0.063 mm; E. Merck, Darmstadt, Germany) was used for flash chromatography. The four common nucleoside phosphoramidites were purchased from Millipore Corp. (Milford, MA), and the wide-pore silica support is a product of BioGenex Laboratories (San Ramon, CA). Oligonucleotides were synthesized using nucleoside phosphoramidite derivatives and an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC) was carried out on an ODS-Hypersil column (0.46 × 25 cm, Shandon Southern, England), using a Beckman HPLC system. Fast-performance liquid chromatography (FPLC) was carried out on a Mono Q column (0.5 × 5 cm), using a Pharmacia FPLC system. ^1H NMR spectra were obtained at 300 or 500 MHz on a Varian XL-300 or -500 multinuclear spectrometer. ^{31}P NMR spectra were obtained at 121 MHz on the varian XL-300. Absorption spectra were recorded by a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. S1 nuclease is a product of United States Biochemical Corp. (Cleveland, OH). Calf intestinal alkaline phosphatase and snake venom phosphodiesterase were obtained from Boehringer (Mannheim, Germany).

Methods

Synthesis of N^6-Benzoyltubercidin (N^6-Benzoyl-7-deazaadenosine) (**1**). Protection of the exocyclic amino group of tubercidin as the benzamide derivative was accomplished using the general procedures described previously (Ti et al., 1982; Sung & Narang, 1982; McLaughlin et al., 1985). Tubercidin (1.0 g, 3.8 mmol, 1 equiv) was coevaporated twice from pyridine and was then dissolved in 40 mL of dry pyridine. To this solution, protected from moisture and cooled in an ice bath, was added 2.5 mL (~5 equiv) of trimethylchlorosilane dropwise. After the reaction mixture was stirred for 30 min, 2.3 mL (~5 equiv) of benzoyl chloride was added dropwise. The reaction mixture was removed from the ice bath and stirred for 2 h at ambient temperature. It was then cooled in an ice bath, and 7.5 mL of cold water was added, followed after 15 min by 7.5 mL of concentrated aqueous ammonia, to give a solution approximately 2 M in ammonia. After 30–60 min the mixture was concentrated to an oil by rotary evaporation. The residue was dissolved in 10 mL of methanol and added to 5 g of silica gel. After mixing well, the solvent was removed, and the dry residue was added to the top of a column containing 30 g of silica gel. The product was eluted with a dichloromethane/methanol gradient. Yield: 1.3 g (90%) as a foam. R_f(solvent a): 0.38. UV (methanol): λ_{max} = 199, 220, 274 nm; λ_{min} = 210, 257 nm. ^1H NMR (DMSO-d_6 + trace of D_2O): δ = 3.55 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.90 (m, 1 H, $H_{4'}$), 4.10 (m, 1 H, $H_{3'}$), 4.40 (dd, 1 H, $H_{2'}$), 6.23 (d, 1 H, $H_{1'}$, J = 6.0 Hz), 6.65 (d, 1 H, H_7), 7.40–8.20 (m, 6 H, H_8, Ar-H), 8.60 (s, 1 H, H_2) ppm.

Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-N^6-benzoyltubercidin (**2**). Compound **1** (0.80 g, 2.2 mmol, 1 equiv) was coevaporated twice from pyridine and was then dissolved in 15 mL of dry pyridine. The solution was cooled in an ice bath, and 4,4′-dimethoxytrityl chloride (0.90 g, 2.6 mmol, 1.2 equiv, dissolved in ~1 mL of dry pyridine) was added in portions over an 8-h period. The reaction mixture was stirred overnight with cooling in an ice bath. TLC analysis (solvent B) indicated that about 50% of starting material had reacted. Increasing the reaction time or increasing the quantity of 4,4′-dimethoxytrityl chloride did not enhance the yield of this step, the incubation of the reaction at room temperature induced the formation of a significant amount of the bis-DMT derivative. The reaction was stopped by cooling in an ice bath and by adding 2 equiv of imidazole followed by 1 mL

of methanol. The solvent was removed in vacuo to give a yellow oil. The resulting residue was coevaporated twice with toluene (20 mL) and was dissolved in 10 mL of methanol. This solution was mixed with 5 g of silica gel and the solvent removed. The silica gel mixture was added to the top of a column of 30 g of silica gel, and the product and the remaining starting material were isolated using a dichloromethane/methanol gradient. The yield of reaction was about 25–30%. The recovered starting material was tritylated again following the same procedure. Usually after three cycles of tritylation, the final yields of **2** ranged from 40% to 50%. R_f (solvent B): 0.28. UV (methanol): λ_{max} = 200, 270, 278 nm; λ_{min} = 257, 274, nm. ^{1}H NMR ($CDCl_3$ + trace of D_2O): δ = 3.25–3.60 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.80 (s, 6 H, OCH_3), 4.40 (m, 2 H, $H_{3'}$, $H_{4'}$), 4.63 (dd, 1 H, $H_{2'}$), 6.20 (d, 1 H, $H_{1'}$, J = 5.9 Hz), 6.70–8.20 (m, 20 H, H_7, H_8, Ar-H), 8.50 (s, 1 H, H_2) ppm.

Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N⁶-benzoyltubercidin (**3**). Synthesis of 2′-*t*-BDMS derivative was performed using essentially standard procedures (Ogilvie et al., 1979, 1980; Sung & Narang, 1982). Compound **2** (0.45 g, 0.67 mmol, 1 equiv) in pyridine solution (10 mL) was treated with *tert*-butyldimethylchlorosilane (0.20 g, 1.34 mmol, 2 equiv) and imidazole (0.14 g, 2.0 mmol, 3 equiv) while being cooled in an ice bath. After 15 min, the reaction mixture was removed from the ice bath and stirred overnight at ambient temperature. TLC analysis (solvent D) indicated the complete disappearance of starting material. The reaction was diluted with 40 mL of dichloromethane and then stopped with water (40 mL). The organic phase was separated, and the aqueous phase was extracted with another 20 mL of dichloromethane. The organic phases were combined, the solvents were removed, and the residue was coevaporated three times from toluene to yield an oil. This oil was dissolved in solvent C and chromatographed on a column of silica gel (20 g). The column was eluted with hexane/ethyl acetate in a stepwise gradient. Separation of the 2′- and 3′-silylated isomers was monitored by TLC in solvent D. Fractions containing the 2′,3′-bis-*t*-BDMS derivative were obtained first. A second fraction containing the 2′-silylated isomer was then collected. The last fraction containing mainly the 3′-silylated isomer was contaminated with some of the 2′ derivative. The last fraction was treated with couple drops of triethylamine in methanol and stirred overnight at ambient temperature to give an equilibrium mixture of the 2′ and 3′ isomers (Sung & Narang, 1982). In some cases, this isomeric mixture was resolved using preparative TLC plates in solvent D instead of short-column chromatography. The identification and the isomeric purity of 2′ and 3′ derivatives were confirmed by 1D and 2D NMR. Yield of 2′-*t*-BDMS derivative: 311 mg (59%). R_f (solvent D): 0.78 (2′,3′-bis-*t*-BDMS), 0.50 (2′-*t*-BDMS), 0.39 (3′-*t*-BDMS). UV (methanol): λ_{max} = 199, 220, 271, 278 nm; λ_{min} = 213, 258, 275 nm. ^{1}H NMR ($CDCl_3$ + trace of D_2O): δ = 0.0 (CH_3Si), 0.85 (s, 9 H, *t*-Bu), 3.30–3.60 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.79 (s, 6 H, OCH_3), 4.25 (m, 1 H, $H_{4'}$), 4.35 (m, 1 H, $H_{3'}$), 4.75 (dd, 1 H, $H_{2'}$), 6.42 (d, 1 H, $H_{1'}$, J = 6.0 Hz), 6.80–8.10 (m, 20 H, H_7, H_8, Ar-H), 8.52 (s, 1 H, H_2) ppm.

A comparison of the carbohydrate resonances for the 2′-*t*-BDMS and 3′-*t*-BDMS derivatives is as follows:

isomer	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$
2′-*t*-BDMS	6.42	4.75	4.35	4.25	3.30–3.60
3′-*t*-BDMS	6.35	4.55	4.55	4.15	3.25–3.55

Synthesis of 5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-3′-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphino]-N⁶-benzoyltubercidin (**4**). This reaction was performed essentially as described for the common nucleosides (Scaringe et al., 1990). Anhydrous compound **3** (0.18 g, 0.23 mmol, 1 equiv) was suspended in 1.2 mL of dichloromethane (dried over molecular sieves). Collidine was added until the compound dissolved (approximately 0.4 mL, 12 equiv). The reaction mixture was placed in an ice bath, and *N*-methylimidazole (0.12 mmol, 0.50 equiv) was added followed by β-cyanoethyl *N,N*-diisopropylphosphonamidic chloride (1.2 mmol, 5 equiv). After the mixture was stirred at room temperature for 1 h, TLC analysis in solvent E indicated that the reaction was complete. The reaction mixture was cooled, and 0.1 mL of collidine was added, followed by slow addition of 0.1 mL of methanol to destroy excess phosphitylating reagent. The solvent was removed in vacuo to give a yellow oil. The resulting residue was coevaporated from toluene (6 × 5 mL). The residue was then dissolved in 2 mL of solvent F and purified by chromatography on 20 g of silica gel packed with petroleum ether (1% triethylamine) and using a gradient of dichloromethane. The fractions containing product were evaporated to dryness, dissolved in a small amount of dichloromethane, and precipitated into 80 mL of petroleum ether. Yields varied in this reaction. In the described example 180 mg of **3** could be converted to 200 mg of **4** (88%). R_f (solvent E): 0.80, 0.83. ^{31}P NMR ($CDCl_3$): δ = 149.0, 150.6 ppm. ^{1}H NMR ($CDCl_3$): δ = 6.39 (d, $H_{1'}$), 6.45 (d, $H_{1'}$) ppm.

Oligonucleotide Synthesis. The oligonucleotides were synthesized from 1 μmol of bound nucleoside on wide-pore silica supports using phosphoramidite chemistry (Matteucci & Caruthers, 1981; Usman et al., 1989; Wu et al., 1989) and an Applied Biosystems 381A DNA synthesizer. After assembly of each sequence, the glass beads were suspended in 4 mL of concentrated ammonium hydroxide/ethanol (3:1) for 6 h at 50 °C (Chou et al., 1989). The glass beads were removed, the ammonia and ethanol were evaporated to dryness, and the residue was evaporated from anhydrous pyridine (3×) and toluene (1×). To the residue was added 2 mL of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran, and the reaction was protected from light and shaken for 16 h at ambient temperature. The crude mixture of oligonucleotides was desalted (Sephadex G-10), reduced in volume by lyophilization, and then purified by ion-exchange chromatography using FPLC and a 0.5 × 5 cm Mono Q column, with a flow rate of 1.5 mL/min in 5 mM sodium cacodylate (pH 6.0) and a gradient of sodium chloride. With a 0–0.45 M gradient of sodium chloride over 30 mL followed by 0.45–0.55 M over 60 mL, the 19-mers typically eluted in the range of 40–47 mL. With a linear gradient of 1 M sodium chloride over 52.5 mL, the 24-mers eluted in the range of 30–40 mL. After isolation, the fragments were desalted (Sephadex G-10) and lyophilized to dryness. HPLC purification followed in some cases, employing reversed-phase HPLC on an ODS-Hypersil column (0.46 × 25 cm) in 20 mM sodium phosphate (pH 5.5) and with a gradient of methanol (0–70% in 60 min). Small portions of the chromatographically purified oligonucleotides were further purified by gel electrophoresis in 20% polyacrylamide/7 M urea gels. In each case, the product band was visualized by UV shadowing, excised, and extracted with 0.1 M ammonium acetate. The resulting oligomers were desalted with a C_{18} Sep-Pak cartridge (Waters) and lyophilized to dryness.

