Importance of Exocyclic Base Functional Groups of Central Core Guanosines for Hammerhead Ribozyme Activity†

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Received July 12, 1993; Revised Manuscript Received August 19, 1993®

ABSTRACT: The three guanosines of the central core of a hammerhead ribozyme were replaced by 2-aminopurine ribonucleoside, xanthosine, isoguanosine, inosine, and deoxyguanosine. These analogues were incorporated by automated solid-phase synthesis, with the exception of isoguanosine. This was introduced by ligating a donor, which carried the isoguanosine at its 5′-end, and an acceptor oligoribonucleotide by a T4 DNA ligase-catalyzed reaction. Most of these modifications lowered the rate constant of cleavage by the hammerhead ribozyme drastically. Inspection of the possible hydrogen-bonding interactions disturbed by these modifications suggests that there is no $G^{12}A^9$ or $A^{13}G^8$ mismatched base pair in the central region. Increasing the Mg^{2+} concentration from 10 to 50 mM did not enhance these rates appreciably. This makes it improbable that the guanosines, including their 2′-hydroxyl groups, are involved in the binding of the catalytically active Mg^{2+}. Transition-state destabilizing energies of 0.6–4.7 kcal mol^{-1} suggest that essentially all guanosines are involved in a hydrogen-bonding network.

Hammerhead ribozymes acting in *trans* contain two single-stranded regions which anneal to the substrate with the formation of two helices, helices I and III (Symons, 1992). A third helix (helix II) which is normally closed by a tetraloop does not interact with the substrate but contributes to the stabilization of the transition state in the catalytic reaction (Tuschl & Eckstein, 1993). A fourth structural element is the core region, which is usually represented as single stranded. In contrast to those in the three helices, the nucleotides in this region cannot be replaced by others, except for the nucleoside at position 7 (Figure 1). The way in which this core region confers catalytic competence on the ribozyme is an as yet unanswered question. The central core structure of the ribozyme/substrate/metal ion complex has so far escaped elucidation either by spectroscopic methods such as NMR or by X-ray crystallography. In an effort to unravel structural information about this complex, several studies have been undertaken. These include looking at potential intramolecular interactions through hydrogen bonding or complexation with the obligatory metal ion. All of these employ nucleoside analogues to probe regions which cannot be changed without a penalty in catalytic efficiency. Such studies include replacement of the 2′-hydroxyl group by a hydrogen or fluorine atom or by amino or alkoxy groups (Fu & McLaughlin, 1992a; Olsen et al., 1991; Paolella et al., 1992; Perreault et al., 1990, 1991; Pieken et al., 1991; Williams et al., 1991). The conclusion from all these studies is that the 2′-hydroxyl groups of guanosines 5 and 8 are important for optimal catalytic activity.

Another line of investigation has concentrated on investigating the possible role of the purine bases in the core region. Guanosines 5, 8, and 12 have been replaced by inosine (Fu & McLaughlin, 1992a; Odai et al., 1990; Slim & Gait, 1992). Purine nucleoside (Fu & McLaughlin, 1992a; Slim & Gait, 1992) and 7-deazaadenosine (Fu & McLaughlin, 1992b) have been introduced for adenosines 6, 9, 13, 14, and 15. Slim and Gait (1992) suggested, without proposing a particular structure, that there might be a G/A double-mismatch base pair at positions 8/13 and 9/12. Fu and McLaughlin (1992a,b) have published a model for the Mg^{2+} binding involving nucleotides at positions 5, 6, 7, and 8.

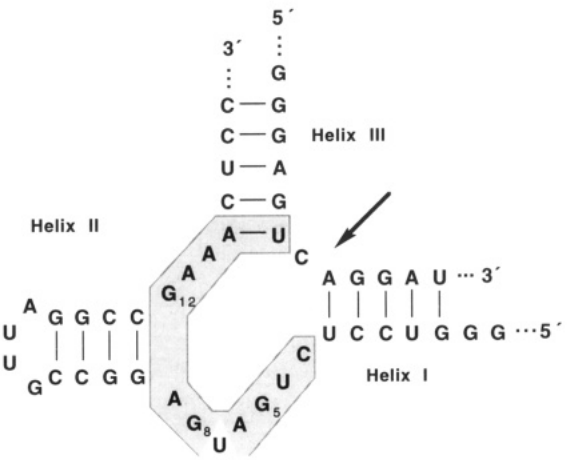

FIGURE 1: Structure of ribozyme: shaded area, central core region.

