Activity of the Hammerhead Ribozyme upon Inversion of the Stereocenters for the Guanosine 2′-Hydroxyls†

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ABSTRACT: Two guanosine 2′-hydroxyls in the hammerhead RNA complex at positions G5 and G8 are critical for efficient cleavage by this RNA catalyst. These two functional groups are likely involved in the binding of the metal cofactor, or they are involved in specific interresidue hydrogen-bonding interactions. The importance of the stereochemical positioning of both critical 2′-hydroxyls was investigated by comparing the cleavage rates of three arabinosylguanine-substituted complexes (in which the positions of specific guanosine 2′-hydroxyls were stereochemically altered by inverting the C2′ stereocenter) with that of the native complex, as well as with the rates of the dG- and dFG-substituted complexes [in which the 2′-hydroxyls are absent as the result of substitution by 2′-deoxyguanosine (dG) or 2′-deoxy-2′-fluoroguanosine (dFG)]. The G5araG and G8araG complexes exhibit dramatically different cleavage rates. The G5araG complex is essentially inactive, at least 10^5-fold slower than the native complex. RNA cleavage by this analogue ribozyme is also 1000-fold slower than cleavage by either the G5dG or the G5dFG ribozyme, both of which lack the 2′-hydroxyl at G5. By comparison, catalytic efficiency of the G8araG complex as expressed by k_{cat}/K_m is comparable with that of the native complex and some 2 orders of magnitude more active than either the G8dG or the G8dFG complex.

The hammerhead RNAs represent a small class of catalytically active nucleic acid complexes capable of accelerating the transesterification of a specific phosphodiester residue [for a review, see Bruening (1989)]. The active complex consists of three helical stems and a series of eleven conserved nucleoside residues comprising the core region (see Figure 1). Nine of the conserved residues are nominally single-stranded, but they presumably fold into a specific structure in order to bind and/or optimally position the metal cofactor (Mg^{2+} or Mn^{2+}) required for catalytic activity (Usman et al., 1989; Dahm & Uhlenbeck, 1991; Olsen et al., 1991; Dahm et al., 1993). The substitution of a number of nucleoside analogues into the hammerhead sequence has permitted the identification of specific functional groups critical to the efficiency of the cleavage reaction [see Fu et al. (1993) and Tuschl et al. (1993) and references therein]. These functional groups, located on individual bases, carbohydrates, or internucleotide phosphates, may be involved in specific hydrogen-bonding or metal-coordinating interactions [see Ruffner et al. (1989)], or they may function in general acid/base chemistry [see Chowira et al. (1991) and Slim and Gait (1992)], such that the cleavage reaction proceeds rapidly. Three conserved guanine residues are present in the core sequence of nine nucleoside residues. On the basis of 2′-deoxyguanosine (dG) (Perreault et al., 1991; Williams et al., 1992; Fu & McLaughlin, 1992) and 2′-deoxy-2′-fluoroguanosine (dFG) (Williams et al., 1992) substitutions at these positions, the 2′-hydroxyls of G5 and G8 (see Figure 1) have been implicated in critical interactions necessary for effective RNA cleavage.

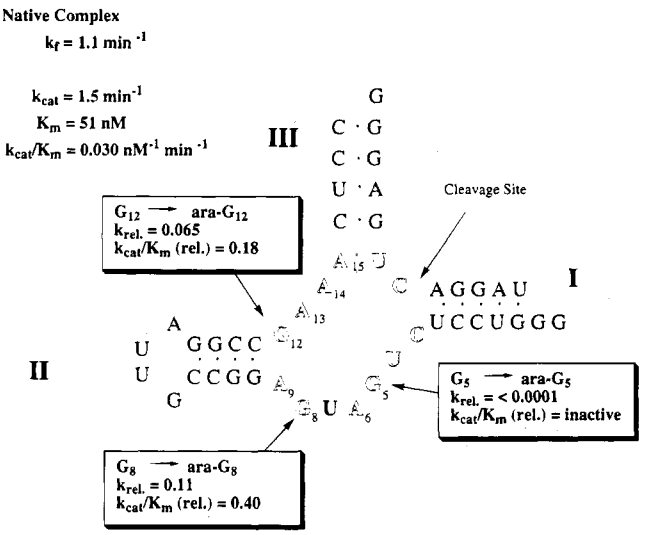

FIGURE 1: Structure of the hammerhead RNA complex with the sites of the araG modifications noted and a summary of the kinetic parameters obtained for each modified complex.

While the 2′-hydroxyls at G5 and G8 are necessary for efficient cleavage by the hammerhead complex, little is known about the optimal positioning of these functional groups within the active catalytic complex. We have examined the stereochemical flexibility of both critical hydroxyls, as well as that of G12 (also present in the core sequence), by simply altering the absolute configuration of the corresponding guanosine C2′ carbons within the ribozyme−substrate complex. Inversion of the configuration at these sites was achieved by simple replacement of the desired guanosine residue with arabinosylguanine (araG). Three RNA complexes were prepared in which G5, G8, or G12 was replaced by the araG residue (G5araG, G8araG, and G12araG, respectively), and the activity of these

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complexes has been compared with that of the corresponding native complex, as well as with the activity of six dG- and dFG-substituted complexes.

