Importance of Specific Guanosine N^7-Nitrogens and Purine Amino Groups for Efficient Cleavage by a Hammerhead Ribozyme†

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ABSTRACT: Seven modified hammerhead ribozyme/substrate complexes have been prepared in which individual purine nitrogens, the guanine N^7-, the guanine N^2-, or the adenine N^6-nitrogen, have been excised. The modified complexes were chemically synthesized with the substitution of a single 7-deazaguanosine (c^7G), inosine (I), or nebularine (purine riboside, P) base analogue as appropriate for residues G_5, G_8, G_{12}, A_{13}, A_{14}, or A_{15}. Two of the base analogues, c^7G_5 and c^7G_8, occur in a 19-mer ribozyme, while the remaining three residues are present in a 24-mer substrate. Under stoichiometric conditions, four of the complexes, G5c^{7}G, G8c^{7}G, G12c^{7}G, and A14P, are cleaved with relatively little change in rate when compared with the native complex. Two complexes, A13P and A15P, are cleaved some 6–8-fold slower than the native complex, while the G12I complex is reduced in rate by 50-fold. Steady-state kinetic analyses indicate that the cleavage efficiencies, as measured by k_{cat}/K_M values, for the G5c^{7}G, G8c^{7}G, and G12c^{7}G complexes are only marginally reduced relative to the native complex. The values for the A13P, A14P, and A15P complexes are reduced by 25-, 15-, and 60-fold, respectively. These reductions in cleavage efficiency are primarily a result of lower k_{cat} values. By comparison, the k_{cat}/K_M value for the G12I complex is decreased 450-fold relative to the native complex and is characterized by an 8-fold increase in K_M and a k_{cat} value that is reduced nearly 60-fold. These results indicate that the N^2-amino group of G_{12} in the hammerhead ribozyme/substrate complex is critical for efficient cleavage activity. The loss of the amino groups from A_{13}, A_{14}, and A_{15} impact the cleavage efficiency, but these complexes remain at least 30-fold more active than the G12I complex. The N^7-nitrogens of the conserved guanine residues do not appear to take part in any critical hydrogen-bonding/metal-ion-coordinating interactions or to have any other significant role in the observed catalytic transesterification reaction.

Self-cleavage reactions of RNAs have been observed in a number of systems [see Perotta and Been (1991), Cech et al. (1992), and Symons (1992)]. The hammerhead ribozymes (Forster & Symons, 1987; Uhlenbeck, 1987; Haseloff & Gerlach, 1988) are derived from a structure present in the genomes of several plant satellite RNAs, where it is believed that the cleavage reactions are an essential step in the replication pathway [for reviews, see Symons (1989) and Bruening (1989)]. The hammerhead 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 by the 2′-hydroxyl at the cleavage site 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). Although in nature these structures result from the folding of a single RNA molecule, synthetic complexes composed of two or even three fragments also exhibit cleavage activity (Haseloff & Gerlach, 1988; Koizumi et al., 1988; Jefferies & Symons, 1989; Koizumi et al., 1989). Recent kinetic studies have provided a detailed view of complex assembly and product dissociation processes (Fedor & Uhlenbeck, 1992). 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). One, two, or more metal cofactors may be necessary for activity (Koizumi & Otsuka, 1991).

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; Fedor & Uhlenbeck, 1990; Ruffner et al., 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. A number of functional group alterations within the ribozyme complex have been made by the incorporation of the appropriate nucleoside analogues; 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, 1992a). The role of some 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 of single guanosine residues by inosine (Odai et al., 1990; Fu & McLaughlin, 1992a; Slim & Gait, 1992). Replacement of the adenosine residues by 7-deazaadenosine has resulted in the location of a specific N^7-nitrogen critical for efficient cleavage by the complex (Fu & McLaughlin, 1992b). Additionally, the substitution of 2′-fluoro-, 2′-amino- or 2′-*O*-methylriboses into ribozyme complexes (Koizumi et al., 1989; Olsen et al., 1991; Williams et al., 1992) has been accomplished. These latter derivatives increase the stability of the ribozyme to intracellular nucleases (Pieken et al., 1991). Studies employing the R_p and S_p phosphorothioate diastereomers at the cleavage site

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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 three conserved guanosine residues and selected purine amino groups in the self-cleavage reaction. We have incorporated single residues of 7-deazaguanosine (c^7G) into each conserved site normally occupied by guanosine. The nucleoside analogue nebularine (P) has been incorporated into the A_{13}, A_{14}, and A_{15} sites, while G_{12} has been replaced by inosine (I). We report here the construction of the "deletion-modified" ribozymes and substrates and the kinetic characterization of the various analogue-containing ribozyme complexes.

EXPERIMENTAL PROCEDURES

Materials

The (4,4′-dimethoxytrityl)(β-cyanoethyl)nucleoside phosphoramidite of inosine was prepared by a procedure similar to that described elsewhere (Green et al., 1991). 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 Varian XL-300 or-500multinuclear spectrometers. ^{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. Nuclease S1 is a product of United States Biochemical Corporation (Cleveland, OH).

