Importance of Specific Adenosine N^3-Nitrogens for Efficient Cleavage by a Hammerhead Ribozyme†

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ABSTRACT: Five modified hammerhead ribozyme/substrate complexes have been prepared in which individual adenosine N^3-nitrogens have been excised and replaced with carbon. The modified complexes were chemically synthesized with the substitution of a single 3-deazaadenosine (c^3A) base analogue for residues A_6, A_9, A_{13}, A_{14}, or $A_{15.1}$. Steady-state kinetic analyses indicate that the cleavage efficiencies, as measured by k_{cat}/K_M, for the c^3A_6, c^3A_9, and c^3A_{14} complexes were only marginally reduced ($\leq$5-fold) relative to the native complex. By comparison, the cleavage efficiencies for the c^3A_{13} and $c^3A_{15.1}$ complexes were reduced by 9-fold and 55-fold, respectively. These reductions in cleavage efficiency are primarily a result of lower k_{cat} values. Profiles of pH and cleavage rate suggest that the chemical cleavage step is the rate-limiting reaction for these complexes. These results suggest that the N^3-nitrogen of the A_{13} residue and particularly the $A_{15.1}$ residue in the hammerhead ribozyme/substrate complex are critical for transition state stabilization and efficient cleavage activity. We have additionally compared the locations of these critical functional groups, as well as those identified from other studies, with recent crystallographic analyses. In some cases, the critical functional groups are clustered around proposed metal binding sites and may reflect functional groups critical for binding the metal cofactor. In other cases, clusters of functional groups may form a network of hydrogen bonds necessary for transition state stabilization.

Self-cleavage reactions of RNA by internal transesterification reactions have been observed in the genomes of several plant pathogens, where it is believed that these reactions are an essential step in replication (Symons, 1989, 1991). 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 3 helices (1 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 & Symons, 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 (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Koizumi et al., 1988, 1989a; Jefferies & Symons, 1989). Divalent metal ions such as Mg^{2+} or Mn^{2+} are required for the cleavage reaction (Uhlenbeck, 1987; Dahm & Uhlenbeck, 1991; Olsen et al., 1991). At least one (but possibly more) metal cofactor is necessary for cleavage activity, but the role of the metal cofactor(s) remains unclear (Kuimelis & McLaughlin, 1995a; Sawata et al., 1995). Thorough kinetic experiments have provided a detailed view of complex assembly and product dissociation processes (Fedor & Uhlenbeck, 1992). Recent X-ray crystallographic (Pley et al., 1994; Scott et al., 1995) and solution studies (Heus & Pardi, 1991; Tuschl et al., 1994; Bassi et al., 1995) have confirmed the early secondary structure predictions and provided a wealth of additional information about the ground state conformation.

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; Pley et al., 1994; Scott et al., 1995), 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 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., 1991; Pieken et al., 1991; Fu & McLaughlin, 1992b; Williams et al., 1992), 2′-deoxy-2′-fluoronucleosides (Olsen et al., 1991; Pieken et al., 1991; Williams et al., 1992), or arabinosyl nucleosides such as ara-G (Fu et al., 1994). The role of the exocyclic amino groups of the conserved purines and pyrimidines in the hammerhead domain has been examined by replacement of single adenosine residues by nebularine, replacement of single guanosine residues by inosine, 2-aminopurine riboside, or O^6-methylguanosine (Odai et al., 1990; Koizumi & Ohtsuka, 1991; Fu & McLaughlin, 1992b; Slim & Gait, 1992; Fu et al., 1993; Grasby et al., 1993; Tuschl et al., 1993), or replacement of cytidine by a 2-pyrimidinone derivative (Adams et al., 1994; Murray et al., 1995). The

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importance of the purine N^7-nitrogens has been probed by using complexes containing the corresponding 7-deazapurine derivatives (Fu & McLaughlin, 1992a; Fu et al., 1993; Seela et al., 1993). Additionally, the substitution of 2′-fluoro-, 2′-amino-, or 2′-*O*-methylriboses into ribozyme complexes (Koizumi et al., 1989b; Olsen et al., 1991; Goodchild, 1992; Williams et al., 1992), in some cases, has stabilized the ribozyme to nuclease activity without dramatically altering cleavage activity (Pieken et al., 1991; Beigelman et al., 1995). Studies employing the R_p and S_p phosphorothioate diastereomers at the cleavage site suggest that the Mg^{2+} (Mn^{2+}) cofactor(s) is (are) bound to the *pro-R* oxygen in the unmodified complex and that transesterification occurs by an in-line mechanism (van Tol et al., 1990b; Koizumi & Ohtsuka, 1991; Slim & Gait, 1991). A two-metal transition state has been proposed (Steitz & Steitz, 1993), but recent work employing a substrate containing a 5′-bridging phosphorothioate argues against such a scenario (Kuimelis & McLaughlin, 1995a,b). Three other specific phosphodiesters within the conserved central core sequence appear to be necessary for efficient folding of the complex, or for cofactor binding (Buzayan et al., 1990a; Ruffner & Uhlenbeck, 1990).