EXPERIMENTAL PROCEDURES

Materials. 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/triethylamine, 49.5:49.5:1; solvent D, hexane/ethyl acetate/triethylamine, 49.5:49.5:1; solvent E, dichloromethane/ethyl acetate/triethylamine, 49.5:49.5:1. 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 Corporation (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. ^{1}H 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 on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. Nuclease S1 is a product of United States Biochemical Corporation (Cleveland, OH). Calf intestinal alkaline phosphatase and snake venom phosphodiesterase were obtained from Boerhinger (Mannheim, FRG).

Methods

N^2-Isobutyrylarabinosylguanine (**1**). Guanine arabinoside (Hanna et al., 1988) was protected as the *N*-isobutyryl derivative using the transient protection approach described elsewhere (Ti et al., 1982; McLaughlin et al., 1985). Yield: 0.99 g, 90%, as an off-white solid. R_f (solvent A): 0.1. UV (methanol): λ_{max} = 205, 254, 275 nm; λ_{min} = 222, 267 nm. ^{1}H-NMR (DMSO + trace of D_2O): δ = 1.10 (d, 6H, CH-(CH_3)$_2$), 2.75 (m, 1H, C*H*(CH_3)$_2$), 3.62 (m, 2H, $H_{5'}$, $H_{5''}$), 3.80 (m, 1H, $H_{4'}$), 4.10 (m, 2H, $H_{3'}$,$H_{2'}$), 6.15 (d, 1H, $H_{1'}$), 8.10 (s, 1H, H_8) ppm.

N^2-Isobutyryl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)arabinosylguanine (**2**). Compound **1** (0.49 g) was converted to the corresponding 3′,5′-*O*-(1,1,3,3,-tetraisopropyldisiloxane) derivative using essentially the procedures described eleswhere (Gait, 1984). Yield: 500 mg, 76%. R_f (solvent B): 0.32. UV (methanol): λ_{max} = 201, 252, 275 nm; λ_{min} = 222, 267 nm. ^{1}H-NMR ($CDCl_3$ + trace of D_2O): δ = 1.00−1.40 (m, 34H,4 CH(CH_3)$_2$ of tetraisopropyl, CH (C*H*$_3$)$_2$ of isobutyryl), 2.62 (m, 1H, C*H*(CH_3)$_2$ of isobutyryl), 3.80−4.12 (m, 3H, $H_{5'}$, $H_{5''}$, $H_{4'}$), 4.50 (m, 1H, $H_{3'}$), 4.75 (m, 1H, $H_{2'}$), 6.05 (d, 1H, $H_{1'}$), 7.86 (s, 1H, H_8) ppm.

N^2-Isobutyryl-2′-O-acetyl-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)arabinosylguanine (**3**) *and N^2-Isobutyryl-2′-O-acetylarabinosylguanine* (**4**). Compound **2** (0.4 g, 0.67 mmol, 1 equiv) in pyridine solution (10 mL) was treated with 4.4 mL of THF/lutidine/acetic anhydride (8:1:1) to which 4.4 mL of a solution of 10% *N*-methylimidazole in THF was added. After the mixture was shaken for 20 min, TLC analysis in solvent B indicated the disappearance of starting material and the appearance of a higher spot (a small-scale run of this reaction was conducted first, and the newly formed higher spot was purified by silica column chromatography. Proton NMR confirmed that the new spot is compound **3**). The reaction was stopped with ice, and the mixture was diluted with 20 mL of CH_2Cl_2 followed by extraction with H_2O and saturated $NaHCO_3$. The organic phase containing compound **3** was dried with Na_2SO_4 and was coevaporated three times from pyridine. This residue was treated with 2 mL of 1.0 M tetrabutylammonium fluoride in THF for 7 min at ambient temperature. TLC in solvent A showed that the reaction was complete. The reaction mixture was diluted with 2 mL of CH_2Cl_2 and immediately loaded on a 20-g short silica column. Compound **4** was eluted with a MeOH/CH_2Cl_2 gradient. Yield of compound **4**: 87%. R_f (solvent A): 0.59 (compound **3**), 0.35 (compound **4**). UV (methanol) for **4**: λ_{max} = 202, 252, 275 nm; λ_{min} = 220, 266 nm. ^{1}H-NMR (DMSO + trace of D_2O) of compound **4**: δ = 1.10 (d, 6H, CH(CH_3)$_2$), 1.80 (s, 3H, $COCH_3$), 2.75 (m, 1H, C*H*(CH_3)$_2$), 3.60−3.80 (m, 2H, $H_{5'}$, $H_{5''}$), 3.90 (m, 1H, $H_{4'}$), 4.18 (m, 1H, $H_{3'}$), 5.22 (t, 1H, $H_{2'}$), 6.34 (d, 1H, $H_{1'}$), 8.14 (s, 1H, H_8) ppm.