Methods

Chromatographic Solvent Systems. The solvent systems employed in this study were as follows: solvent A, dichloromethane:methanol, 90:10; solvent B, dichloromethane:methanol, 95:5; solvent C, dichloromethane:ethyl acetate:triethylamine, 49.5:49.5:1; solvent D, hexane:dichloromethane:triethylamine, 49.5:49.5:1; solvent E, dichloromethane:petroleum ether:triethylamine, 59.5:39.5:1; solvent F, dichloromethane:ethyl acetate:triethylamine, 79.5:19.5:1; solvent G, dichloromethane:petroleum ether:triethylamine, 33:66:1.

Nucleoside Syntheses. (A) 5′-O-(4,4′-Dimethoxytrityl)-nebularine (**1**). Nebularine (1 g, 4.0 mmol) was coevaporated twice from pyridine and was then dissolved in 100 mL of anhydrous pyridine. Dimethoxytrityl chloride (1.6 g, 4.8 mmol, 1.2 equiv) was added over 8 h (0.6 mmol per addition). After the mixture was stirred overnight, an additional 1.4 g of dimethoxytrityl chloride was added over a period of 8 h. The reaction mixture was stirred a total of 48 h at ambient temperature. TLC analysis (solvent A) indicated that the reaction was complete. Methanol (5 mL) was added to stop the reaction, and the solvent was removed *in vacuo* to give a yellow oil. The resulting residue was coevaporated twice from toluene (20 mL) and was redissolved in dichloromethane (50 mL). The organic solution was washed with 5% $NaHCO_3$ (50 mL) and with water (50 mL). The organic layer was dried, the solvent was removed, and the residue was purified by short-column chromatography on silica gel (30 g) using a dichloromethane/methanol gradient. Yield: 1.9 g (3.4 mmol), 84%. R_f(solvent A): 0.55. UV (methanol): λ_{max} = 205, 233, 263 nm; λ_{min} = 221, 255 nm. ^1H NMR ($CDCl_3$ + trace of D_2O): δ = 3.32–3.50 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.78 (s, 6 H, OCH_3), 4.42 (m, 1 H, $H_{4'}$), 4.80 (m, $H_{3'}$, HOD), 5.29 (m, 1 H, $H_{2'}$), 6.08 (d, 1 H, $H_{1'}$), 6.70–7.30 (m, 13 H, Ar-H), 8.19 (s, 1 H, H_6), 8.88 (s, 1 H, H_8), 9.20 (s, 1 H, H_2) ppm.

(B) 5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)nebularine (**2**). Compound **1** (1.8 g, 3.3 mmol) in anhydrous pyridine (20 mL) was treated with *tert*-butyldimethylchlorosilane (0.7 g, 4.7 mmol, 1.4 equiv) and imidazole (0.64 g, 9.4 mmol, 2.8 equiv) at ambient temperature. After 24 h, the reaction was stopped with water (30 mL) and dichloromethane (100 mL) was added. The aqueous layer was separated and washed with dichloromethane (20 mL). The combined organic layers were evaporated *in vacuo* to yield a gum, which was triturated once with petroleum ether (20 mL). The precipitate was then dissolved in dichloromethane and purified by short-column chromatography on silica gel (30 g), eluting with dichloromethane/methanol in a stepwise gradient. Three major fractions were collected: fraction 1 contained the 2′, 3′-disilylated derivative, fraction 2 contained the purest samples of the 2′-silylated isomer (0.74 mg), and fraction 3 contained largely the 3′-silylated isomer contaminated with some of the 2′-derivative (0.6 g). The third fraction was treated with triethylamine (0.2 mL) in methanol (60 mL) overnight at room temperature. After removing the solvent *in vacuo*, the resulting gum was chromatographed on a silica gel column as described above to yield a further 0.3 g of the 2′-protected isomer. Yield of 2′ derivative: 1.04 g (1.5 mmol), 45%. R_f (solvent B): 0.39. UV (methanol): λ_{max} = 205, 263 nm; λ_{min} = 221 nm. ^1H NMR ($CDCl_3$ + trace of D_2O): δ = 0–0.08 (m, CH_3), 0.81 (s, 9 H, *tert*-Bu), 3.37–3.60 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.79 (s, 6 H, OCH_3), 4.30 (m, 1 H, $H_{4'}$), 4.38 (m, 1 H, $H_{3'}$), 4.81 (HOD), 5.10 (m, 1 H, $H_{2'}$), 6.14 (d, 1 H, $H_{1'}$), 6.79–7.50 (m, 13 H, Ar-H), 8.32 (s, 1 H, H_6), 8.90 (s, 1 H, H_8), 9.16 (s, 1 H, H_2) ppm.

(C) 5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-3′-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphino]nebularine (**3**). Compound **2** (0.3 g, 0.45 mmol) was dried overnight in a desiccator under high vacuum and was then suspended in 2 mL of dichloromethane (dried over molecular sieves). Collidine was added until the compound dissolved (approximately 0.7 mL, 5.4 mmol, 12 equiv). The reaction mixture was placed on an ice bath. *N*-methylimidazole (18 μL, 0.23 mmol, 0.5 equiv) was added, followed by (*N*,*N*-diisopropylamino)(β-cyanoethyl)phosphonamidic chloride (0.5 mL, 2.3 mmol, 5 equiv) (Scaringe et al., 1990). A pale white precipitate appeared within seconds; the mixture was stirred for 1 h at room temperature to ensure that the reaction went to completion. TLC analysis in solvent C showed the formation of two new faster moving bands and the absence of starting material. The reaction mixture was cooled, and 150 μL collidine was added, followed by the slow addition of 60 μL of methanol to destroy excess phosphitylating reagent. The mixture was diluted with dichloromethane (20 mL), and washed with 5% $NaHCO_3$ (20 mL) followed by saturated $NaHCO_3$ (20 mL). The aqueous layers were separated and then reextracted with dichloromethane (20 mL). The organic layers were combined, dried over Na_2SO_4, evaporated to dryness, and then coevaporated from toluene (6 × 5 mL). The residue was then dissolved in 2 mL of solvent D and purified by chromatography on 20 g of silica gel packed with petroleum