The present work focuses on the role of the N^3-nitrogens of the five conserved adenosine residues and their importance in the self-cleavage reaction. We have incorporated single residues of 3-deazaadenosine into each conserved site normally occupied by adenosine, thus replacing a single nitrogen atom with a carbon atom and eliminating a potential hydrogen bonding or metal binding interaction. We report here the syntheses of the appropriately protected 3-deazaadenosine phosphoramidite derivative, the construction of the "deletion-modified" ribozymes, and the kinetic characterization of these 46-nucleotide ribozyme complexes, each lacking a single adenosine N^3-nitrogen.

EXPERIMENTAL PROCEDURES

Materials

Oligonucleotides were synthesized using nucleoside phosphoramidite derivatives obtained from Milligen (New Bedford, MA) and an Applied Biosystems 381A DNA synthesizer. High-performance liquid chromatography (HPLC)[1] was carried out on an ODS-Hypersil column (0.46 × 25 cm, Shandon Southern, England), using a Beckman HPLC system. Absorption spectra were recorded by a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. Nuclease S1 is a product of United States Biochemical Corp. (Cleveland, OH). Radioactivity of polyacrylamide gels was quantified using a Molecular Dynamics Phosphorlmager:425.

Methods

N^6-Benzoyl-3-deazaadenosine. To 1.0 g (3.76 mmol) of 3-deazaadenosine (Montgomery et al., 1977) coevaporated from anhydrous pyridine (×2) was added 25 mL of anhydrous pyridine, and the solution was cooled in an ice−water bath. To the cooled mixture was added dropwise 4 mL of trimethylsilyl chloride, and the cooled solution was stirred for 30 min. To this mixture was added dropwise 2.5 mL benzoyl chloride, and after the addition was complete, the cold bath was removed. It was stirred at ambient temperature for 2 h and then cooled again with the ice−water bath. The reaction was stopped by the dropwise addition of 7 mL of cold water and the mixture stirred for 15 min with cooling before the addition of 7.5 mL of concentrated ammonium hydroxide, and this final mixture was stirred for 30 min. The solution was evaporated to dryness *in vacuo* and coevaporated with toluene twice. The residue was applied to column of silica gel and eluted with a gradient of dichloromethane/methanol. The desired fractions were pooled, and the solvent was removed to yield 1.2 g (87%) of the desired compound. R_f (dichloromethane/methanol, 8:2), 0.55; ^{1}H NMR (DMSO-d_6): δ 3.66 (m, 2H, $2H_{5'}$), 4.00 (m, 1H, $H_{4'}$), 4.14 (m, 1H, $H_{3'}$), 4.40 (m, 1H, $H_{2'}$), 5.20 (t, 1H, $OH_{5'}$), 5.28 (d, 1H, $OH_{3'}$), 5.58 (d, 1H, $OH_{2'}$), 5.92 (d, 1H, $H_{1'}$), 7.5−8.0 (5H, C_6H_5), 7.74 (d, 1H, ArH), 8.18 (d, 1H, ArH), 8.54 (s, 1H, ArH).

5′-O-(4,4′-Dimethoxytrityl)-N^6-benzoyl-3-deazaadenosine. To 1.0 g (2.7 mmol) of vacuum-oven-dry *N^6*-benzoyl-3-deazaadenosine coevaporated from anhydrous pyridine (×2) and cooled in an ice−water bath was added 1.2 g (3.55 mmol) of dimethoxytrityl chloride in a small portion under an argon atmosphere. The mixture was stirred at 4 °C overnight, and the pyridine was removed *in vacuo*. The residue was coevaporated with toluene twice and then was applied to a column of silica gel and was eluted with 5% methanol in dichloromethane to yield 1.4 g (77%). R_f (dichloromethane/methanol, 9:1), 0.58; ^{1}H NMR (DMSO-d_6) δ 3.28 (m, 2H, $2H_{5'}$), 3.74 (s, 6H, 2 OCH_3), 4.14 (m, 1H, $H_{4'}$), 4.24 (m, 1H, $H_{3'}$), 4.52 (m, 1H, $H_{2'}$), 5.98 (d, 1H, $H_{1'}$), 6.8−8.1 (20H, ArH), 8.44 (s, 1H, ArH).

2′-O-tert-Butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-N^6-benzoyl-3-deazaadenosine and 3′-O-tert-Butyldimethylsilyl-5′-O-(4,4′-dimethoxytrityl)-N^6-benzoyl-3-deazaadenosine. To 673 mg (1.0 mmol) of 5′-*O*-(4,4′-dimethoxytrityl)-*N^6*-benzoyl-3-deazaadenosine and 0.24 g of imidazole coevaporated from anhydrous pyridine (×2) was added 25 mL of anhydrous pyridine. This solution was cooled in an ice−water bath, and 0.26 g (1.73 mmol) of *tert*-butyldimethylsilyl chloride was added. The solution was kept cold for 15 min and then was stirred at ambient temperature overnight. The solvent was removed *in vacuo*, and the residue was coevaporated from toluene twice. Column chromatography using 5% methanol in ethyl acetate yielded 350 mg (44%) of the 2′-regioisomer and 300 mg (38%) of the 3′-regioisomer.