N^2-Isobutyryl-5′-O-(4,4′-dimethoxytrityl)-2′-O-acetylarabinosylguanine (**5**). Compound **4** (0.22 g, 0.55 mmol, 1 equiv) was coevaporated twice from pyridine and was then dissolved in 10 mL of dry pyridine. The reaction mixture was chilled in an ice bath, and dimethoxytrityl chloride (0.25 g; 0.73 mmol; 1.3 equiv) was added. The reaction mixture was stirred overnight in an ice bath. TLC analysis (solvent B) indicated that some starting material had not reacted. Another 1.6 equiv of dimethoxytrityl chloride was added to the reaction mixture over a 5-h time period, and TLC in solvent B indicated that the reaction was nearly complete and that a trace amount of bis(DMT) side product had appeared. The reaction was stopped by cooling in an ice bath and adding 100 mg of imidazole followed by 2 mL of MeOH. After standard workup, the mixture was dissolved in a small amount of solvent C and added to a short column silica gel (20 g) which was packed in hexane. The product was eluted with an ethyl acetate/hexane gradient. Yield: 300 mg, 80%, as a white solid. R_f (solvent B): 0.26. UV (methanol): λ_{max} = 202, 231, 274 nm; λ_{min} = 218, 265 nm. ^{1}H-NMR ($CDCl_3$ + trace of D_2O): δ = 1.25 (m, 6H, CH-(CH_3)$_2$), 1.76 (s, 3H, $COCH_3$), 2.62 (m, 1H, C*H*(CH_3)$_2$), 3.40−3.58 (m, 2H, $H_{5'}$, $H_{5''}$), 3.77 (s, 6H, OCH_3), 4.10 (m, 1H, $H_{4'}$), 4.60 (m, 1H, $H_{3'}$), 5.21 (m, 1H, $H_{2'}$), 6.33 (d, 1H, $H_{1'}$), 6.80−7.42 (m, 13H, Ar-H), 7.75 (s, 1H, H_8) ppm.

N^2-Isobutyryl-5′-O-(4,4′-dimethoxytrityl)-2′-O-acetyl-3′-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphinyl]arabinosylguanine (**6**). Anhydrous compound **5** (0.25 g, 0.39 mmol, 1 equiv) was suspended in 5 mL of anhydrous CH_2Cl_2 (filtered though alumina basic sieves), and 400 μL of diisopropylethylamine (6 equiv) was added. The reaction mixture was placed in an ice bath, and *N*-methylimidazole (0.15 mmol, 0.5 equiv) was added, followed by (*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphonamidic chloride (0.78 mmol, 2 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 2 mL of ice-cold 10% Na_2CO_3 was added slowly to destroy excess phosphitylating reagent. The reaction mixture was diluted in 20 mL of EtOAc and extracted with 20 mL of 10% Na_2CO_3. The organic phase was dried with Na_2SO_4, and the solvent was removed. The residue was then dissolved in 2 mL of solvent D and was purified by chromatography on 20 g of silica gel packed with hexane (1% triethylamine) using a gradient of ethyl acetate. The fractions containing product were evaporated and precipitated into 140 mL of ice-cold hexane to yield crystals. Yield: 270 mg, 75%, as an off-white powder. R_f (solvent E): 0.57, 0.62. ^{31}P-NMR ($CDCl_3$), δ = 150.4, 150.8 ppm. ^{1}H-NMR ($CDCl_3$): H_1: δ = 6.26 (d) and 6.32 (d) ppm.

RNA Preparation. The RNA fragments were synthesized using phoshoramidite-based technology (Matteucci & Caruthers, 1981) modified as described for RNA (Usman et al., 1987; Wu et al., 1989). Each sequence was treated with 2 mL of 3:1 (v/v) concentrated aqueous NH_4OH/ethanol for 16 h at 55 °C in a screw-cap glass vial. The resulting solution was evaporated to complete dryness and re-evaporated from a small amount of ethanol, and the residue was taken up in 0.5−1.0 mL of 1.0 M TBAF in THF and incubated for 17−24 h at ambient temperature protected from light. Then 1.5 mL of 1.5 M sodium acetate (pH 6.0) was added, and the resulting solution was extracted twice with 1.5 mL of ethyl acetate. The aqueous phase containing RNA was concentrated to about 0.3 mL, and then 0.9 mL of chilled absolute ethanol was added. The ethanol precipitation was performed after incubation of the solution overnight at −20 °C. The RNA precipitate was collected by centrifugation at 13 000 rpm for 20 min at 4 °C, and the pellet was dissolved in 200−500 μL of distilled water. The crude RNA material was purified by gel electrophoresis using 20% polyacrylamide/1% bis(acrylamide)/7 M urea gels (14 × 32 cm) in 89 mM Tris−borate buffer and 2 mM Na_2EDTA, pH 8.0 (1× TBE buffer), at 15 mA overnight. The product bands were visualized by UV shadowing and excised. The gel pieces were either electroeluted using a Elutrap or extracted with 0.3 M sodium acetate (pH 6.0). The resulting RNA solution was ethanol precipitated or was desalted with a C_{18} Sep-Pak cartridge. Quantities of RNA were estimated using an extinction coefficient of 6.6×10^3 M^{-1} cm^{-1} per nucleotide as described previously (Fedor & Uhlenbeck, 1990).