ether (1% triethylamine). The product eluted with solvent E. The purified nebularine phosphoramidite could not be precipitated into hexane and was evaporated to a pale yellow foam. Yield: ~0.25 g of product (~0.27 mmol), approx. 60%. R_f(solvent C): 0.61, 0.67. R_f (solvent C) of **2**: 0.53. ^{31}P NMR ($CDCl_3$): δ = 146.6, 148.8 ppm.

(*D*) *N^2-[(Phenylacetyl)amino]-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidin-4(3H)-one* [*N^2-(Phenylacetyl)-7-deazaguanosine*] (**4**). 2-Amino-7-β-D-ribofuranosylpyrrolo-[2,3-*d*]pyrimidin-4(3*H*)-one was prepared by the glycosylation of 7-deazaguanine with 1-chloro-2,3-*O*-isopropylidene-5-*O*-(*tert*-butyldimethylsilyl)-α-D-ribofuranose, and subsequently the sugar was deprotected, both as described by Ramasamy et al. (1987). To 600 mg (2.1 mmol) of 7-deazaguanosine, which was evaporated from pyridine (3×) and then dissolved in 15 mL of dry pyridine, was added trimethylsilyl chloride (1.3 mL, 10.2 mmol), and the reaction mixture was stirred for 0.5 h. To 1-hydroxybenzotriazole (450 mg, 3.3 mmol) suspended in dry acetonitrile (1 mL) was added phenylacetyl chloride (0.450 mL, 3.18 mmol). Dry pyridine (0.5 mL) was then added to the mixture to dissolve any precipitated salt. This colorless solution was added dropwise to the ice-cooled solution of silylated 7-deazaguanosine [see Benseler and McLaughlin (1986)]. The reaction mixture was stirred for 18 h at room temperature under an argon atmosphere. The trimethylsilyl protecting groups were removed by adding water (1 mL) followed by concentrated ammonium hydroxide (3 mL) to the mixture for 30 min. The solvent was evaporated to dryness and purified by short-column chromatography (dichloromethane/methanol) to give **4**. Yield: 750 mg (85%) as a pale yellow solid. R_f(solvent A): 0.45. UV (CH_3OH): λ_{max} = 207, 268; λ_{min} = 245. ^{1}H NMR (DMSO-d_6) δ = 3.4–3.6 (m, 2 H, $H_{5'}$, $H_{5''}$), 3.79 (s, 2 H, CH_2), 3.8 (m, 1 H, $H_{4'}$), 4.0 (m, 1 H, $H_{3'}$), 4.2 (m, 1 H, $H_{2'}$), 4.9 (t, 1 H, OH), 5.1 (d, 1 H, OH), 5.34 (d, 1 H, OH), 6.0 (d, 1 H, $H_{1'}$), 6.45 (d, 1 H, H_7), 7.2–7.4 (m, 6 H, Ar-H, H_8), 11.6 (s, 1 H, NH), 11.8 (s, 1 H, N H) ppm.

(*E*) *5′-O-(4,4′-Dimethoxytrityl)-N^2-(phenylacetyl)-7-deazaguanosine* (**5**). To compound **4** (0.53 g, 1.3 mmol) dried at 50 °C under vacuum over night and then dissolved in freshly distilled THF (30 mL/mmol of starting material) was added 10 equiv of anhydrous pyridine (1.1 mL) and 2 equiv of $AgNO_3$ (0.44 g) [see Hakimelahi et al. (1982)]. The reaction mixture was stirred for 5 min to ensure that all the $AgNO_3$ dissolved. The reaction was cooled in an ice bath while dimethoxytrityl chloride (0.68 g, 2.0 mmol, 1.5 equiv) was added. The reaction mixture was removed from the ice bath 10 min later and stirred for 3 h at ambient temperature. TLC analysis (solvent B) indicated that most of starting material had reacted. The reaction mixture was cooled in an ice bath, and the reaction was stopped by the addition of 2 equiv of imidazole followed by 1 mL of methanol. The reaction mixture was filtered through celite, and the solvent was removed *in vacuo* to yield a yellow oil. The resulting residue was coevaporated twice from toluene (20 mL) and was dissolved in 10 mL of methanol. This solution was mixed with 5 g of silica gel, and the solvent was removed. The product was purified by short-column silica gel (30 g) chromatography and was eluted with a dichloromethane/methanol gradient. The yield of **5** was 0.56 g (0.79 mmol), 60%. R_f (solvent B): 0.23. UV (CH_3OH): λ_{max} = 207, 267 nm; λ_{min} = 248 nm. ^{1}H NMR ($CDCl_3$ + trace of D_2O): δ = 3.2–3.4 (m, 4 H, $H_{5'}$, $H_{5''}$, CH_2-Ar), 3.72 (m, 6 H, OCH_3), 4.22 (m, 1 H, $H_{4'}$), 4.38 (m, 1 H, $H_{3'}$), 4.75 (m, 1 H, $H_{2'}$), 5.97 (d, 1 H, $H_{1'}$), 6.42 (d, 1 H, H_7), 6.75–7.50 (m, 19 H, H_8, Ar-H) ppm.