Nucleoside Analyses. Nucleotide (or nucleoside) composition was determined after S1 nuclease (or S1 nuclease and calf intestinal alkaline phosphatase) hydrolysis. Snake venom

phosphodiesterase/calf intestinal alkaline phosphatase hydrolysis was also performed in some cases to confirm nucleoside composition.

A 10-μL reaction mixture containing 0.5 A_{260} unit of oligomer in 200 mM sodium chloride/5 mM $MgCl_2$/0.1 mM $ZnSO_4$/25 mM sodium acetate, pH 5.5, was incubated for 5 min at room temperature with 267 units of S1 nuclease. A 3-μL aliquot was analyzed by HPLC using a 0.46 × 25 cm column of ODS-Hypersil in 20 mM potassium phosphate, pH 5.5, and a gradient of 0–35% methanol (60 min). For nucleoside analyses, 5 μL of 0.1 M Tris-HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase were added to the remaining 7 μL of reaction mixture. Following incubation for 60 min at ambient temperature, a 5-μL aliquot was analyzed by HPLC as described above. Under the S1 digestion conditions, the following retention times were observed (260 nm): 2.2 min (Cp), 2.4 min (Up), 3.1 min (Gp), 4.5 min (C^7Ap), 5.8 min (Ap), and 12.2 min (G). After treatment with bacterial alkaline phosphatase the retention times for the nucleosides were 4.2 min (C), 5.3 min (U), 12.2 min (G), 17.3 min (c^7A), and 19.0 min (A). In some cases, a 14-μL reaction mixture containing 0.3 A_{260} unit of oligomer in 100 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 3 units of snake venom phosphodiesterase, and 2 units of calf intestinal alkaline phosphatase was incubated at 55 °C for 2 h and then at 37 °C overnight. A 7-μL aliquot was analyzed by HPLC as described above.

T_m Values. T_m values were obtained in 10 mM sodium phosphate (pH 7.0) and 1 M sodium chloride at duplex concentrations in the low micromolar range. The heating rate for the melting experiments was 0.5 °C/min. Absorbance values were measured with a Perkin-Elmer Lambda 3B UV/visible spectrophotometer equipped with a digital temperature control. The solution temperatures were measured directly with a thermister probe (OMEGA Engineering, Stanford, CT). Absorbance and temperature data were collected after analog to digital conversion (DT-2800; Data Translation, Marlboro, MA) using an IBM-XT computer and the ASYST (version 1.53) scientific software package (MacMillian Software, New York, NY). T_m values were determined from first- and second-order derivatives of the absorbance vs temperature plots.

Radioisotopic Labeling. Each 24-mer was 5′-end-labeled with [γ-^{32}P]ATP as follows: A 100-μL reaction mixture containing 2 A_{260} units of 24-mer (~0.1 mM), 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM Na_2EDTA, 0.1 mM ATP, 300–600 μCi of [γ-^{32}P]ATP, and 20 units of T4 polynucleotide kinase was incubated for 60 min at 37 °C. The product was isolated by absorption on a C_{18} Sep-Pak cartridge. The cartridge was washed with water and then with 40–50% aqueous methanol to elute the product. The labeled 24-mer was repurified by electrophoresis in a 20% polyacrylamide/7 M urea gel. The product band was excised, extracted with 0.1 M ammonium acetate, pH 7.0, and desalted with a C_{18} Sep-Pak cartridge. The specific activity of the 24-mer was typically 0.01 μCi/pmol.

Stoichiometric Cleavage Analysis. Two 50-μL solutions containing either 0.6 μM ribozyme or 0.4 μM substrate in 50 mM Tris-HCl (pH 8.0) with 10 mM $MgCl_2$ were each heated to 95 °C for 1 min and cooled at 37 °C for 15 min. The reaction was initiated by mixing the two solutions. Aliquots of 10 μL were withdrawn, and the reaction was quenched by the addition of an equal volume of 50 mM Na_2EDTA/7 M urea/10% glycerol/0.05% xylene cyanol/0.05% bromophenol blue. The extents of cleavage were analyzed by electrophoresis in 20% polyacrylamide/1% bis(acrylamide)/7 M urea gels (14 × 16 cm) in 89 mM Tris-borate buffer and 2 mM Na_2-EDTA, pH 8.0. After autoradiography, the substrate and product bands were excised and lyophilized to dryness, and the radioactivity was determined by scintillation counting. The logarithm of the unreacted fraction was plotted against time, and the data points were fitted using a linear least squares analysis. The cleavage half-lives ($t_{1/2}$) were used to obtain first-order rate constants ($k = 0.693/t_{1/2}$).

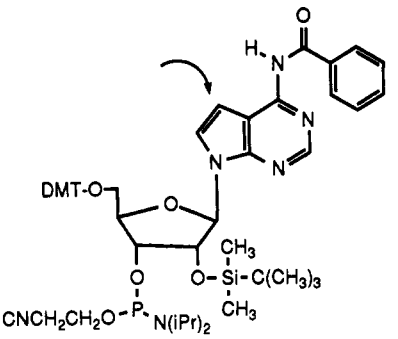
Catalytic Cleavage Analysis. A 10-μL solution of the ribozyme and a 30-μL solution of the radiolabeled substrate RNAs in 10 mM $MgCl_2$/50 mM Tris-HCl (pH 8.0) were each heated separately to 95 °C for 1 min and cooled to 55 °C for 15 min. The reaction was initiated by mixing the two solutions. The ribozyme concentration in these reactions was 0.1 μM (native and c^7A_{14} ribozyme) or 0.2 μM for the catalytically less efficient ribozyme (c^7A_{11}). Four to eight substrate concentrations were used varying from 0.5 to 12 μM depending on the individual sequence. Aliquots of 4 μL were taken from the reaction mixture at various times, quenched, and analyzed as described above. Values of up to 15% cleavage were used in the calculation of the kinetic parameters. K_M and V_{max} values were obtained from linear Lineweaver–Burk and Eadie–Hofstee plots.