We have extended this approach by determining the activities of ribozymes in which the guanosines in the core region are substituted by 2-aminopurine nucleoside, xanthosine, isoguanosine, inosine, or deoxyguanosine. The results obtained with these analogues are consistent neither with the existence of G/A mismatches nor with an involvement of these guanosines in the binding of the catalytic metal ion. They suggest that the guanosines might be part of a hydrogen-bonding network.

MATERIALS AND METHODS

HPLC grade methanol and acetonitrile were purchased from Baker (Deventer, Holland); pro analysis solvents acetone, dichloromethane, chloroform, ethyl acetate, *n*-hexane, and toluene were obtained from Merck (Darmstadt, FRG); and triethylamine and *N*,*N*-dimethylformamide were from Fluka AG (Neu-Ulm, FRG). Dry pyridine, 1,4-dioxane, and THF

† This work was financially supported in part by the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie.

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® Abstract published in *Advance ACS Abstracts,* October 1, 1993.

0006-2960/93/0432-11658$04.00/0

(all less than 0.01% H_2O) from Merck were stored over 4-Å molecular sieves (Merck). Dichloromethane used in the phosphitylation reaction was stored over sodium–lead alloy (10% Na; Merck) and passed down a column of basic alumina (Alumina B-Super I, ICN Biomedicals, Eschwege, FRG) immediately prior to use. TBDMSCl,[1] DMTCl, TMSCl, *N*-ethyldiisopropylamine, *N*-methylimidazole, DMAP, and Raney nickel (slurry in water) were obtained from Fluka; 2,4,6-collidine, β-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, 1.1 M TBAF in THF, 1-(trimethylsilyl)imidazole, triphenylphosphine, 2-(4-nitrophenyl)ethyl alcohol, diethyl azodicarboxylate, and xanthosine dihydrate were obtained from Aldrich (Steinheim, FRG); silver nitrate, isobutyryl chloride, anhydrous sodium sulfate, sodium hydrogen carbonate, and urea were obtained from Merck; and 6-thioguanosine and inosine were obtained from Pharma Waldhof (Mannheim, FRG). Hydrofluoric acid (40%) was purchased from Merck.

T4 polynucleotide kinase and T4 DNA ligase were obtained from United States Biochemical Corp. (USB). [γ-^{32}P]ATP (10 μCi/μL) was obtained from Amersham. X-ray film (X-OMAT XAR-5) was purchased from Kodak.

^{1}H NMR spectra were recorded at 360.13 MHz on a Bruker WH 360 spectrometer with tetramethylsilane as the internal standard. CD_3OD was added to ^{1}H NMR samples for the identification of exchangeable protons. ^{31}P NMR spectra were recorded on the same instrument at 145.79 MHz with ^{1}H decoupling and 85% H_3PO_4 as the external standard. Chemical shift values that are downfield of the respective standards possess a positive value.

TLC plates of silica gel 60 F_{254} (Merck) were developed by use of one of the following solvent systems: S1, water-saturated $CHCl_3$/MeOH, 8/3 (v/v); S2, $CHCl_3$/MeOH, 9/1 (v/v); S3, CH_2Cl_2/ethyl acetate/acetone, 5/5/1 (v/v); S4, CH_2Cl_2/ethyl acetate/triethylamine, 47.5/47.5/5 (v/v); S5, CH_2Cl_2/ethyl acetate, 7/3 (v/v); S6, hexane/CH_2Cl_2/Et_2O, 2/4/4 (v/v); S7, CH_2Cl_2/MeOH, 9/1 (v/v); or S8, CH_2Cl_2/MeOH, 8/2 (v/v). The compounds were visualized by the use of UV light or by spraying the plate with 5% aqueous sulfuric acid followed by heating. Flash column chromatography was performed on silica gel 60 (Merck) with particle size less than 0.063 mm. HPLC was carried out on a Waters Associates System with a Model 6000A pump, a Model 680 automated gradient controler, a Model 730 data module, and a Model 481 LC spectrophotometer. Separations were performed on reversed-phase material, 5-μm ODS Hypersil (Shandon), in a 250 × 4 mm column. Injecting 0.1–0.2 A_{260} units of oligonucleotide and employing a linear gradient of acetonitrile (3.5–28% in 15 min) in 50 mM triethylammonium acetate (pH 7.0) with a flow rate of 2 mL/min give typical retention times of 6–7 min.