The 300 mg of the 3′-regioisomer was dissolved in 50 mL of 10% methanol in dichloromethane, and 1 mL of triethylamine was added. This solution was stirred overnight, and the solvent was removed *in vacuo*. Column chromatography was used to yield an additional 150 mg of the 2′-regioisomer. Final yield for the 2′-derivative: 500 mg (63%).

2′-Silyl derivative: R_f (dichloromethane/methanol, 20:1), 0.45; ^{1}H NMR (DMSO-d_6): δ −0.26 (s, 3H, $SiCH_3$), −0.10 (s, 3H, $SiCH_3$), 0.72 (s, 9H, 3 CH_3), 3.25 (m, 2H, $2H_{5'}$), 3.70 (s, 6H, $2OCH_3$), 4.16 (m, 1H, $H_{4'}$), 4.24 (m, 1H, $H_{3'}$), 4.62 (m, 1H, $H_{2'}$), 5.30 (d, 1H, $OH_{3'}$), 6.00 (d, 1H, $H_{1'}$), 6.8−8.1 (20H, ArH), 8.46 (s, 1H, ArH).

3′-Silyl Derivative: R_f (dichloromethane/methanol, 20:1), 0.43; ^{1}H NMR (DMSO-d_6): δ 0.04 (s, 3H, $SiCH_3$), 0.08 (s, 3H, $SiCH_3$), 0.84 (s, 9H, 3 CH_3), 3.25 (m, 2H, $2H_{5'}$), 3.70 (s, 6H, $2OCH_3$), 4.08 (m, 1H, $H_{4'}$), 4.32 (m, 1H, $H_{3'}$), 4.48

[1] Abbreviations: c^3A, 3-deazaadenosine; HPLC, high-performance liquid chromatography.

(m, 1H, $H_{2'}$), 5.58 (d, 1H, $OH_{2'}$), 5.92 (d, 1H, $H_{1'}$), 6.8−8.1 (20H, ArH), 8.44 (s, 1H, ArH).

2′-O-tert-Butyldimethylsilyl-3′-O-[(N,N-diisopropylamino)-(β-cyanoethoxy)phosphino]-5′-O-(4,4′-dimethoxytrityl)-N^6-benzoyl-3-deazaadenosine. To 200 mg (0.25 mmol) of 2′-*tert*-butyldimethylsilyl-5′-*O*-(4,4′-dimethoxytrityl)-*N*^{6}-benzoyl-3-deazaadenosine dissolved in 2 mL of freshly distilled dichloromethane were added 0.25 mL of diisopropylethylamine, 0.1 mL of *N*-methylimidazole, and then 150 mg of 2-cyanoethyldiisopropylchlorophosphoramidite (dropwise). The mixture was stirred at room temperature for 1 h, and then one drop of methanol was added to stop the reaction. The mixture was washed with dilute sodium bicarbonate and then water. The organic solution was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was applied to a column of silica gel and eluted with 5% methanol in dichloromethane to yield 210 mg (84%) of product. R_f (dichloromethane/methanol, 95:5), 0.38; ^{31}P NMR ($CDCl_3$): δ 148.8, 151.9.

Oligonucleotide Synthesis. Oligonucleotides were synthesized from 1 μmol of bound nucleoside on wide-pore silica supports using phosphoramidite chemistry (Matteucci & Caruthers, 1981; Usman et al., 1987; 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 16 h at 55 °C. The glass beads were removed, and the ammonia and ethanol were evaporated to dryness. 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 18 h at ambient temperature. To the crude mixture of oligonucleotides was added 1 mL of 0.9 M sodium acetate (pH 6.0), and the mixture was extracted twice with ethyl acetate. To the aqueous phase was added 100% ethanol at 3 times the volume, and the oligonucleotides were precipitated at −20 °C for 12 h. The oligonucleotides were centrifuged at 13,000 rpm for 20 min at 4 °C. The ethanol was decanted, and the oligonucleotide pellet was lyophilized to dryness and resuspended in water. The oligonucleotides were purified by gel electrophoresis in 20% polyacrylamide/1% bis(acrylamide)/7 M urea gels. The product band was visualized by UV-shadowing, excised, and extracted by electroelution with a Schleicher & Schuell Elutrap electro-separation system in 0.0045 M Tris−borate and 0.001 M EDTA (pH 8). To 250 μL of the eluted oligonucleotide solution was added 50 μL of 3 M sodium acetate and 900 μL of 100% ethanol. The pure oligonucleotides were precipitated at −20 °C for 12 h. The oligonucleotides were centrifuged at 13 000 rpm for 20 min at 4 °C. The ethanol was decanted, and the oligonucleotide pellet was lyophilized to dryness and resuspended in water.