(*F*) *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^2-(phenylacetyl)-7-deazaguanosine* (**6**). Compound **5** (0.56 g, 0.79 mmol) in pyridine solution (10 mL) cooled in an ice bath was treated with *tert*-butyldimethylchlorosilane (0.18 g, 1.2 mmol, 1.5 equiv) and imidazole (0.16 g, 2.4 mmol, 3 equiv). After 15 min, the reaction mixture was removed from ice and stirred overnight at ambient temperature. TLC analysis (solvent B) indicated that most of the starting material had disappeared. The reaction was stopped with water (40 mL), and the mixture was extracted with CH_2Cl_2 (40 mL followed by 20 mL). The organic phases were combined, evaporated, and coevaporated three times from toluene to yield an oil. This material was purified by short-column silica gel (20 g) chromatography, and the product was eluted with a dichloromethane/methanol gradient. Separation of the 2′- and 3′-silylated isomers was monitored by TLC in solvents B and D. Fractions containing the 2′-silylated isomer eluted first and were collected. A second fraction containing largely the 3′-silylated isomer was contaminated with some of the 2′-derivative. This later fraction was treated with a few drops of triethylamine in methanol at ambient temperature for 1 h to generate an equilibrium mixture of the 2′- and the 3′-isomer (a longer time period resulted in additional side reactions as judged by TLC). This isomeric mixture was separated by short-column chromatography. The identification and the isomeric purity of the 2′- and the 3′-derivative were confirmed by 2D NMR. Yield of 2′-derivative: 0.25 g (0.31 mmol), 40%. R_f (solvent B): 0.66 (2′-derivative), 0.58 (3′-derivative). UV (CH_3OH): λ_{max} = 199, 268 nm; λ_{min} = 248 nm. ^{1}H NMR ($CDCl_3$ + trace of D_2O): δ = –0.25 (s, 3 H, CH_3Si), –0.02 (s, 3 H, CH_3Si), 0.78 (s, 9 H, *t*-Bu), 3.20 (m, 1 H, $H_{5'}$), 3.28 (d, 2 H, CH_2-Ar), 3.54 (m, 1 H, $H_{5''}$), 3.76 (m, 6 H, OCH_3), 4.18 (m, 1 H, $H_{4'}$), 4.27 (m, 1 H, $H_{3'}$), 4.80 (m, 1 H, $H_{2'}$), 5.88 (d, 1 H, $H_{1'}$), 6.61 (d, 1 H, H_7), 6.75–7.70 (m, 19 H, H_8, Ar-H) ppm.

(*G*) *5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-3′-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphino]-N^2-(phenylacetyl)-7-deazaguanosine* (**7**). To compound **6** (0.13 g, 0.16 mmol) suspended in 2 mL of anhydrous dichloromethane (dried over molecular sieves) was added enough collidine to dissolve the compound (approximately 0.17 mL, 8 equiv). The reaction mixture was placed in an ice bath, and *N*-methylimidazole (7 μL, ~0.5 equiv) was added, followed by (*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphonamidic chloride (~76 μL, 0.32 mmol, 2 equiv). After the mixture was stirred at room temperature for 1 h, TLC analysis in solvent F indicated that the reaction was complete. The reaction mixture was cooled in an ice bath, and 0.2 mL of collidine was added, followed by the slow addition of 0.2 mL of methanol to destroy the excess phosphitylating reagent. The solvent was removed *in vacuo* to give a yellow oil, which was coevaporated from toluene (6 × 5 mL). The residue was then dissolved in 2 mL of solvent G and purified by chromatography on 20 g of silica gel packed with petroleum ether (1% triethylamine). The product was eluted with a gradient of dichloromethane. The fractions containing product were evaporated and precipitated into 80 mL of hexane to yield white crystals. Yields in this reaction varied (typically 60–80%). R_f (solvent F): 0.67 (elongated spot containing both diasteromers). ^{31}P NMR ($CDCl_3$): δ = 148.84, 148.77 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 12 h at 55 °C. 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 a 0.45–0.55 M gradient 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 (0–35% 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) or with an Econo-Pac 10 DG cartridge (Bio-Rad) and lyophilized to dryness.

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

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): 2.4 (Cp), 2.8 (Up), 3.6 (Ip), 3.6 (Gp), 3.6 (c^7Gp), 7.0 (Pp), 7.0 (Ap), and 11.9 min (G). After treatment with bacterial alkaline phosphatase, the retention times for the nucleosides were 4.9 (C), 6.6 (U), 11.4 (I), 12.0 (G), 12.7 (c^7G), 16.7 (P), and 19.8 min (A).

Thermal Stability of the Ribozyme/Substrate Complexes. Thermal stability studies were performed 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). Pseudo-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 C18 Sep-Pak cartridge or an Econo-Pak 10 DG 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 an Econo-Pak 10 DG 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% bromphenol 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 (0.2 μM for the catalytically less efficient substrate (I_{12}). 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 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 plots, from Eadie–Hofstee plots, and by fitting the velocity and substrate concentration data to a hyperbolic function.