RNA Labeling. The substrate 12-mer was 5′-end-labeled with [γ-^{32}P]ATP as follows: A 100-μL reaction mixture containing 1 A_{260} unit of 12-mer substrate (~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 (5 mCi in 33 μL), 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 labeled substrate was repurified by electrophoresis in a 20% polyacrylamide/7 M urea gel (14 × 32 cm). The product band was detected by UV shadowing or autoradiography, cut out, and extracted with 0.1 M ammonium acetate or 0.3 M sodium acetate (pH 6.0) by a thawing−freezing method (Pieken et al., 1991) followed by desalting through a C_{18} Sep-Pak cartridge. The specific activity of the labeled substrate was typically 0.01 μCi/pmol. Concentrations of RNA solutions were determined by assuming an extinction coefficient per nucleotide at 260 nm of 6.6×10^3 M^{-1} cm^{-1} (Fedor & Uhlenbeck, 1990).

Conditions for Stoichiometric Cleavage Analyses. Two 25-μL solutions containing either 1.2 μM ribozyme or 0.2, 0.12, or 0.06 μM substrate in 50 mM Tris-HCl, pH 7.5, were heated 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 reaction was initiated by mixing the two solutions (final ribozyme concentration = 0.6 μM; final substrate concentration = 0.1, 0.06, or 0.03 μM). Aliquots of 5−7 μL were withdrawn at appropriate time intervals, 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_2EDTA, pH 8.0. After autoradiography, the substrate and product bands were excised and lyophilized to dryness and the amount of radioactivity was determined by scintillation counting. For each complex the fraction of cleaved substrate was normalized to the percentage of cleavage after extended incubation (which was usually longer than 10 half-lives, and the final extent of cleavage ranged from 80 to 90%, with the exception of the G5araG complex) before plotting. 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 ($k = 0.693/t_{1/2}$).

Steady-State Cleavage Analyses. The procedures described below are similar to those reported previously (Fedor & Uhlenbeck, 1990; Pieken et al., 1991). Solutions of varying substrate concentrations were prepared separately in a 25-μL volume containing 50 mM Tris-HCl (pH 7.5), and each was heated separately to 95 °C for 1 min and cooled to 25 °C for 15 min.[1] A solution of 4 nM ribozyme in 50 mM Tris-HCl (pH 7.5) was also preheated and cooled. Then each solution was adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. The reaction was initiated by addition of 25 μL of ribozyme solution to the 25-μL substrate solutions, which resulted in a final ribozyme concentration of 2 nM. Six substrate concentrations were used, varying from 10 to 300 nM depending on the individual sequences. 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. The experimental error for different isolated batches of ribozyme is about a factor of 2.

Mg^{2+} Rescue Experiments. Three 25-μL solutions containing 1.2 μM ribozyme and different concentrations of $MgCl_2$ (20 mM, 200 mM, or 1 M) were prepared. A substrate solution at 0.2 μM was prepared separately in 50 mM Tris-HCl, pH 7.5. Both ribozyme and substrate solution were heated to 95 °C for 1 min and cooled at 25 °C for 30 min. The reaction was initiated by mixing the two solutions (final ribozyme concentration = 0.6 μM; final substrate concentration = 0.1 μM; final [$MgCl_2$] = 10, 100, or 500 mM). Aliquots of 5−7 μL were withdrawn at appropriate

[1] The G8araG ribozyme solution could be incubated with 10 mM Mg^{2+} for either 15 min or overnight before reaction. The kinetic parameters derived under both conditions were similar.

time intervals ranging from 0 to 60% total cleavage. The 10 mM Mg^{2+} 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 100 mM Mg^{2+} reaction was stopped by adding 2 vol of the above quenching buffer; and the 500 mM Mg^{2+} reaction was stopped by adding 2 vol of the above quenching buffer, except the concentration of Na_2EDTA was increased to 0.45 M. The extents of cleavage were analyzed as described above.