Nucleoside Analyses. Nucleoside composition was determined after 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. To the 10 μL reaction mixture were added 5 μL of 0.1 M Tris-HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase. Following incubation for 2 h at ambient temperature, a 5 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). The retention times for the nucleosides were 4.4 (C), 5.8 (U), 9.6 (G), 10.4 (c^{3}A), and 13.9 (A) min.

Radioisotopic Labeling. Each 12-mer was 5'-end-labeled with [γ-^{32}P]ATP as follows: a 25 μL reaction mixture containing 1 A_{260} unit of 12-mer, 40 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.02 mM EDTA, 450 μCi of [γ-^{32}P]ATP, and 120 units of T4 polynucleotide kinase was incubated for 60 min at 37 °C. The product was purified by electrophoresis in a 20% polyacrylamide/7 M urea gel. The product band was excised, extracted with 0.3 M sodium acetate, pH 6.0, and desalted with a Waters Sep-Pak C18 cartridge. The specific activity of the 12-mer was typically 0.05 μCi/pmol.

Single-Turnover Cleavage Analyses Defining the pH−Rate Profiles. Twenty-five microliter solutions of ribozyme and radiolabeled substrate RNAs in 50 mM buffer were each heated separately to 95 °C for 1 min and cooled at 25 °C for 15 min. Each solution was adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. The reactions were initiated by mixing the two solutions. The ribozyme concentration was in excess of substrate concentration which was approximately 10 nM. The following buffers were used at the various pHs: MES (pH 6.0), PIPES (pH 6.5), HEPES (pH 7 and 7.5), TAPS (pH 8, 8.5, and 9), and CHES (pH 9.5). Reactions were terminated at appropriate time intervals by withdrawing 7 μL aliquots and quenching the mixture in an equal volume of 50 mM Tris/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 in 50 mM Tris−borate buffer and 2 mM Na_2EDTA, pH 8.0. The radioactivity of the substrate and product bands was quantified with a Molecular Dynamics Phosphorlmager. The logarithm of the unreacted fraction was plotted against time, and the data points were fitted to a straight line. The cleavage half-lives ($t_{1/2}$) were used to obtain first-order rate constants ($k = 0.693/t_{1/2}$). Each point of the pH−rate profiles is the average of at least two assays.

Steady-State Cleavage Analyses. A 50 μL solution of the ribozyme and a 50 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 at 25 °C for 15 min. The heating step was necessary to obtain reproducible kinetic parameters, and likely results in the removal of aggregates, and does not result in any signficant substrate of ribozyme cleavage. The reaction was initiated by mixing the two solutions. The ribozyme concentration in these reactions was typically 1 nM. Six to eight substrate concentrations were used varying from 20 to 400 nM depending on the individual sequence. Aliquots of 12 μL were taken from the reaction mixture at various times and quenched by the addition of an equal volume of 50 mM Na_2-EDTA/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_2EDTA, pH 8.0. Values up to 20% 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. The parameters obtained for each analysis were averaged, and each reported value is the

average of at least three determinations. Experimental errors for the parameters were minimized by performing each set of experiments with a single batch of isolated substrate or ribozyme sequence. Experimental errors under these conditions were ±15%.

RESULTS AND DISCUSSION

The N^3-nitrogen of an adenosine residue has not typically been observed to take part in inter- or intrastrand hydrogen bonding in nucleic acid complexes. In selected mononucleoside crystals, such interactions have been documented. For example, when forced into the *syn* confromation, the N^3-nitrogen of adenosine has been observed to function as a hydrogen bond acceptor for the 5′-hydroxyl in selected complexes (Birnbaum & Shugar, 1978; Pless et al., 1978). This purine N^3-nitrogen does function as a hydrogen bond acceptor in double-stranded DNA complexed to sequence-specific proteins such as the eukaryotic TATA transcription factor (Kim, J. L., et al., 1993; Kim, Y., et al., 1993) that bind in the minor groove. Additionally, crystal structure analyses suggest that complexes containing certain minor groove-specific ligands, such as netropsin, distamycin, and Hoechst 33258 (Kopka et al., 1985a,b; Coll et al., 1987; Teng et al., 1988; Carrondo et al., 1989; Larsen et al., 1989), bound to double-stranded DNA are mediated by intermolecular hydrogen bonding interactions between the ligand and the N^3-nitrogens of the dA residues functioning as hydrogen bond acceptors. The N^3-nitrogen is generally considered a weak ligand for the formation of inner sphere metal coordination complexes even in mononucleotides, although the structure of ADP crystallized in the presence of potassium ions (Adamiak & Saenger, 1980) exhibits simultaneous coordination of the sugar 2′-hydroxyl, the adenosine N^3-nitrogen, the unesterified phosphate oxygens, and water by a potassium ion. In the ground state structures (Pley et al., 1994; Scott et al., 1995) of the hammerhead ribozyme complexed to a DNA or RNA inhibitor, the N^3-nitrogens of the conserved adenosine residues present in the core sequence do not appear to be involved in any critical interactions. However, functional groups that may appear unimportant in the ground state structure could well take part in critical interactions in the transition state. How the metal cofactor(s) is(are) bound in the ground state structure and its role in transition state stabilization remain to be clarified.