RESULTS

The imidazole N^7-nitrogens and exocyclic amino groups of purine nucleosides are known to take part in non-Watson–Crick hydrogen-bonding interactions [see Saenger (1984)]. The N^7-nitrogens can participate in either direct metal chelation or interligand interactions [see de Meester et al. (1974), (1976), Pezzano and Podo (1980), and Marzilli et al. (1980)]. The exocyclic amino groups have not been observed to chelate metals, but can take part in water-mediated interligand interactions as observed in a number of metal nucleoside/nucleotide structures [see Collins et al. (1975),

Sletten and Ruud (1975), Kistenmacher et al. (1976), Aoki (1976), and Marzilli et al. (1980)]. In the structure of yeast $tRNA^{Phe}$, derived by X-ray crystal analysis, the anticodon loop is stabilized by the presence of a hydrated magnesium ion coordinated to a phosphodiester and hydrogen bonded to the N^7-nitrogen of the Y base (a hypermodified guanine nucleoside), as well as the exocyclic amino groups of A_{31}, C_{32}, and A_{38} through water-mediated interligand interactions (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 second magnesium hydrate (Holbrook et al., 1977; Jack et al., 1977; Teeter et al., 1980). Crystalization of $tRNA^{Phe}$ in the presence of manganese results in a complex in which the Mn^{2+} is chelated to the N^7-nitrogen of G_{20} (Jack et al., 1977). The nitrogen and oxygen functional groups of the conserved nucleosides in the hammerhead ribozyme complex could be involved in similar interactions that would permit the structural organization of the catalytically competent complex and/or assist in the effective positioning of the magnesium (manganese) cofactor(s) to assist in catalyzing the transesterification reaction. To examine the importance of specific nitrogen functional groups of the conserved purine residues in the hammerhead RNA cleavage reaction, we have prepared seven deletion-modified complexes; in each complex one of the purine exocyclic amino groups or guanine N^7-nitrogens has been excised from either the ribozyme or the substrate sequence.

Oligonucleotide Syntheses. To examine the importance of specific guanosine N^7-nitrogens or purine amino groups in ribozyme-catalyzed RNA cleavage, we prepared two modified ribozymes and five modified substrates. In each case a single guanosine N^7-nitrogen or purine amino group was deleted by the replacement of the native residue by a single 7-deazaguanosine (c^7G), nebularine (P), or inosine (I) residue. The three fully protected nucleoside phosphoramidites necessary to introduce the desired modifications were prepared using procedures similar to those described (Green et al., 1991; SantaLucia et al., 1991; Seela & Mersmann, 1992), but we have used the *tert*-butyldimethylsilyl protecting group for the 2′-hydroxyl and the β-cyanoethyl protecting group for the phosphoramidite in all three cases. The N^2-amino group of c^7G was protected as the phenylacetamide derivative (Benseler & McLaughlin, 1986). The structures of the synthesized derivatives are illustrated in Figure 1.

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 nitrogen functionality at a preselected site. With this structure, the substrate is a 24-mer that is cleaved into a 18-mer and a 6-mer and the ribozyme is a 19-mer (see Figure 2). 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^7G, P, or I phosphoramidite into the growing oligonucleotide chain, after a reaction time of 30–60 min, generally 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 we were able to purify approximately 1–1.5 mg (30–40 A_{260} units) in most cases. Purification proceeded primarily by anion-exchange chromatography, but a second isolation using reversed-phase chromatography and/or polyacrylamide gel electrophoresis was required for some sequences. Complete S1 nuclease hydrolysis confirmed the integrity of the 3′-5′ phosphodiester linkages, and nucleoside

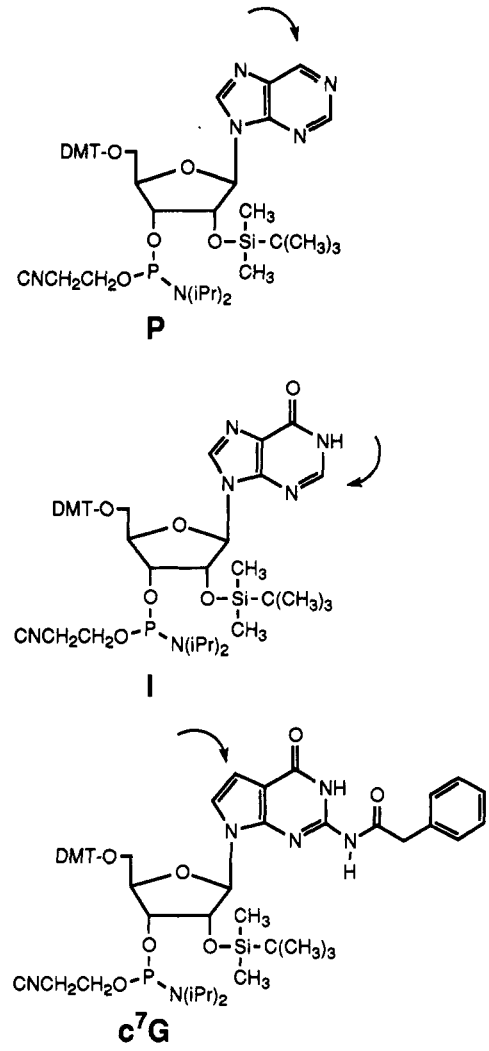

FIGURE 1: Structures of the fully protected phosphoramidite nucleoside building blocks for nebularine (purine riboside, P), inosine (I), and 7-deazaguanosine (c^7G). Arrows mark the sites of the "excised" functional groups.

analysis confirmed the presence of the modified residue in each synthetic RNA.