Molecular Modeling. The structure of the $G_{10}A_{11}U_{12}G_{13}$–Mg^{2+} complex was optimized using the CAChe Molecular Mechanics application (version 2.8), a product of Tektronix. The molecular mechanics application employs an MM2 force field (Allinger, 1977) and augments it with a generalized force field for interactions not parameterized by MM2.

RESULTS

The imidazole nitrogens of purine nucleosides are known to take part in non-Watson–Crick hydrogen-bonding interactions [see Saenger (1984a)] as well as participate in direct metal chelation [see Pezzano and Podo (1980)]. In the structure of yeast $tRNA^{Phe}$, derived by X-ray crystal analysis, the N^7-nitrogen of the Y base assists in positioning a hydrated magnesium in the anticodon loop by functioning as a hydrogen bond acceptor for one of the coordinated water molecules (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). In similar fashion, the N^7-nitrogen of G_{20} in the D-loop interacts with a seond magnesium hydrate (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). Some of the N^7-nitrogens of the five conserved adenosines in the hammerhead ribozyme complex could be involved in similar interactions that would permit the structural organization of the catalytically competent complex involving the ribozyme and substrate molecule and/or assist in the effective positioning of the magnesium (manganese) cofactor to assist in catalyzing the transesterification reaction. To examine the importance of this functional group at the five conserved adenosine residues, we have prepared the corresponding five "deletion-modified" complexes; in each complex one of the adenosine N^7-nitrogens has been excised from either the ribozyme or substrate sequence.

Synthesis of the Nucleoside Phosphoramidite Building Block. In order to prepare the desired complexes by chemical syntheses, we began by converting tubercidin (c^7A) into the appropriately protected phosphoramidite derivative **4**:



4

Preparation of the *N*-benzoyl derivative **1**, the 5′-DMT compound **2**, and the corresponding 2′-*t*-BDMS derivative **3** was accomplished using essentially standard procedures (Ti et al., 1980; Sung & Narang, 1982; McLaughlin et al., 1985). After column purification of the 2′-silyl derivative **3**, it was converted to the corresponding phosphoramidite as described (Scaringe et al., 1990). ^{31}P NMR analysis of the phosphoramidite product indicated the presence of a single pair of phosphorus diastereomers.

Oligonucleotide Syntheses. To examine the importance of specific adenosine N^7-nitrogens in ribozyme-catalyzed RNA cleavage, we prepared two modified ribozymes and three modified substrates. In each case a single adenosine N^7-nitrogen was deleted by the replacement of the adenosine residue by a single 7-deazaadenosine (c^7A) residue. The native ribozyme/substrate complex formed is identical to that described by Uhlenbeck (1987) and Ruffner et al. (1990), and each modified complex lacks a single adenosine N^7-nitrogen at a preselected site. With this structure, the substrate is a 24-mer that is cleaved into an 18-mer and a 6-mer, and the ribozyme is a 19-mer (see Figure 1). The oligonucleotides were prepared by solid-phase phosphite triester synthesis on a wide-pore silica support (Matteucci & Caruthers, 1981; Usman et al., 1989; Wu et al., 1989). Incorporation of the c^7A phosphoramidite into the growing oligonucleotide chain, after a reaction time of 60 min, occurred with yields comparable to those obtained for the "normal" monomers, based upon the color of the liberated DMT cation. From an initial 1.0 μmol of 3′-bound nucleoside (C) we were able to purify approximately 1–1.5 mg (30–40 A_{260} units) of each strand.

Purification was achieved primarily by anion-exchange chromatography, but a second isolation using reversed-phase chromatography was required for some sequences. For example, after FPLC isolation of the c^7A ribozyme, nucleotide analysis (after treatment with S1 nuclease) generated the expected five peaks for Cp, Up, Gp, c^7Ap, Ap, and G (the G residue is the 5′-terminal nucleoside), but additional peaks eluted from the column (see ?, Figure 2). The hydrolysis mixture obtained after treatment with snake venom phosphodiesterase also produced unidentified peaks with relatively long retention times (data not shown). The most likely explanation for these peaks is the presence of some nucleoside residues that had not been fully deprotected. The presence of residual benzoyl or silyl protecting groups on a 19-mer or 24-mer sequence should result in a more hydrophobic molecule (relative to the fully deprotected sequence). In these cases, the sequences were repurified by reversed-phase HPLC. In some cases the sequences were further purified by extraction from denaturing polyacrylamide gels. Nucleotide analyses of the FPLC/HPLC isolated fragments no longer exhibited any unidentified residues. The lack of any unidentified

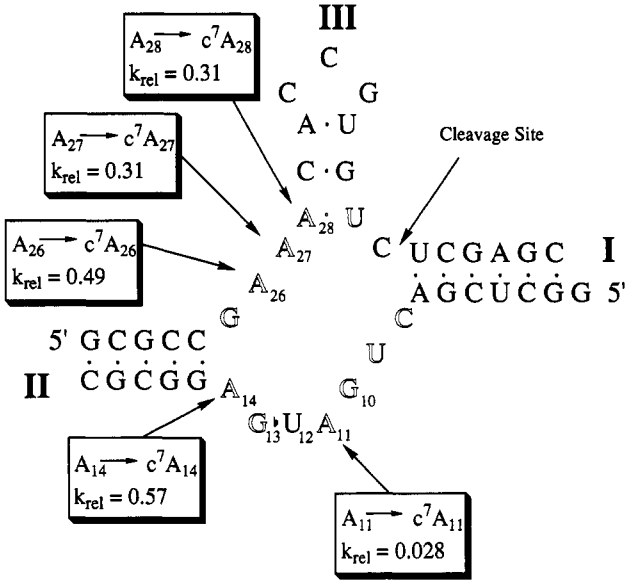


FIGURE 1: Structure of the hammerhead RNA complex illustrating the sites for the c^7A substitutions and the relative cleavage efficiencies under stoichiometric conditions ("outlined" letters mark the locations of the 11 conserved nucleoside residues).