Determination of Equilibrium Dissociation Constant for the G5araG Hammerhead Complex. A gel retardation assay was employed to measure the equilibrium dissociation constant for essentially inactive G5araG complex. The procedures described below are essentially the same as those reported previously (Fedor & Uhlenbeck, 1992). Substrate 12-mer RNA was 5′-end-labeled as described above. The end-labeled substrate was separated from its unlabeled counterpart by denaturing gel electrophoresis so that the specific activity of labeled RNA was the same as that of the [γ-^{32}P]ATP (about 6000 Ci/mmol). Separate solutions of [5′-^{32}P]substrate and G5araG ribozyme were heated to 95 °C for 1 min and cooled at 25 °C for 15 min in 50 mM Tris-HCl, pH 7.5, 5% sucrose, 0.02% xylene cyanol, and 0.02% bromophenol blue. Each solution was adjusted to 10 mM $MgCl_2$ and incubated at 25 °C for 15 min. After the ribozyme and substrate solutions were combined, the mixture was incubated at 25 °C for 17 h to 2 days. Eight to 12 ribozyme concentrations ranging from 0.8 to 500 nM were used, while substrate concentration was at least 5-fold lower than the lowest ribozyme concentration. Bound and unbound end-labeled substrate were separated by nondenaturing polyacrylamide gel electrophoresis in 15% polyacrylamide (19:1 ratio of acrylamide to bis(acrylamide), 14 × 24 × 0.15 cm) in 50 mM Tris−acetate buffer, pH 7.5, and 10 mM magnesium acetate. The nondenaturing gels were first prerun for 2 h at 10 W, and then the samples were loaded to electrophorese overnight at 7 W and 15 °C. In our hands, bound and unbound substrate could not be resolved by nondenaturing gel electrophoresis at 25 °C, but such resolution could be obtained at 15 °C. To attain this temperature, the gel was immersed in a water bath and a refrigerated recirculating water pump was used to maintain temperature. After autoradiography, the bound and unbound substrate were excised and lyophilized to dryness, and the radioactivity was determined by scintillation counting. The values of bound substrate fraction at each ribozyme concentration were normalized to the value of bound substrate fraction obtained at a ribozyme concentration that was at least 100 times higher than the K_d. The fraction of bound substrate was plotted against ribozyme concentration, and the K_d value was determined by computing hyperbolic fitting of the binding curve.

RESULTS

Incorporation of an araG residue into the core sequence (Figure 1) shifts the position of the associated 2′-hydroxyl relative to the corresponding ribose derivative by approximately 109° as a result of the inversion of the C2′ stereocenter (see Figure 2), but associated changes in sugar puckering could result in more extensive changes in location of this hydroxyl when present in an RNA sequence. Repositioning each of the 2′-hydroxyls critical for efficient

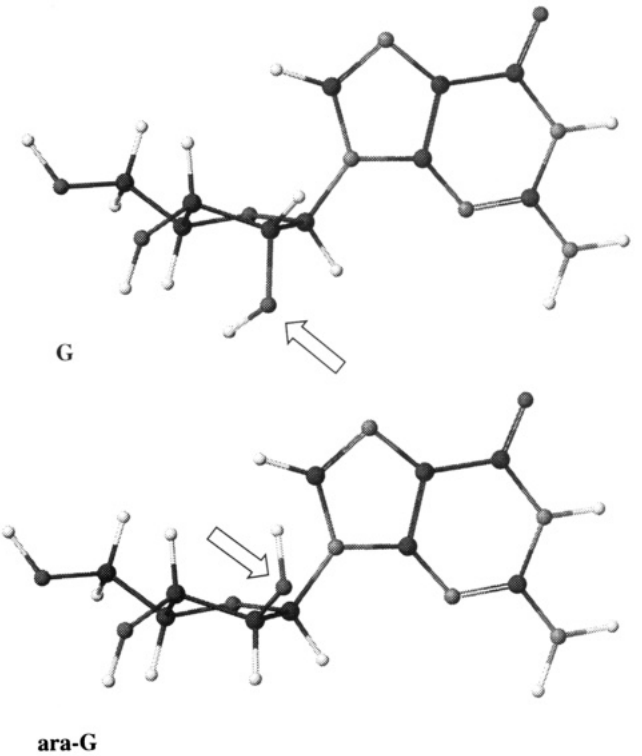

FIGURE 2: Structures of guanosine (G) and arabinosyl guanine (araG). Arrows note the relative positions of the 2′-hydroxyls.

cleavage by the hammerhead ribozyme (at G5 and G8) could result in one of two obvious but diametrically opposed catalytic effects in the hammerhead RNA: (i) the hydroxyl of the arabinosyl residue could still participate in critical interactions involving direct magnesium binding or the formation of specific hydrogen-bonding interactions that result in a conformation with high magnesium affinity or (ii) movement of the hydroxyl could disrupt or otherwise alter such interactions critical for efficient cleavage by the ribozyme and reduce the corresponding catalytic activity to a level similar to that observed for complexes lacking this critical functional group (i.e., the dG- or dFG-substituted complexes).

Stoichiometric Cleavage Analyses. The three complexes of interest, G5araG, G8araG, and G12araG, each containing a single arabinosylguanine residue within the core sequence, were prepared chemically using the appropriately protected araG phosphoramidite and were purified and analyzed as described previously (Pieken et al., 1991; Benseler et al., 1993). Under stoichiometric conditions, 25 °C and 10 mM Mg^{2+}, and with substrate concentrations 6-, 10-, and 20-fold less than the ribozyme concentration, the native complex exhibited first-order rate constants that varied from 1.1 to 1.4 min^{-1}. Under these conditions, and irrespective of the substrate concentration, two of the modified complexes, G8araG and G12araG, exhibited relative cleavage rates that were reduced by roughly an order of magnitude (average k_{rel} = 0.11 and 0.065) relative to the native complex. By comparison, no cleavage activity could be detected for the G5araG complex even after an incubation period of 24 h.