Thermal Stability Studies. To determine if the analogues introduced into the ribozyme or substrate sequence resulted in a dramatic change in complex stability, we examined absorbance vs temperature plots for the ribozyme/substrate complexes in the absence of magnesium. Each plot exhibited a broad cooperative transition beginning at about 35 °C and finishing near 65 °C. Although the observed transition is likely to be composed of several individual transitions for each of the helices as well as one for the nominally single-stranded core region, there was no indication of any biphasic or multiphasic character in the absorbance vs temperature plots. The midpoint of each transition has been reported as a pseudo-T_m value (Table I) since its relationship to the true thermodynamic T_m value (the temperature at which folded and unfolded states are equal in population) is presently unclear.

All of the measured transitions of the modified complexes were generally similar to that of the native complex (Table I). The least stable complex is A15P, in which the purine residue has replaced adenine in the presumed Watson–Crick base pair at the base of helix III. Substitution of purine for adenine deletes one of the Watson-Crick hydrogen bonds normally found between the adenine N^6-amino group and the uridine O^4-carbonyl. The loss of this interbase hydrogen bond

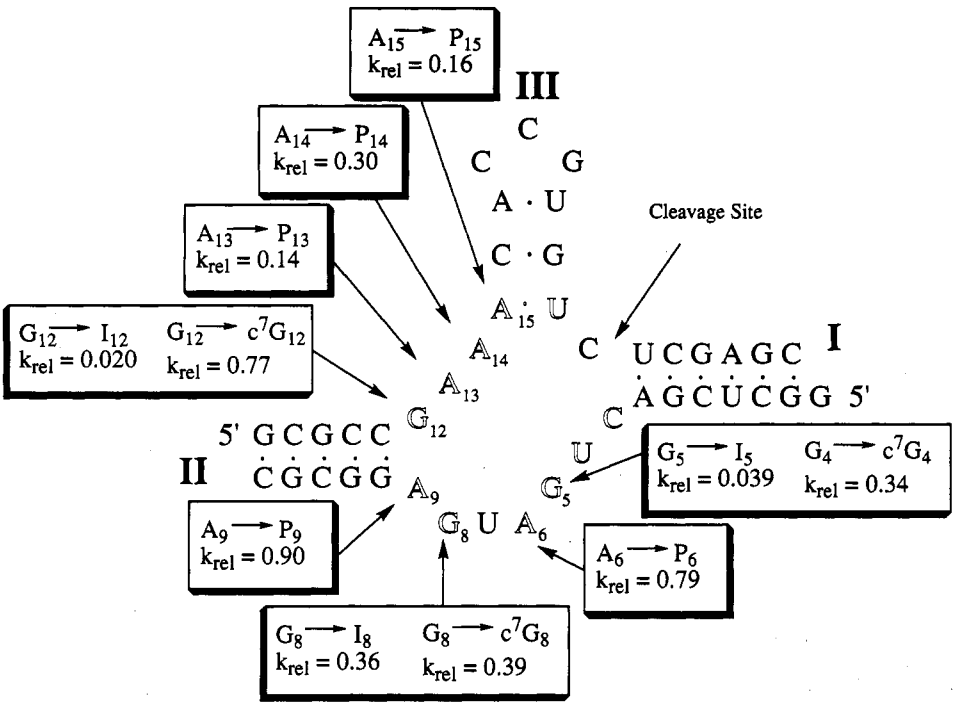

FIGURE 2: Structure of the hammerhead RNA complex illustrating the sites for the P, I, and c^{7}G substitutions and the relative cleavage efficiencies under stoichiometric conditions (outlined letters mark the locations of the eleven conserved nucleoside residues). The values for the G5I, G8I, A6P, and A9P complexes are shown for comparison only; the data was obtained from previous work (Fu & McLaughlin, 1992a).

Table I: Steady-State Kinetic Parameters of Native and Analogue-Containing Hammerhead Ribozyme Complexes

ribozyme	substrate	pseudo-T_m[a] (°C)	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\times 10^{-3}$)	k_{cat}/K_M (rel)
native	native	54.2	1.4	0.69	500	1.0
G5c^{7}G	native	53.5	3.1	0.19	61	0.12
G8c^{7}G	native	52.8	1.1	0.096	86	0.17
native	G12c^{7}G	54.5	1.2	0.47	380	0.76
native	G12I	52.1	11	0.012	1.1	0.0022
native	A13P	50.7	1.9	0.039	20	0.040
native	A14P	52.8	1.7	0.056	33	0.066
native	A15P	49.8	1.8	0.015	8.3	0.017

[a] The pseudo-T_m is the midpoint in the thermally induced transition obtained by plotting absorbance (260 nm) vs temperature.

could account for the roughly 4 - 4.5 °C decrease in the midpoint of the thermal transition observed for the A15P complex.