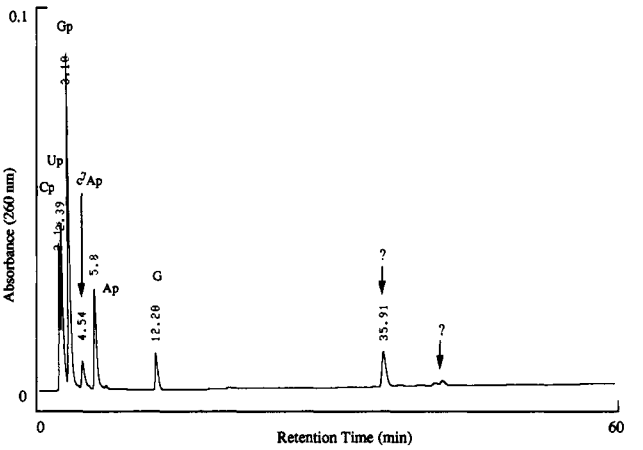


FIGURE 2: S1 nuclease digest of the partially (see text) purified c^7A_{11} ribozyme sequence. After treatment of the c^7A-containing 19-mer with S1 nuclease, the resulting hydrolysate was analyzed on a reversed-phase column using 20 mM potassium phosphate, pH 5.5, and a gradient of methanol.

residues after treatment with S1 nuclease also confirmed the fidelity of the 3′–5′ internucleotide linkages (2′–5′ linkages are not substrates for S1 nuclease). Quantitative analysis of the base composition agreed with that expected for each of the sequences prepared.

Stoichiometric Cleavage Analyses. To analyze relative cleavage activity under single turnover conditions, we prepared solutions that contained the ribozyme sequence in approximately a 50% excess with respect to the substrate sequence. These reactions were performed at 37 °C and pH 8.0. The assay temperature was 15–20 °C below the measured T_m values for the complexes (in the absence of Mg^{2+}) (see Table I). The native complex exhibited a half-life of 13 min with a first-order rate constant of 0.053 min^{-1}. Although the precision of the data for the cleavage rates was quite good for identical batches of ribozyme and substrate, we have observed that the rate constants will vary by as much as a factor of 2 for different batches of purified sequences. Similar variations have been noted for other ribozyme sequences prepared by enzymatic syntheses (Herschlag & Cech, 1990). The values reported in this study were obtained from a single batch of native ribozyme

Table I: Single Turnover Kinetic Parameters for Native and c^7A-Substituted Hammerhead Ribozyme Complexes[a]

ribozyme	substrate	$t_{1/2}$ (min)	k (min^{-1})	k_{rel}[b]	T_m (°C)[c]
native	native	13	0.053	1.0	56.1
c^7A_{11}	native	470	0.0015	0.028	54.8
c^7A_{14}	native	23	0.031	0.57	54.7
native	c^7A_{26}	27	0.027	0.49	54.2
native	c^7A_{27}	42	0.017	0.31	54.3
native	c^7A_{28}	41	0.017	0.31	51.7

[a] Reactions were conducted in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 μM substrate, and 0.3 μM ribozyme at 37 °C. Data precision is approximately ±10%. [b] k_{rel}: relative cleavage activity. [c] T_m values were recorded in the absence of Mg^{2+}.

Table II: Steady-State Kinetic Parameters for Native and c^7A-Substituted Hammerhead Ribozyme Complexes[a]

ribozyme	substrate	K_M (μM)	k_{cat} (min^{-1})	$10^3 k_{cat}/K_M$
native	native	1.4	0.78	574
c^7A_{11}[b]	native	2.7	0.0075	2.7
c^7A_{14}	native	2.4	0.078	32
native	c^7A_{26}	3.2	0.19	59
native	c^7A_{27}	3.2	0.054	17
native	c^7A_{28}	2.6	0.095	36

[a] Reactions were conducted in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 0.1 μM ribozyme at various substrate concentrations at 55 °C. Data precision is approximately ±10%. [b] Ribozyme concentration was 0.2 μM.

and substrate. Two conserved adenosine residues, A_{11} and A_{14}, in the ribozyme sequence were replaced by 7-deazaadenosine. The c^7A_{11} complex resulted in a dramatic decrease in rate ($t_{1/2}$ = 470 min) with a relative cleavage activity that was some 35-fold lower than that observed for the native complex (Table I, Figure 1). By comparison, the c^7A_{14} complex exhibited a half-life only slightly longer than that of the native complex and a relative cleavage activity within 2-fold of that observed with the native ribozyme/substrate complex. All three of the complexes formed by replacing either A_{26}, A_{27}, or A_{28} in the substrate sequence with c^7A exhibited half-lives and relative cleavage activities that were within 3-fold of that observed for the native complex (Table I, Figure 1).

Steady-State Cleavage Analyses. In our hands, it was necessary to examine the steady-state kinetics at a temperature near the T_m of the complex in order to obtain reproducible results. In other studies (Uhlenbeck, 1987; Ruffner et al., 1990), steady-state kinetic parameters for similar native and modified complexes have been obtained at 55 °C, and we have employed this temperature in the present study. In each assay, we have used a single batch of native and/or modified sequence to obtain the kinetic parameters.

The native complex exhibited a K_M of 1.4 μM and a k_{cat} of 0.78 min^{-1} (Table II), and these parameters are similar to those reported earlier by Uhlenbeck (1987) as well as those described in our previous publication (Fu & McLaughlin, 1992). The c^7A_{11} complex was characterized by a K_M value that was only slightly increased relative to the native complex (2.7 μM) but a k_{cat} that was reduced by 2 orders of magnitude (0.0075 min^{-1}). The remaining modified complexes, c^7A_{14}, c^7A_{26}, c^7A_{27}, and c^7A_{28}, all exhibited K_M values that were similar to that of the native complex and k_{cat} values that were reduced by 4-fold (c^7A_{26}), 8-fold (c^7A_{28}), 10-fold (c^7A_{14}), and 16-fold (c^7A_{27}) (see Table II).