Electroelution was performed with Schleicher & Schuell BIOTRAP elution chambers and BT1 and BT2 membranes for quantitative elution of nucleic acids longer than 14 bases. Radioanalytic scanning of gels was performed with a Fuji BAS2000 Bio-Imaging analyzer.

Preparation of Oligoribonucleotides. Oligoribonucleotides were prepared on an Applied Biosystems 380B DNA synthesizer on a 1-μmol scale with ribonucleotide phosphoramidites from MilliGen/Biosearch; standard phosphoramidite chemistry was employed. The coupling yields of inosine and 2-aminopurine ribonucleoside phosphoramidite, monitored by trityl group release, were indiscernible from commercial phosphoramidites and measured to be better than 99%. Xanthosine phosphoramidite coupling yields were around 95%, and those for the isoguanosine phosphoramidite were 80–85%. The oligoribonucleotides were base-deprotected by incubation in 3 mL of aqueous concentrated ammonia (32%)/ethanol (3:1) for 16 h at 55 °C in a screw-cap glass vial. After complete removal of the solvent by Speed-Vac evaporation, the residue was taken up in 0.5 mL of 1.1 M tetrabutylammonium fluoride in THF (Aldrich) and left at 25 °C overnight. After addition of 0.5 mL of 1.8 M NaOAc (pH 5.8), the solution was concentrated to a volume of 0.5–0.6 mL on a Speed-Vac. This solution was extracted twice with 0.8 mL of ethyl acetate followed by RNA precipitation at −20 °C after the addition of 1.6 mL of absolute ethanol. The RNA was collected as a pellet after spinning, and the supernatant was checked for complete RNA precipitation by UV spectroscopy at 260 nm. The pellet was dissolved in water and further purified on denaturing 20% polyacrylamide (8 M urea) gels. The product bands were detected by UV shadowing and cut out. The gel pieces were either electroeluted in Tris–borate–EDTA buffer using a BIOTRAP or extracted using the freeze–thaw method (Pieken et al., 1991). The resulting RNA solution was loaded onto a Sep-Pak C18 cartridge (Waters-Millipore) which was prewashed with 10 mL of acetonitrile followed by 10 mL of 100 mM triethylammonium bicarbonate (pH 7.5). After loading, the cartridge was first washed with 20 mL of 4% acetonitrile in 100 mM triethylammonium bicarbonate (pH 7.5) to remove a UV-absorbing impurity which could be detected as an extra peak in the HPLC analysis. The RNA was then eluted with acetonitrile/methanol/water (35:35:30, v/v) and collected within 1 mL after the void volume of 0.4 mL had passed through the column. The solution was dried down in a Speed-Vac; the residue was taken up in 0.2 mL of methanol and dried again. The pellet was dissolved in 0.1–0.5 mL of water, and the solution was stored frozen at −20 °C. The purity of the oligoribonucleotides was checked by PAGE after 5′-^{32}P-labeling and by reversed-phase HPLC analysis.

Nucleoside composition analysis of base-modified oligoribonucleotides to ascertain the incorporation of the modified nucleoside was carried out according to Connolly (1991). Short model oligonucleotides 6–12 nucleotides in length were synthesized and purified as described above. After digestion with snake venom phosphodiesterase and alkaline phosphatase, the nucleoside mixture was analyzed by reversed-phase HPLC employing a linear gradient of acetonitrile (0–28%) in 50 mM triethylammonium acetate (pH 7.0).