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 13–17 °C below the measured stability values for the complexes (in the absence of Mg^{2+}) (see Table I). The native complex exhibited a half-life of 8.8 min with a first-order rate constant of 0.079 min^{-1}. This value is in agreement with that obtained for the indentical complex prepared by enzymatic methods (Uhlenbeck, 1987; Ruffner et al., 1990). 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 for different batches of purified sequences (but by no more than 2-fold). 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 and substrate. The three conserved guanosine residues, G_5, G_8, and G_{12}, in the ribozyme/substrate complex were each replaced by 7-deazaguanosine. All three complexes (G5c^{7}G, G8c^{7}G, and G12c^{7}G) exhibited cleavage rates that were within 3-fold (see Figure 2) of the rate obtained for the native complex (k_f = 0.027, 0.031, and 0.061 min^{-1} respectively). Of the three nebularine-containing complexes, two exhibited cleavage rates some 6–8-fold slower than the native complex (A13P, k_f = 0.011, and A15P, k_f = 0.012), while the rate for the A14P complex was reduced only 3-fold (k_f = 0.030 min^{-1}; see also Figure 2). The inosine-containing complex (G12I) exhibited the slowest cleavage rate of the complexes studied (k_f = 0.0016 min^{-1}), some 50-fold lower than the value obtained for the native ribozyme/substrate complex.

Steady-State Cleavage Analyses. In our hands, it was necessary to examine the steady-state kinetics at a temperature near the pseudo-T_m value of the complex in order to obtain reproducible results. In other studies (Uhlenbeck, 1987; Ruffner et al., 1990), steady-state kinetic parameters for the native and sequence-altered 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 0.69 min^{-1} (Table I), and these parameters are similar to those reported earlier by Uhlenbeck (1987). The three c^{7}G-containing complexes were characterized by K_M values that were largely unchanged relative to those of the native complex and k_{cat} values that were reduced only marginally (Table I). The three nebularine-containing complexes, A13P, A14P, and A15P, were also characterized by K_M values that were essentially the same as that obtained for the native complex, but the k_{cat} values were decreased by 18-, 12-, and 46-fold, respectively. The G12I complex was the least efficient complex, with a K_M value increased some 8-fold above that measured for the native complex and a k_{cat} value decreased by nearly 60-fold (Table I).

The final column of Table I compares the overall cleavage efficiency of the ribozyme/substrate complexes as expressed by the apparent bimolecular rate constant, k_{cat}/K_M. The c^{7}G

complexes were generally similar to the native complex with a reduction in k_{cat}/K_M values from 8-fold to less than 2-fold. The differences in catalytic efficiency were primarily a function of a reduced turnover number (k_{cat}). By comparison, the G12I complex exhibited an apparent bimolecular rate constant some 450-fold lower than that obtained for the native complex (Table I). Of the three nebularine complexes, A15P was the least efficient with a k_{cat}/K_M value reduced from that of the native complex by 60-fold. The activities of A13P and A14P were slightly better with relative k_{cat}/K_M values 25- and 15-fold lower than that obtained for the native sequence.

DISCUSSION

Although there are a number of examples in which RNA functions in a catalytic manner, at present, other than some preliminary NMR studies (Heus et al., 1990; Heus & Pardi, 1991a), little detailed structural information is available describing a catalytically competent complex. Some molecular modeling of the hammerhead complex has been reported (Mei et al., 1989). The hammerhead RNAs appear to require three, essentially native, helical regions (see Figure 2), but the structure of the nominally single-stranded regions, C_3–A_9 and G_{12}–A_{14}, remains undefined. The substitution of base analogues into nucleic acid sequences can be a powerful technique in the identification of specific functional groups critical for efficient catalysis. With the identification of a series of critical functional groups, it should then be possible to describe a structure that is based upon the 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 blocks of three nucleoside analogues, nebularine, inosine and 7-deazaguanosine, for incorporation into RNA sequences. The c^7G analogue, like the corresponding c^7A analogue (Fu & McLaughlin, 1992b), permits the site-specific deletion of the purine N^7-nitrogen, while the nebularine and inosine derivatives permit the study of complexes lacking specific purine amino groups.

Metal Coordination. Of the seven deletion-modified complexes prepared in the present study, the G5c^{7}G, G8c^{7}G, and G12c^{7}G complexes exhibited kinetic parameters that suggest cleavage efficiencies (as expressed by first-order rate constants or k_{cat}/K_M values) very similar to that of the native sequence. Although the N^7-nitrogen of guanosine (or inosine) has been commonly observed coordinated to metal ions in various metal/nucleotide complexes (see, for example, the Mn^{2+}/GMP complex; de Meester et al, 1974), the absence of significant effects in the cleavage reaction after the excision of each of these nitrogens argues against the formation of a magnesium/manganese coordination complex critical for the observed cleavage reaction at any of these sites.

By comparison, there is a dramatic loss in cleavage efficiency with the excision of a single guanosine N^2-amino group from G_{12} (Figure 2; Table I), and this result is in agreement with that reported for a similar complex (Slim & Gait, 1992). Nucleoside amino groups have not been observed to coordinate to metal ions (except under conditions of ionization), but they can take part in interligand hydrogen-bonding interactions to coordinated water molecules [see Marzilli and Kistenmacher (1980)]. Although such interligand interactions have not been observed, to our knowledge, with the N^2-amino group in any metal/nucleotide structures involving guanosine, similar interactions have been suggested for the amino group of adenosine (Collins et al., 1975). Additionally, in the crystal structure of $tRNA^{Phe}$, the exocyclic amino groups of A_{31}, C_{32}, and A_{38} all appear to be involved in interligand interactions with the pentahydrated magnesium ion bound in the anticodon loop (Holbrook et al., 1977; Teeter et al., 1980). A similar interaction with the N^2-amino group at G_{12} could assist in optimally positioning a hydrated magnesium/manganese cofactor for efficient catalysis in the hammerhead ribozyme.