The final column of Table II compares the overall cleavage efficiency of the ribozyme complexes as expressed by k_{cat}/K_M. The c^7A_{11} complex resulted in a 200-fold reduction in k_{cat}/K_M, primarily as a function of a reduced turnover number (k_{cat}). By comparison, the remaining modified complexes had k_{cat}/K_M values that were reduced 10-fold (c^7A_{26}) to 34-fold (c^7A_{27}).

DISCUSSION

Although there are now a number of cases in which RNA functions in a catalytic manner, at present, other than some preliminary NMR studies (Heus et al., 1990; Heus & Pardi, 1991), there is little detailed structural information available describing a catalytically competent complex. The hammerhead RNAs appear to require three, essentially native, helical regions (see Figure 1), but the structure of the ostensibly single-stranded regions, C_8–A_{14} and G_{25}–A_{27}, remains undefined. The substitution of base analogues into nucleic acid sequences is a powerful technique that can be used for the identification of critical functional groups, providing that conservative modifications are introduced into the analogue. With the identification of a series of critical functional groups, it should then be possible to describe a structure that is based upon the specific orientation of these functional groups in a manner that accounts for structural or catalytic properties of the complex. In the present work we have synthesized the phosphoramidite building block of 7-deazaadenosine for incorporation into RNA sequences. This analogue (c^7A), like the corresponding 2′-deoxy derivative [see, for example, Mazzarelli et al. (1992)], permits the site-specific deletion of the purine N^7-nitrogen. Five deletion-modified complexes were prepared in the present study. Four of the complexes (c^7A_{14}, c^7A_{26}, c^7A_{27}, and c^7A_{28}) exhibited kinetic parameters that suggest cleavage efficiencies (as expressed by k_{cat}/K_M values) not drastically different from that of the native sequence. However, the c^7A_{11} complex exhibited a k_{cat}/K_M at least 200-fold lower than that obtained for the native complex, and this observation suggests that the presence of the N^7-nitrogen at A_{11} is critical for efficient cleavage by the ribozyme complex.

In addition to the N^7-nitrogen at A_{11} identified in the present work, another four critical functional groups have been identified in a localized portion of the ribozyme sequence involving the tetramer $G_{10}A_{11}U_{12}G_{13}$. The other four critical functional groups present in this tetrameric sequence include the amino group (Odai et al., 1990; Fu & McLaughlin, 1992) and the 2′-hydroxyl (Perreault et al., 1990, 1991; Williams et al., 1992; Fu & McLaughlin, 1992) of G_{10}, the 2′-hydroxyl of G_{13} (Williams et al., 1992; Fu & McLaughlin, 1992),[1] and the phosphodiester residue 3′ to G_{13} (Buzayan et al., 1990; Ruffner & Uhlenbeck, 1990). The importance of these functional groups in binding or catalytic activity may reflect their involvement in specific interactions. For example, individual functional groups may be involved in specific interbase hydrogen-bonding interactions, direct chelation to the magnesium, or interligand interactions involving the water

[1] There is some discrepancy concerning the relative importance of the 2′-hydroxyls at G_{13} and A_{14}. Perreault et al. (1991) have reported that the 2′-hydroxyl at A_{14} is critical for cleavage while the deletion of the 2′-hydroxyl at G_{13} has no significant effect on catalysis. The work of Olsen et al. (1991) and Williams et al. (1992) as well as our own (Fu & McLaughlin, 1992) suggests a reversal in the relative importance of these two hydroxyls, at least in the described complex. The loss of the hydroxyl from G_{13} results in a catalytic activity that is reduced by a factor of at least 150 (Williams et al., 1992) in one complex or by a factor of 300 in the complex identical to the one described in the present study (Fu & McLaughlin, 1992). The results of Olsen et al. (1991) and our own (Fu & McLaughlin, 1992) did not indicate significant changes in activity when the 2′-hydroxyl is deleted from A_{14}.

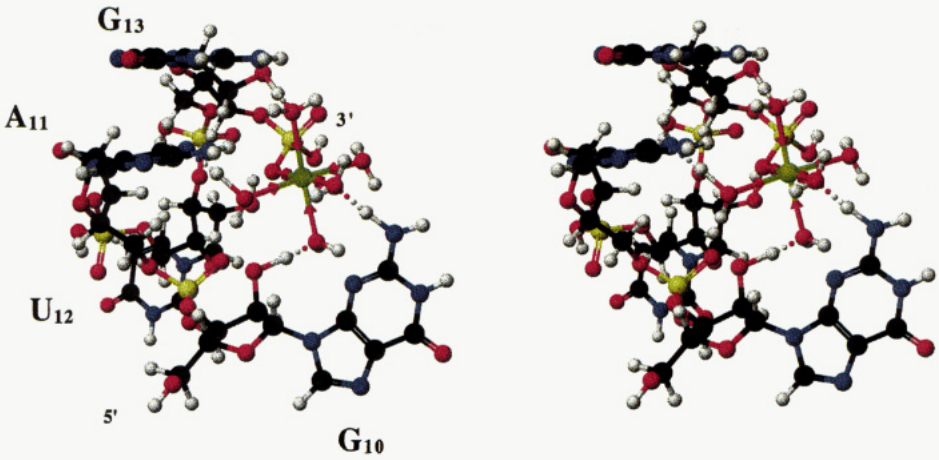


FIGURE 3: Stereoview of the proposed metal cofactor binding site involving the tetrameric sequence $G_{10}A_{11}U_{12}G_{13}$ and a tetrahydrated magnesium (green).

molecules of the inner hydration sphere of the metal cofactor. These interactions could be important both to maintain tertiary structure and to bind the magnesium cofactor.