Equilibrium Binding Constant for the G5araG Complex. Two likely possibilities existed to explain the total lack of cleavage activity by the G5araG ribozyme: (i) the ribozyme failed to bind the substrate sequence or (ii) the ribozyme substrate sequence was formed, but was simply an inactive

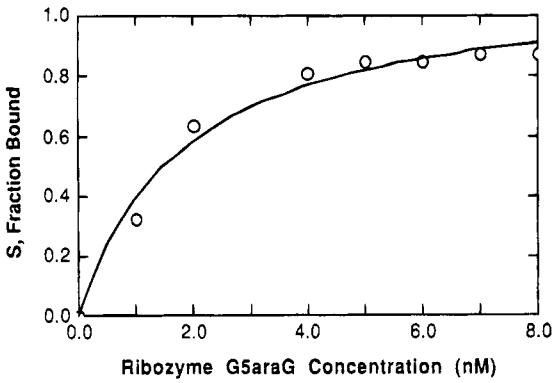

FIGURE 3: Determination of the equilibrium dissociation constant for the catalytically inactive G5araG ribozyme. The fraction of substrate (S) bound is shown as a function of ribozyme concentration.

Table 1: Kinetic Parameters for Native and Modified Hammerhead RNA Complexes

complex	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (nM^{-1} min^{-1})	k_{cat}/K_m (rel)
native	51	1.5	0.030	1.0
G5araG	*a*	*a*	*a*	*a*
G8araG	11	0.13	0.012	0.40
G12araG	40	0.22	0.0055	0.18
G5dG				0.0067[b]
G8dG				0.0067[b]
G12dG				1.3[b]
G5dFG				0.0034[b]
G8dFG				0.0027[b]
G12dFG				0.093[b]

[a] Inactive under the conditions used (see Experimental Procedures). [b] Values taken from Williams et al. (1992).

or dead-end complex. To discriminate between the two possibilities, we examined complex formation using a gel shift assay (Pyle et al., 1990; Fedor & Uhlenbeck, 1992). Binding isotherms were obtained for three different experiments using the gel retardation assay, and one example is illustrated in Figure 3. The K_d values obtained from this assay varied from 1.0 to 2.4 nM with an average value for the three assays of 1.7 nM. This value is generally in good agreement with that obtained previously by Fedor and Uhlenbeck (1992). In that work a ribozyme complex containing 12 interstrand (ribozyme−substrate) base pairs was characterized by a K_d of 0.5 nM using a gel shift assay at 25 °C. The present complex contains only 10 interstrand base pairs and results in a K_d value only 3-fold higher.

Steady-State Cleavage Analyses. Under steady-state conditions, kinetic parameters could be obtained for both the G8araG and the G12araG complex (Table 1). Relative to the native complex, the G8araG complex exhibits a k_{cat} value reduced by 1 order of magnitude, but this reduction in turnover number is compensated by a corresponding reduction in the K_m value such that the overall catalytic efficiency as expressed by k_{cat}/K_m is altered by less than 3-fold. The G12araG complex exhibits a K_m value nearly identical with that of the native complex, but a slight reduction in the turnover number results in a k_{cat}/K_m relationship that is reduced by 5−6-fold. The observed k_{cat} value for this complex (0.22 min^{-1}) is some 3-fold higher than the corresponding first-order rate obtained under stoichiometric cleavage conditions. Although some of this variation can be attributed to experimental error (different isolations of the same ribozyme sequence commonly result in cleavage variations by as much as a factor of 2), possible aggregation of the ribozyme when is it present in large excess could additionally account for this variation.

The steady-state kinetic parameters for these araG-containing complexes can be compared with those reported (Williams et al., 1992) for the dG and dFG complexes in which the guanosine 2′-hydroxyls have been eliminated from the complex at the corresponding 8 and 12 positions (values are reproduced in Table 1). Both the G8dG and the G8dFG complex are much poorer catalysts with k_{cat}/K_m values that are reduced by 150-fold and 370-fold, respectively, relative to the native complex (Williams et al., 1992). Those results have implicated the 2′-hydroxyl at G8 as a critical functional group for efficient cleavage of a substrate sequence by the hammerhead ribozyme. The G8araG complex, which contains a 2′-hydroxyl but is of inverted stereochemistry, cleaves the target RNA nearly as well as does the native ribozyme. This catalytic efficiency suggests that repositioning the 2′-hydroxyl within the catalytic core by inversion of the C2′-carbon does not eliminate the critical interactions present in the native complex involving this 2′-hydroxyl.