In order to probe the importance of specific N^3-nitrogens of the conserved adenosine residues in the hammerhead complex in the cleavage reaction, we wished to delete individual N^3-nitrogens from the otherwise native 46-nucleotide complex. To accomplish such single-atom deletions, we prepared five analogue complexes; each complex contained a single 3-deazaadenosine residue replacing one of the native adenosines present in the conserved core sequence. The 3-deazaadenosine nucleoside analogue should exist in the same tautomeric form as does adenosine. Each 3-deazaadenosine can then partake in all of the "normal" hydrogen bond donor and acceptor interactions (metal coordination or interligand interactions) as does adenosine, with one exception—interactions involving the N^3-nitrogen will be eliminated (see Figure 1a). In cases where the N^3-nitrogen is involved in critical interaction(s) necessary for efficient cleavage by the hammerhead ribozyme, a significant reduction in catalytic efficiency should be observed for the relevant analogue complex.

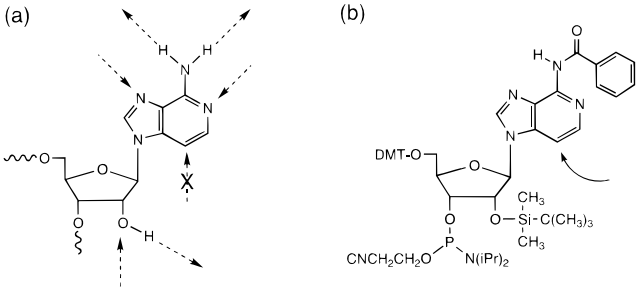

FIGURE 1: (a) Structure of 3-deazaadenosine (c^3A) with the hydrogen bonding donor and acceptor functional groups noted with arrows. The native adenosine would have an N^3-nitrogen hydrogen bond acceptor; this site is lost (X) with the c^3A substitution. (b) Fully protected phosphoramidite building block of 3-deazaadenosine.

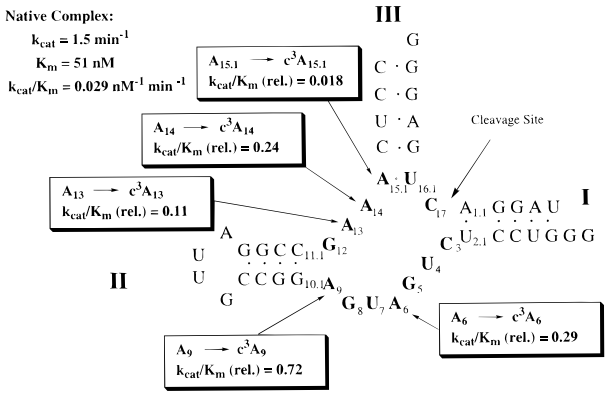

FIGURE 2: Proposed secondary structure for the hammerhead ribozyme/substrate complex with relative k_{cat}/K_M values noted for the various analogue complexes.

Oligonucleotide Synthesis. The 3-deazaadenosine nucleoside analogue was prepared as described previously (Montgomery et al., 1977). The free nucleoside was converted to the fully protected phosphoramidite derivative (Figure 1b) by (i) protection of the exocyclic amino group as an aromatic amide, (ii) conversion to the 5′-*O*-dimethoxytrityl derivative, (iii) reaction with *tert*-butyldimethylsilyl chloride, (iv) separation of the 2′-*O*- and 3′-*O*-silyl isomers, and (v) conversion of the 2′-*O*-silyl derivative to the desired 3′-*O*-phosphoramidite (Figure 1b) using essentially standard procedures.

The native ribozyme/substrate complex that we have employed is similar to the one described by Uhlenbeck (Fedor & Uhlenbeck, 1992) and others [see Tuschl et al. (1995) and references cited therein], and each modified complex lacks a single N^3-nitrogen functionality at a preselected site. With this system, the substrate is a 12-mer that is cleaved into a 7-mer and a 5-mer, and the ribozyme is a 34-mer (see Figure 2). After assembly, deprotection, and purification of each 34-mer ribozyme, a small portion was completely hydrolyzed using S1 nuclease to confirm the integrity of the 3′−5′ phoshodiester linkages. After treatment of this mixture with phosphatase, resolution of the nucleoside mixture confirmed the presence of the c^3A residue and the absence of any unidentified species (Figure 3).