Ligation of Isoguanosine-Containing Oligoribonucleotides by T4 DNA Ligase. The protocol by Moore and Sharp (1992) with experimental details taken from Strobel and Cech (1992) was followed. The oligodeoxynucleotide required for this reaction as a splint was prepared on an Applied Biosystems 380B DNA synthesizer with deoxyribonucleotide phosphoramidites from Applied Biosystems. For the ligation reaction the 5′-^{32}P-labeled donor oligoribonucleotide (3 μM), the 3′-OH acceptor oligoribonucleotide (10 μM), the DNA splint (7 μM), EDTA (1 mM), and Tris-HCl (10 mM, pH 7.5) were combined in a final volume of 10 μL, and the reaction solution was heated to 90 °C for 1 min, after which it was cooled to room temperature and left for 2 h. Ligation buffer (USB;

[1] Abbreviations: DMTCl, 4,4′-dimethoxytrityl chloride; DMAP, 4-(dimethylamino)pyridine; TBAF, tetrabutylammonium fluoride; TBDMSCl, *tert*-butyldimethylchlorosilane; THF, tetrahydrofuran; TMSCl, trimethylchlorosilane; I, inosine; 2AP, 2-aminopurine nucleoside; X, xanthosine; isoG, isoguanosine; E-I[5], ribozyme containing inosine at position 5 (other modifications are denoted accordingly; for example, E-X[8]); Y, pyrimidine nucleoside; R, purine nucleoside; N, any nucleoside.

10×, 2 μL), ATP (to a final concentration of 0.5 mM), and T4 DNA ligase (10 units) were added, and the reaction mixture (total volume 20 μL) was incubated at room temperature for 14 h. The reaction mixture was purified by denaturing PAGE. The desired product was excised and eluted as described above.

Synthesis of Nucleosides and Their Phosphoramidites. 2-Aminopurine ribonucleoside (**1**) was prepared by aqueous Raney nickel reduction of commercially available 6-thioguanosine according to the procedure of Fox et al. (1958) with yields greater than 68%. Employing an excess of Raney nickel can dramatically reduce the yield of 2-aminopurine ribonucleoside due to surface adsorbtion on the nickel sponge. The reaction was followed by TLC (S1; R_f of starting material 0.0; R_f of product 0.24).

N^2-Isobutyryl-2-aminopurine Ribonucleoside (**2**). To 2-aminopurine ribonucleoside (3.1 g, 11.6 mmol), dried two times by evaporation with 20 mL of anhydrous pyridine and suspended in 80 mL of anhydrous pyridine, was added TMSCl (9.45 g, 87 mmol). After the solution was stirred for 2 h at room temperature, isobutyryl chloride (6.1 mL, 58 mmol) was added, and the reaction mixture was maintained for 3 h at room temperature. The mixture was then cooled in an ice bath, and 10 mL of methanol was added. The reaction mixture was then evaporated to near dryness and dissolved in 60 mL of water. The solution was extracted two times with 60 mL of ethyl acetate, and the combined organic phases were evaporated to dryness. The residue was dissolved in 60 mL of 85% aqueous ethanol, and 6 mL of 25% aqueous ammonia was added. This solution was stirred for 20 min at room temperature, and the desilylation was followed by TLC (S1; R_f(**2**) 0.41). The solvent was then evaporated, and residual water and pyridine were removed by coevaporation two times each with absolute ethanol and toluene. The product was purified by flash chromatography on silica gel using CH_2Cl_2 with a 1–7.5% MeOH gradient. Product-containing fractions (eluted at 7.5% MeOH) were pooled and evaporated to dryness: yield 2.0 g (51%); ^{1}H NMR (D_2O) δ = 8.98 (s, 1 H, H6), 8.60 (s, 1 H, H8), 6.18 (d, $J_{HH2'}$ = 5.2 Hz, 1 H, H1′), 4.86 (m, overlapping with HDO signal, H2′), 4.53 (dd, 1 H, H3′), 4.28 (m, 1 H, H4′), 3.86 (dd, 1 H, H5′B), 3,88 (dd, 1 H, H5′A), 2.81 (septet, 1 H, J = 6.9 Hz, $CH(CH_3)_2$), 1.26 (d, 6 H, J = 6.9 Hz, $CH(CH_3)_2$) ppm.