Replacement of G12 by dG has little effect upon catalytic activity [k_{cat}/K_m (rel) = 1.3] (Williams et al., 1992) (Table 1), while the corresponding replacement by dFG results in a 10-fold decrease in activity [k_{cat}/K_m (rel) = 0.093]. The G12araG complex exhibits a relative k_{cat}/K_m value of 0.18, suggesting a moderate effect upon catalysis, although the G12araG ribozyme appears to be a slightly less efficient catalyst than the G8araG ribozyme, and the 5-fold reduction in the k_{cat}/K_m value suggests that the arabinosyl analogue may have some modulating effect on catalytic activity. But neither the results for the dG and dFG substitutions reported earlier (Williams et al., 1992) nor those for the araG substitutions reported here implicate the involvement of the guanosine 2′-hydroxyl at G12 in a critical interaction necessary for efficient cleavage activity.

Magnesium Rescue Experiments. The absence of catalytic activity in the G5araG complex, which differs from the native sequence by the inversion of single stereocenter, is surprising and suggests that the G5 hydroxyl is intimately involved in the cleavage mechanism. The only other analogue complexes which exhibit such a complete lack of cleavage activity are those in which the active-site nucleophile (the 2′-hydroxyl at C17) has been deleted (Koizumi et al., 1989; Yang et al., 1990). One explanation for the inactivity of the G5araG ribozyme is the inability of the complex to effectively bind and/or position the magnesium cofactor such that it participates in catalysis. If the 2′-hydroxyl at G5 is involved in binding the magnesium cofactor through coordination or by the formation of interligand hydrogen bond(s), loss of this interaction (by the substitution of dG, dFG, or araG) might simply reduce the Mg^{2+} affinity of the ribozyme−substrate complex, whereas shifting the position of the 2′-hydroxyl (by inversion of the C2′ stereocenter) might also shift the position of the magnesium cofactor within the active site and have more dramatic effects upon catalysis. To compare magnesium effects for the dG5 and araG5 complexes, we have monitored the cleavage activity for both complexes at increased concentrations of magnesium under stoichiometric cleavage conditions. For the G5dG complex (lacking the 2′-hydroxyl) cleavage efficiency was characterized by first-order rate constants of 0.0028, 0.022,

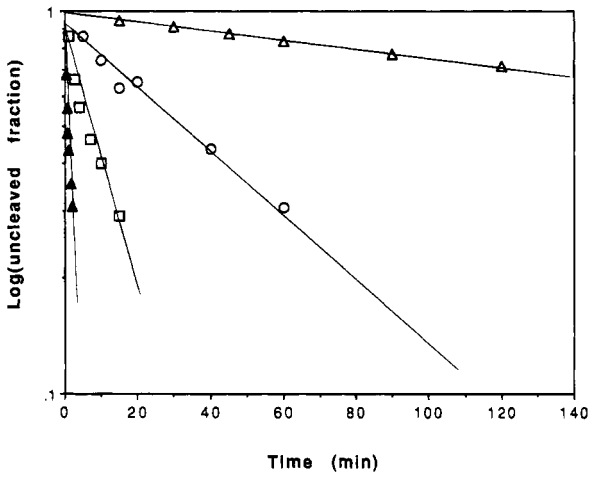

FIGURE 4: First-order kinetic plots for the native complex at 10 mM Mg^{2+} (▲) and the G5dG complex at 10 (△), 100 (○), and 500 mM Mg^{2+} (□).

and 0.1 min^{-1} at 10, 100, and 500 mM Mg^{2+}, respectively (Figure 4). The 36-fold increase in cleavage rate with increasing cofactor concentration indicates that the activity of the dG5 complex could be largely rescued by simply providing a higher concentration of Mg^{2+}. At 500 mM Mg^{2+}, the dG5 complex remains some 10-fold less active than the native complex in the presence of 10 mM Mg^{2+}. This rescue of cleavage activity with increased concentrations of metal cofactor is more dramatic than those recently reported (Tuschl et al., 1993) for an increase in Mg^{2+} concentration from 10 to 50 mM, but these variations in effects are likely the result of different ranges of magnesium concentrations used in the previous and present reports.

Increased concentrations of Mg^{2+} had some effect on the activity of the G5araG ribozyme, but these effects were more difficult to quantitate (reported values have been corrected for background cleavage). No cleavage was observed for the G5araG ribozyme at 10 mM Mg^{2+}. At 100 mM Mg^{2+}, 2% of the substrate was cleaved after 6 h, and this value had risen to about 5% after 24 h. At 500 mM Mg^{2+}, approximately 6% cleavage was detected after 3 h, about 10% was detected after 6 h, and nearly 19% was observed after a 24-h period of incubation. From this last set of cleavage data, a half-life of 96 h can be estimated and a corresponding first-order rate constant of 0.00012 min^{-1} can be approximated. This rate, obtained at the highest concentration of magnesium, remained some 1,000-fold slower than the corresponding G5dG complex under the same conditions [$k_f = 0.10$ (dG5) vs $k_f = 0.00012$ (araG5), both at 500 mM Mg^{2+}]. At this high concentration of metal cofactor, cleavage by the G5araG complex is still about 10^4-fold slower than that of the native complex at 10 mM Mg^{2+}. We estimate that the cleavage rate for the G5araG complex at 10 mM Mg^{2+} is at least 10^5-fold slower than that of the native complex ($k_{rel} < 0.00001$).