The binding of magnesium to RNA sequences has been described in some detail, most notably in the crystal structure of yeast $tRNA^{Phe}$ (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). In the X-ray analysis of this molecule, four magnesium ions are present; one magnesium ion is located in the anticodon loop and a second has been identified in the dihydrouridine loop. They appear to assist in the organization of the largely single-stranded (loop) structure in these two areas of the molecule. In both cases, the magnesium is directly coordinated to a phosphodiester residue, and it binds to other sites in the loop structure through a network of hydrogen-bonding interactions involving specific base functional groups and the water molecules of the pentahydrated magnesium ion (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). Similar interligand interactions, which employ hydrogen bonds between base functional groups and the water molecules of a hydrated metal, have been suggested on the basis of a number of metal–nucleoside crystal structures [for examples, see Sletten and Ruud (1975), Kistenmacher et al. (1976), and Aoki (1976)] as well as on the basis of computational modeling (Mei et al., 1989). Using these general concepts, many reports have suggested that the magnesium ion(s) of the ribozyme/substrate complex could be coordinated to one or more phosphate residues, including the scissile phosphodiester, as well as make a series of interligand hydrogen bonds through coordinated water molecules [see, for example, Mei et al. (1989), Perreault et al. (1991), Dahm and Uhlenbeck (1991), and Koizumi and Otsuka (1991)].

We now suggest one possible model in which the five critical functional groups of the tetrameric $G_{10}A_{11}U_{12}G_{13}$ sequence orient themselves about a hydrated magnesium cofactor as illustrated in the partial ribozyme structure in Figure 3. We recognize that other models are possible in which some, or most, of these functional groups are involved in other types of hydrogen-binding interactions, but the possibility that many of the identified critical functional groups are involved in binding the magnesium cofactor was the assumption made for construction of the present model. This preliminary structure does not account for all known hammerhead ribozyme properties, but it is consistent with much of the reported data. There are a number of characteristics of this model that should be considered (Figure 3):

(i) The model permits the orientation of the five critical functional groups about a partially hydrated magnesium ion; the phosphodiester residue 3′ to G_{13} is chelated to the magnesium ion, and the remaining four functional groups partake in interligand hydrogen-bonding interactions similar to those observed for the binding of magnesium in the anticodon loop or dihydrouridine loop of $tRNA^{Ph3}$ (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). With respect to the interligand hydrogen-bonding interactions, the N^7-nitrogen of A_{11} functions as a hyrogen bond acceptor while the amino group (G_{10}) and two hydroxyl groups (G_{10} and G_{13}) are shown as hydrogen bond donors. In principle, the 2′-hydroxyls could also participate in direct magnesium chelation; however, with the present model, all five critical functional groups could not be effectively positioned about the metal cofactor if the two 2′-hydroxyls were located in the inner-sphere coordination complex.

As noted above, we have shown the two 2′-hydroxyls of G_{10} and G_{13} as hydrogen bond donors to the water molecules of the inner coordination sphere, but they could also function as hydrogen bond acceptors and result in a very similar structure. The involvement of the 2′-hydroxyls (or the guanine amino group) in water-mediated interactions is not unknown. A water-mediated U 2′-OH to G 2-NH_2 interaction has been observed in a recent crystal structure analysis of a mismatched RNA duplex (Holbrook et al., 1991). The importance of 2′-hydroxyls in other ribozyme recognition processes has also been described (Pyle & Cech, 1991; Bevilacqua & Turner, 1991).

The phosphodiester residue 3′ to G_{13} is shown with an unesterified phosphate oxygen as part of the inner-sphere coordination complex, but a slight rotation of this residue would permit a second structure in which an interligand hydrogen-bonding interaction is present between the phosphate residue and an inner-sphere water molecule. Hydrogen bonding between a metal-coordinated water molecule and the unesterified oxygen of a phosphate residue has been reported previously in nucleotide–metal ion complexes [see Aoki (1976)].

(ii) Three of the critical functional groups are present on two adjacent nucleoside residues, G_{10} and A_{11}. In order for the phosphodiester 3′ to G_{13} to form an inner-sphere chelate, for the 2′-hydroxyl of G_{13} to make an interligand hydrogen bond with the magnesium hydrate, and for both interactions to form without disrupting the interligand interactions involving the three critical functional groups on G_{10} and A_{11}, the U_{12} residue is displaced out away from the central core of the complex. In this position, U_{12} appears unable to participate in any obvious hydrogen-bonding or base-stacking

(hydrophobic) interactions. This position for the U_{12} residue (and the apparent absence of any critical interactions) is consistent with the observation that U_{12} can be replaced by C, G, or A with only minor changes in the efficiency of the cleavage reaction (Ruffner et al., 1990).

(iii) The unesterified phosphodiester residue 3′ to G_{13} can form an inner-sphere chelation complex (or, as noted above, an interligand hydrogen bond with a chelated water molecule). At present, it appears that either the *pro-S* or *pro-R* unesterified oxygen of the phosphodiester residue can form the proposed chelate, and this observation does not entirely account for the reported results. The (R_p)-phosphorothioate, when present 3′ to G_{13}, results in a significant reduction in catalytic efficiency (Ruffner & Uhlenbeck, 1990). Since that study employed enzymatically synthesized sequences, no data are presently available for the effects of the (S_p)-phosphorothioate derivative at this site. However, the conformation imposed upon this internucleotide linkage by other portions of the sequence (e.g., A_{14} and helix II) may ultimately dictate that only the *pro-R* phosphate oxygen can effectively interact with the magnesium cofactor.