Importance of the Adenosine N^3-Nitrogens for Cleavage Activity. All five analogue complexes exhibited RNA cleavage activity although the catalytic efficiencies of the complexes varied significantly. In all cases, the measured K_M values were very similar to that of the native complex (Table 1), suggesting that the absence of the adenosine N^3-

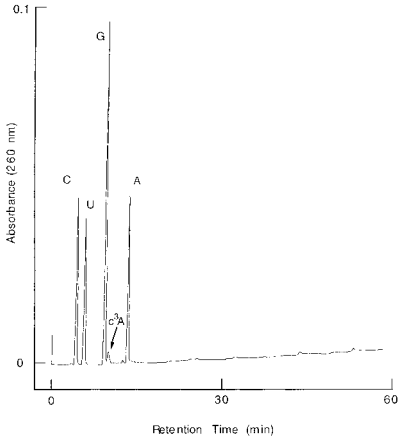

FIGURE 3: HPLC analysis of an S1 nuclease/alkaline phosphatase digest of the c^{3}A$_{15.1}$ ribozyme. For chromatographic conditions, see Experimental Procedures.

Table 1: Steady-State Kinetic Parameters for Cleavage of the c^{3}A-Containing Ribozymes[a]

ribozyme	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\times 10^{-3}$ nM^{-1} min^{-1})	k_{cat}/K_M(rel)
native	51	1.5	29	1.0
c^{3}A$_6$	40	0.33	8.5	0.29
c^{3}A$_9$	48	1.02	21	0.72
c^{3}A$_{13}$	54	0.18	3.3	0.11
c^{3}A$_{14}$	65	0.45	6.9	0.24
c^{3}A$_{15.1}$	34	0.018	0.53	0.018

nitrogen at various sites did not impact upon the affinity of the ribozyme for the substrate RNA sequence. This is not a surprising result since the binding affinity for such complexes is likely determined largely from interstrand Watson−Crick hydrogen bonding interactions between the ribozyme and substrate oligonucleotides and the N^3-nitrogens of the adenosine residues do not partake in such hydrogen bonding schemes.

The observed variations in catalytic efficiency for the five analogue complexes were all a result of decreased k_{cat} values. The c^{3}A$_9$ complex exhibited kinetic parameters that were essentially the same as the native complex, suggesting the absence of any critical role for the N^3-nitrogen at this site. The overall catalytic efficiency as judged from k_{cat}/K_M values for this complex was also similar to the native complex (Figure 2). By comparison, the c^{3}A$_{14}$ and c^{3}A$_6$ complexes were somewhat less efficient catalysts with k_{cat} parameters some 3−4-fold lower than the native complex and overall catalytic efficiencies (k_{cat}/K_M) similarly reduced (Figure 2). The most significant effects on activity were observed with the c^{3}A$_{13}$ complex, exhibiting a k_{cat} value reduced by approximately 1 order of magnitude, and the c^{3}A$_{15.1}$ complex with a k_{cat} value reduced by nearly 2 orders of magnitude. In both cases, overall catalytic efficiencies as judged from k_{cat}/K_M values were correspondingly reduced.

As noted above, none of the adenosine N^3-nitrogens are involved in any observable hydrogen bonding interactions in the ground state hammerhead structure, based upon recent crystallographic analyses (Pley et al., 1993; Scott et al., 1995). The lack of any apparent effects upon K_M values by the deazanucleoside residues in the present study is consistent with the absence of any critical interactions involving the adenosine N^3-nitrogens (at least those that affect binding

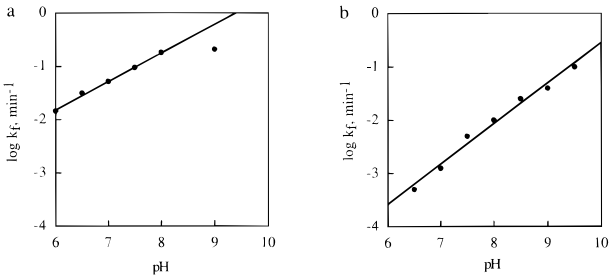

FIGURE 4: Hammerhead ribozyme-mediated cleavage rates for the analogue complexes c^{3}A$_{13}$ (a) and c^{3}A$_{15.1}$ (b) as a function of pH in the presence of Mg^{2+}.

affinity) in the ground state structure. All of the losses in catalytic efficiency are a result of effects in the cleavage event as characterized by k_{cat}. The present complex has been employed in a variety of other studies in part because the chemical cleavage event (transesterification of the scissile phosphodiester bond) is considered to be the rate-limiting step (Fedor & Uhlenbeck, 1992). Thus, reductions in the k_{cat} parameter characterizing the cleavage reaction can most easily be correlated with less effective stabilization of the transition state [see Tuschl et al.)1995)].

To confirm that effects in the chemical step were responsible for the reductions in cleavage activity observed for the c^{3}A$_{13}$ and c^{3}A$_{15.1}$ complexes, we examined the cleavage event under first-order conditions over the pH range 6.0−9.5 (Figure 4). In both cases, we observed a linear relationship with the exception of a single point in one assay at pH 9.0 (Figure 4a). A linear pH−rate profile provides strong evidence that the chemical step, rather than a conformational change, is the rate-determining step (Dahm et al., 1993). All assays were performed in the linear portion of these pH−rate profiles (pH 7.5).