The loss of the N^6-amino groups from position A_{13}, A_{14}, or A_{15} results in intermediate effects on RNA cleavage. The A14P complex is somewhat less active than the corresponding c^7G complexes (Figure 2; Table I) but still remains quite an efficient catalyst relative to the G12I complex. By comparison, the cleavage activities of the A13P and A15P complexes are significantly reduced (as expressed by first-order rate constants or k_{cat}/K_M values), but both complexes are also still quite active (by at least an order of magnitude) relative to the G12I complex. These results for the relative catalytic activities of the nebularine-containing complexes are generally in agreement with those described in a previous preliminary report (Slim & Gait, 1992). Although interligand interactions involving the A13 or A15 amino group may be present in the hammerhead complex and may assist in positioning the metal cofactor(s), the role of these functional groups would appear to be far less critical than that of the G_{12} amino group.

Hydrogen Bonding. Of the possible non-Watson–Crick base-pairing schemes available in the ribozyme/substrate complex, the formation of a pair of G-A "mismatches" at the base of helix II (G_8-A_{13} and A_9-G_{12}; see Figure 2) is an attractive possibility suggested previously (Li et al., 1991b; Slim & Gait, 1992) on the basis of the observed stability of such base pair mismatches in both DNA (Gao & Patel, 1988; Leonard et al., 1990; Li et al., 1991a; Ebel et al., 1992; Lane et al., 1992) and RNA (SantaLucia et al., 1990; Heus & Pardi, 1991b). An additional report suggests that cleavage activity is still present when helix II is replaced by various truncated structures (McCall et al., 1992). A number of base-pairing orientations for the G-A mismatch have been proposed (Gao & Patel, 1988; Leonard et al., 1990; SantaLucia et al., 1990; Li et al., 1991b; Ebel et al., 1992; Heus & Pardi, 1991a,b), all of which involve complementary hydrogen bonding through the exocyclic amino group of A or G and the corresponding purine N^1-, N^3- or N^7-nitrogen. The base analogue results from the present work indicate that loss of either the G_8 or the G_{12} N^7-nitrogen has little effect on the efficiency of the cleavage reaction. Previous observations (Fu & McLaughlin, 1992b) suggest that loss of the corresponding A_9 and A_{13} N^7-nitrogens has only marginal effects upon cleavage activity. Taken together, these observations tend to argue against the presence of complex-stabilizing G_8-A_{13} and A_9-G_{12} mismatches involving hydrogen bonding to the purine N^7-nitrogens. Other G-A mismatch structures have been proposed that do not involve the purine N^7-nitrogens [see Li et al. (1991b)], but in all structures proposed to date, the N^2- or N^6-amino group of G or A, respectively, takes part in the suggested interbase hydrogen bonding. While the loss of the N^2-amino group from G_{12} results in a dramatic reduction in cleavage efficiency, and this deletion strongly suggests a role for this functional group in a structural or transition state stabilizing interaction, the loss of the corresponding amino group from G_8 (Fu & McLaughlin, 1992a) has little effect upon cleavage activity (see Figure 2). Additionally, the loss of the N^6-amino group from A_{13} has a moderate effect upon cleavage activity, but loss of the corresponding amino group from A_9 does not (Fu & McLaughlin, 1992a) (see Figure 2). The results of these base analogue substitutions suggest that structure-stabilizing G-A mismatch base pairs that employ one or both of the N^2-

and N^6-amino goups are unlikely to be present at the base of helix II in this hammerhead RNA complex.

The guanine N^2-amino group (as well as the adenine N^6-amino) is capable of participating in a variety of hydrogen-bonding interactions that could assist in stabilizing the ribozyme/substrate complex or the corresponding transition state. We note here that the guanine N^2-amino group is present in various non-Watson–Crick hydrogen-bonding interactions (Saenger, 1984), it is observed bound to an internucleotide phosphate in the GNRA tetraloop structure (Heus & Pardi, 1991b), and it is involved in a water-mediated hydrogen bond to the 2′-OH of a uridine residue in an RNA duplex (Holbrook et al., 1991). In addition to its potential role in hydrogen-bonding interactions, there are two reports suggesting that the N^2-amino group could participate as a general base to facilitate the catalytic cleavage observed with ribozymes (Chowrira et al., 1991; Slim & Gait, 1992).

CONCLUSIONS

Loss of the N^2-amino group from the guanosine residue at position 12 in the ribozyme/substrate complex results in a dramatic reduction in cleavage activity and implicates this amino group in a critical interaction in the complex and/or the transition state. By comparison, none of the guanosine N^7-nitrogens present in the catalytic core are necessary for efficient catalytic activity. Loss of the A_{13}, A_{14}, and A_{15} amino groups individually results in some moderation of catalytic activity, but each of these deletion-modified complexes is significantly more active than the G12I complex. Although guanine N^7-nitrogens are known to be involved in binding metals in some metal/nucleoside complexes, such interactions do not appear to be present in the present hammerhead ribozyme/substrate complex. The results from the present analogue sequences argue against the presence of adjacent G-A mismatched base pairs at the base of helix II.

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