(iv) With the displacement of U_{12} from the central core of the complex, the presence of the phosphate chelate (or interligand hydrogen bond) 3′ to G_{13}, and the presence of an interligand hydrogen bond between the 2′-hydroxyl of G_{13} and an inner-sphere water molecule, the two purine heterocycles of A_{11} and G_{13} are oriented such that some interbase stacking interactions could occur between them. The distance between the planes of the purine bases for A_{11} and G_{13} is approximately 3.4 Å. Nucleic acid structures have been described previously in which a single pyrimidine nucleoside residue is displaced away from a helix structure such that base-stacking interactions (and complementary hydrogen-bonding interactions) between two nonadjacent base residues result (Morden et al., 1983).

However, this orientation of the two purines and the resulting base-stacking interaction is not entirely consistent with all of the nucleoside substitution studies (Ruffner et al., 1990). Replacement of G_{13} by an adenosine residue results in an active complex, albeit with cleavage efficiency reduced by some 2 orders of magnitude (Ruffner et al., 1990). In our proposed model, the presence of an adenosine residue at position 13 (or, in fact, the presence of cytidine or uridine) should provide a 2′-hydroxyl and a 3′-phosphate diester for interaction with the magnesium hydrate. An adenosine substitution at this location should additionally provide similar stacking interactions with A_{11} (while the corresponding pyrimidine substitution might not). Nevertheless, replacement of G_{13} by the purines, adenosine or inosine, is much less deleterious to ribozyme activity than similar replacements by pyrimidines (Ruffner et al., 1990; Fu & McLaughlin, 1992). A simple stacking interaction between A_{11} and G_{13} does not completely explain the observations for the nucleoside substitution studies reported for A_{11}. Pyrimidine substitutions at position 11 have a drastic effect upon cleavage efficiency (Ruffner et al., 1990), and this observation can be explained by the present work which identifies the adenosine N^7-nitrogen as a critical functional group necessary for efficient catalysis. However, replacement of A_{11} by guanosine should also provide a purine N^7-nitrogen as well as appropriate base-stacking interactions; yet with this substitution, no cleavage activity is observed (Ruffner et al., 1990). Deletion of the exocyclic amino group of A_{11} (replacement by nebularine) does not drastically alter cleavage kinetics (Fu & McLaughlin, 1992). While the present model cannot account for a few of the base substitution results at A_{11} and G_{13}, the noted inconsistencies may simply reflect the presence of other critical interactions (e.g., interbase hydrogen bonding or additional interligand interactions) at these sequence locations involving functional groups that remain to be identified.

(v) Finally, four additional minor aspects concerning this model are noteworthy. (a) All four of the base residues are oriented in the generally preferred [see Saenger (1984b)] anti conformation relative to the corresponding ribose residues. (b) The phosphodiester residues in the complex remain evenly spaced with an average separation of the charged oxygens of ~5.5 Å. This spacing of the internucleotidic phosphodiesters is similar to that observed in duplex RNA structures [see Saenger (1984c)]. (c) The 3′-phosphate of G_{13} and the 5′-hydroxyl of G_{10} are located nearly on opposite sides of the complex. This orientation should permit the magnesium complex to form within the series of ostensibly single-stranded nucleoside residues from C_8 through A_{14} and allow helix I and helix II to provide some structural stabilization on either side of the complex (see Figure 1). (d) The remaining coordination site of the magnesium cofactor is quite open and available for additional interaction(s).

Although we have not attempted to model the entire hammerhead complex as described earlier (Mei et al., 1989), the remaining coordination site for the magnesium cofactor, as noted above, lies on an open face of the structure (in Figure 3, right side, this site is occupied by a water molecule). The open face of the complex would permit an additional chelation interaction, for example, with the scissile phosphodiester residue (Mei et al., 1989; Dahm & Uhlenbeck, 1991; Perreault et al., 1991) or with a water molecule that interacts with the scissile phosphodiester residue (Koizumi & Otsuka, 1991). Some preliminary molecular modeling studies indicate that the scissile phosphodiester could easily approach the magnesium cofactor to form such interactions. Direct coordination, or an interligand interaction, to the scissile phosphodiester would assist both in binding the substrate to the ribozyme and in providing an electrostatic or general acid/base cofactor to catalyze the cleavage reaction. With complexation of the magnesium hydrate to the scissile phosphodiester residue, the model complex has a total of six specific interactions between critical functional groups and the partially hydrated magnesium cofactor. Similar tRNA–magnesium complexes (in the anticodon loop and dihydrouridine loop) have a large network of hydrogen-bonding interactions involving the chelated water molecules (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). Each inner-sphere water molecule, in theory, can form as many as three separate hydrogen-bonding interactions (one accepting and two donating) [see Jeffery and Saenger (1991)]. Therefore, additional interligand interactions involving, for example, the critical 2′-hydroxyl of U_{36} (Yang et al., 1990) or the critical phosphodiesters 5′ to A_{28} and A_{29} (Buzayan et al., 1990; Ruffner & Uhlenbeck, 1990) are not excluded by the present model. The present model accounts for the binding of only a single magnesium at a single site, and ribozyme activity may be mediated by more than one metal cofactor (Koizumi & Otsuka, 1991). In any case, simple complexation of one or more of the magnesium cofactors within the secondary/tertiary structure of the ribozyme/substrate complex is unlikely to be sufficient for the observed catalysis in the absence of other effects. The magnesium hydrate complexed in the anticodon loop or dihydrouridine loop of $tRNA^{Phe}$ (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980) does not result in cleavage of the tRNA, even though one phosphodiester

oxygen is chelated to the magnesium ion. It is likely, as many have suggested, that a general acid and/or base is necessary for effective catalytic activity. One candidate for this function would be one of the coordinated water molecules as suggested by Koizumi and Otsuka (1991). A coordinated water molecule could function both as a general acid and as a general base (Dahm & Uhlenbeck, 1991). A second candidate is the amino group of G_{25} as suggested by Slim and Gait (1992); they reported that the replacement of G_{25} by inosine produced an essentially inactive complex. The amino group of a guanosine residue has been suggested to function as a general base in other ribozyme complexes (Chowrira et al., 1991). An inactive complex is the likely result for one that has lost the requisite general base.

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