The present results suggest that the adenosine N^3-nitrogens at positions 13 and particularly at 15.1 in the native complex are important in the chemical step of the cleavage reaction and are involved in important transition state stabilizing interactions. The adenosine N^3-nitrogen does not generally appear to be an effective inner sphere coordinating ligand such that it could be complexed directly with the metal (Mg^{2+} or Mn^{2+}) cofactor(s), but it could partake in interligand hydrogen bonding interactions with coordinated inner sphere water molecules. Both N^3-nitrogens could also be involved in critical non-Watson−Crick hydrogen bonds that are not present in the ground state structure, but are formed as the result of a conformational change necessary for transition state stabilization. Both of the identified sites would function as hydrogen bond acceptors, and the complementary hydrogen bond donors could be present in the various functional groups previously identified from other studies. Such donor functional groups could include the N^2-amino groups of G$_5$ and/or G$_{12}$ as well as the N^4-amino groups of C$_2$ and/or C$_{17}$ (see Figure 2). The loss of each of these amino groups results in significant reductions in catalytic efficiency for the ribozyme/substrate complex (Odai et al., 1990; Koizumi & Ohtsuka, 1991; Fu & McLaughlin, 1992b; Slim & Gait, 1992; Fu et al., 1993; Adams et al., 1994; Murray et al., 1995). Other potential hydrogen bond donors could include the 2′-hydroxyls of G$_5$, G$_8$, and/or U$_{16.1}$. The loss of each of these hydroxyl groups from the complex also results in significant reductions in the catalytic efficiency for the cleavage reaction (Yang et al., 1990, 1992; Perreault et al.,

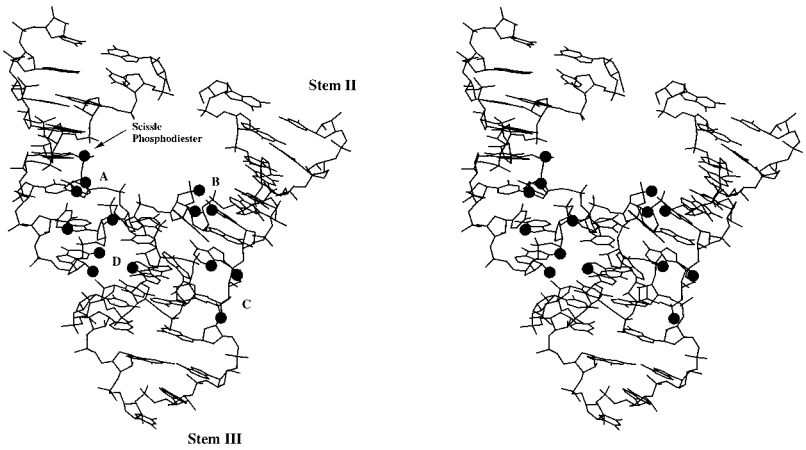

FIGURE 5: Stereoview [generated using SETOR (Evans, 1993)] of the X-ray structure of the hammerhead ribozyme complexed to an RNA substrate analogue in which the active site 2′-OH is blocked as the 2′-*O*-methyl derivative [taken from Scott et al. (1995)]. Overlaid on the structure are the sites of identified critical functional groups (solid circles) obtained from atom-mutagenesis studies described fully in the text. A, B, C, and D represent clusters of critical functional groups. For example, the two most well-defined clusters include B and D. Cluster B includes the 2′-OH of G_8, the phosphodiester linking G_8 with A_9, and the 2-amino group of G_{12}. Cluster D includes the N^3-nitrogen of $A_{15.1}$, the 2′-OH and 2-amino group of G_5, and the 2′-OH of $U_{16.1}$.

1991; Pieken et al., 1991; Fu & McLaughlin, 1992b; Williams et al., 1992).

Critical Functional Groups in the Hammerhead Ribozyme Complex. A number of functional groups critical to the efficiency of the cleavage reaction have been identified on the basis of atom-specific mutations within the conserved residues of the catalytic core. In addition to the adenosine N^3-nitrogens identified in this report, and the various potential hydrogen bond donors noted above, other identified critical functional groups include the N^7-nitrogen of A_6 (Fu & McLaughlin, 1992a; Seela et al., 1993) and the *pro-*R_p oxygens of the phosphodiester residues linking G_8 with A_9, G_{12} with A_{13}, A_{13} with A_{14} (Buzayan et al., 1990b; Ruffner & Uhlenbeck, 1990), and at the site of cleavage (van Tol et al., 1990a; Slim & Gait, 1991; Dahm et al., 1993).