DISCUSSION

The cleavage rates observed for single substitutions by the araG nucleoside do not parallel those reported from substitutions with dG or dFG. In one case, the replacement of G12 by araG results in a cleavage rate very similar to those reported for the G12dG and G12dFG complexes. This result suggests that the inversion of the C2′ carbon and the repositioning of the attached hydroxyl does not result in a local or global change in conformation that significantly impacts the cleavage efficiency of this analogue ribozyme. This result is in good agreement with those reported for the G12dG and G12dFG complexes (Williams et al., 1992). On the other hand, the results obtained for the similar araG substitutions at positions G5 and G8 are quantitatively different from those for the corresponding dG and dFG substitutions. Although the G5dG, G5dFG, and G5araG complexes are all poor cleavage catalysts, the latter complex is remarkably inefficient, exhibiting cleavage activity estimated to be some 3 orders of magnitude lower than that of either the G5dG or the G5dFG complex, suggesting that folding or other specific interactions with the metal cofactor are quantitatively different in this complex. By comparison, the G8araG complex is a dramatically more effective catalyst than either the G8dG or the G8dFG complex (see Table 1). This observation suggests that the hydroxyl at G8 can partake in the critical interactions at this site from either the *R* or the *S* configuration, and further suggests a significant degree of flexibility in the catalytically competent structure.

The extremely poor activity of the G5araG complex could result from either the inability of the analogue complex to effectively bind the substrate sequence or the inability of the bound complex to proceed to substrate cleavage. The gel shift assay indicates that the araG5 ribozyme binds the substrate sequence with an affinity that is very similar to that reported previously for a similar native ribozyme complexed to an inactive substrate (Fedor & Uhlenbeck, 1992). Therefore, the poor cleavage activity by the G5araG complex must result from its inability to effectively bind or position the magnesium cofactor, or from the formation of other interactions involving the 2′-hydroxyl that result in an inactive cleavage complex.

The involvement of the G5 2′-hydroxyl in the binding of the magnesium cofactor or its involvement in some other interaction that is critical to the formation of a complex capable of binding the magnesium cofactor provides one explanation for the sensitivity of the G5dG (or dFG) complex. A 36-fold increase in cleavage rate for the G5dG complex was observed with an increase in the concentration of magnesium ion. On the other hand, the activity of the G5araG complex could not be as effectively rescued at high concentrations of magnesium, in spite of the fact that the arabinose sugar provides a 2′-hydroxyl at the critical G5 site. The G5araG complex differs from the native complex at a single C2′ stereocenter. If the 2′-hydroxyl of the arabinose sugar is unable to participate in a specific interaction necessary for efficient cleavage to occur, one would expect that the activity of the G5araG derivative should be similar to that of the G5dG (or G5dFG) ribozyme(s). The observed inactivity of the G5araG complex (reduced by 10^3 relative to G5dG) indicates that the presence of the arabinose sugar at position 5, and the corresponding repositioning (rather than the deletion) of the 2′-hydroxyl, results in a much poorer catalyst than that obtained simply by removing this same hydroxyl. At least two interpretations could account for these results: (i) Repositioning the 2′-hydroxyl could result in a global change in the folding pathway leading to the structure of the active ribozyme−substrate complex; an inactive or dead-end complex would result. (ii) Alternatively, a more subtle effect could be present in which an essentially normal conformation is formed, but a change in the stereochemical

position of the G5-hydroxyl results in the repositioning of the magnesium cofactor; in this alternative binding mode, the cofactor is largely ineffective in transition-state stabilization and in promoting transesterification of the target phosphodiester residue.

CONCLUSIONS

Previous reports have suggested, on the basis of nucleoside analogue studies, that two of the three guanosine 2′-hydroxyls (at G5 and G8) in the core sequence are critical for efficient cleavage by the hammerhead ribozyme (Perreault et al., 1991; Williams et al., 1992; Fu & McLaughlin, 1992). Repositioning each of these hydroxyls by inverting the stereochemical configuration of the corresponding C2′ carbons has two dramatically different effects. Inversion of this stereochemical center at G5, a single site within the 46-nucleotide complex, virtually eliminates any measurable cleavage activity for the G5araG ribozyme. We interpret this lack of activity as resulting from differences in the folding of the arabinose-containing complex and/or the mode in which the hydroxyl of the arabinose sugar is able to participate in critical interactions neceassary for cleavage activity. By comparison, the G8araG ribozyme is almost as effective in catalytic activity as the native sequence. This latter activity is in stark contrast to that observed with the G8dG and G8dFG ribozymes in which the G8 hydroxyl has been removed, resulting in a reduction in catalytic activity by at least 2 orders of magnitude (Williams et al., 1992), which suggests that the critical interactions involving the G8 2′-hydroxyl can occur in a catalytically active complex regardless of the stereochemical configuration at this C2′ site. The third guanosine 2′-hydroxyl (present at G12) does not appear to be a functional group that is critical for efficient cleavage activity on the basis of dG and dFG substitutions, and incorporation of the arabinose sugar at this site has only moderate effects upon cleavage activity.

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