A loss of activity is also observed when other analogues containing modified base residues such as 2-aminopurine (Tuschl et al., 1993), O^6-methylguanosine (Grasby et al., 1993), or 1-methylguanosine (Limauro et al., 1994) replace the conserved G residues. These studies attest to the importance of the conserved G residues, but are unable by themselves to identify a unique critical functional group. In addition to altering tautomeric equilibria, these analogues introduce a methyl group which could have steric implications for folding pathways without specifying an individual functional group as being critical for catalysis.

The location of each identified critical functional group has been noted (see Figure 5) in the stereoview of the ground state (all RNA) hammerhead structure solved recently by X-ray crystallography (Scott et al., 1995). Although few of these identified functional groups appear to make any interresidue contacts in the ground state, they could still contribute to the formation of important metal binding sites. Additionally, much like a proteinacious enzyme, a conformational change to generate the transition state may require the formation of additional stabilizing interactions involving such critical residues.

The critical functional groups highlighted in Figure 5 are loosely organized into four clusters (A−D). One of these clusters (B) includes the 2′-OH of G_8, the phosphodiester linking G_8 with A_9, and the 2-amino group of G_{12}. Upon diffusion of Mn^{2+} into crystals of the hammerhead complex containing a DNA inhibitor (Pley et al., 1994; Scott et al., 1995), the Mn^{2+} was identified at this same location in the structure bound to the N^7 of $G_{10.1}$ and the phosphodiester linking G_8 with A_9 and is similar to that described as site 2 in the Scott et al. structure (Scott et al., 1995). Two additional observations, the reduced catalytic activity upon replacement of G_{12} by 2-aminopurine riboside (Tuschl et al., 1993) or 1-methylguanosine (Limauro et al., 1994) and the virtual absence of activity when the $G_{10.1}$-$C_{11.1}$ base pair is reversed (Tuschl & Eckstein, 1993), are both consistent with this area of the structure being a possible site for metal complexation. A second cluster of functional groups (D in Figure 5) consists of the 2′-OH and 2-amino group of G_5, the 2′-OH of $U_{16.1}$, and the N^3-nitrogen of $A_{15.1}$. Additionally, the simple inversion of the stereocenter containing the G_5 2′-hydroxyl (Fu et al., 1994), or the replacement of G_5 by other G analogues (Grasby et al., 1993; Tuschl et al., 1993; Limauro et al., 1994), attests to the importance of this nucleoside residue for catalytic efficiency. In addition to these identified critical functional groups, both of the O^2-carbonyls of $C_{15.2}$ and $U_{16.1}$ are oriented toward the center of this group of residues and could complete the cluster of functional groups involved in a potential metal cofactor binding site. This cluster of functional groups lies on the outer edge of the conserved CUGA turn and includes nearby residues of stem III. This location has not been suggested as a metal binding site based upon the analysis of residual solvent peaks (Scott et al., 1995), although such analyses by themselves do not preclude the presence of critical transition state metal binding sites. An alternative role for these residues would be the formation of hydrogen bonds to stabilize a transition state structure, possibly involving the conserved CUGA turn.

The remaining two clusters of functional groups are less well-defined. One is located at the cleavage site (A in Figure 5) and consists of the *pro-*R_p oxygen of the scissile phosphodiester and the 4-amino groups of C_3 and C_{17}, and could also include the N^7-nitrogen of A_6. This site is the same as that identified in the crystal structure as site 3 (Scott et al., 1995). The remaining identified critical functional groups (C in Figure 5) include the two *pro-*R_p oxygens of the phosphodiesters linking G_{12} with A_{13} and A_{13} with A_{14}

as well as the N^3-nitrogen of A_{13}, but these residues are less well-organized about a potential cofactor binding site.

Although we have considered such clusters of critical functional groups as possible sites for metal cofactor binding, it is equally possible that interresidue hydrogen bonding interactions between critical functional groups are necessary in order to generate a hydrogen bonding network in the transition state structure necessary to effect phosphodiester bond cleavage. Recent work on the requirements for the cleavage reaction indicates that with a sufficiently labile leaving group, spermine in the absence of a metal cofactor is capable of organizing the hammerhead into a catalytically competent complex (Kuimelis & McLaughlin, 1995a), and, presumably, facilitating appropriate transition state stabilizing interactions. Nevertheless, to date there have been no clearly identified complementary functional group interactions (e.g., hydrogen bonding) that could be reversed in a manner that results in the full retention of catalytic activity for the complex.

CONCLUSIONS

The loss of either of two potential hydrogen bond acceptors from the 46-nucleotide hammerhead complex, specifically the adenosine N^3-nitrogens at A_{13} or $A_{15.1}$, results in a significant loss in catalytic efficiency. The identification of these critical functional groups, in addition to those identified from other studies, provides clusters of critical sites in the hammerhead complex that generally do not appear to be involved in any stabilizing interactions in the ground state structure. Yet the importance of these clusters of functional groups critical to the efficiency of the catalytic event suggests the presence of a hydrogen bonding network in the transition state, possibly involving the required metal cofactor(s), that is likely quite different from the hydrogen bonding network observed in the ground state crystal structure.

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