5′-O-(4,4′-Dimethoxytrityl)-N^2-isobutyryl-2-aminopurine Ribonucleoside (**3**). To **2** (2.0 g, 6.0 mmol), dried three times by evaporation with 10 mL of anhydrous pyridine and dissolved in 50 mL of anhydrous pyridine, was added a total amount of 2.9 g (8.5 mmol) of DMTCl over a period of 5 h. The reaction was followed by TLC (S2; R_f(**2**) 0.1; R_f(**3**) 0.4) and stopped after completion by the addition of 5 mL of MeOH. The reaction mixture was then evaporated to a gum and redissolved in 60 mL of CH_2Cl_2. The solution was washed once with 60 mL of 5% aqueous $NaHCO_3$. The organic phase was dried over anhydrous Na_2SO_4, evaporated to dryness, and purified by flash chromatography on silica gel. The solvent polarity was increased stepwise from CH_2Cl_2/NEt_3 (100/0.5, v/v) to CH_2Cl_2/MeOH/NEt_3 (99/1/0.5, v/v). Product-containing fractions were pooled and evaporated to dryness to give a white foam: yield 3.3 g (87%);^{1}H NMR ($CDCl_3$) δ = 8.93 (s, 1 H, H6), 8.48 (s, broad, 1 H, exchangeable with CD_3OD, N^2H), 8.28 (s, 1 H, H8), 7.32 (s, broad, 1 H, exchangeable with CD_3OD, OH), 7.28–7.05 (m, 9 H, 4-MeO-*phenyl*, 4 H_{ortho}, and *phenyl*), 6.68 (d, 4 H, J = 8.8 Hz, 4-MeO-*phenyl*, H_{meta}), 5.97 (d, $J_{HH2'}$ = 3.8 Hz, 1 H, H1′), 5.03 (m, 1 H, H2′), 4.51 (dd, 1 H, H3′), 4.41 (d, 1 H, H4′), 3.75 (s, 6 H, 4-MeO-phenyl, OCH_3), 3.46 (s, broad, 1 H, exchangeable with CD_3OD, OH), 3.38 (dd, 1 H, H5′B), 3.19 (dd, 1 H, H5′A), 2.63 (septet, 1 H, $CH(CH_3)_2$), 1.32 (m, 6 H, $CH(CH_3)_2$) ppm.

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^2-isobutyryl-2-aminopurine Ribonucleoside (**4**). Compound **3** (3.3 g, 5.2 mmol) was dissolved in 40 mL of anhydrous THF, followed by the addition of 1.7 mL of pyridine and $AgNO_3$ (0.93 g, 5.5 mmol). The solution was stirred for 1 h until all the $AgNO_3$ had dissolved, and then TBDMSCl (0.94 g, 6.2 mmol) was added. The mixture was stirred in the dark at room temperature overnight, and the progress of the reaction was monitored on TLC (S3; R_f(**3**) 0.11; R_f(**4**) 0.45; R_f(3′-*O*-silylated product) 0.33). The solution was then filtered in 80 mL of 5% aqueous $NaHCO_3$ and extracted with 200 mL of CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4 and evaporated. The 2′-*O*-silylated product was isolated by flash chromatography on silica gel using a stepwise gradient of hexane/CH_2Cl_2/acetone from 110/40/0 to 110/40/40 (v/v) where the 3′-*O*-silylated isomer elutes. Isomerically pure 2′-*O*-silylated product fractions were combined and evaporated to dryness: yield 1.3 g (33%) of 2′-*O*-silylated product **4** and 1.4 g (35%) of mainly 3′-*O*-silylated product contaminated with **4**; ^{1}H NMR ($CDCl_3$) δ = 9.07 (s, 1 H, H6), 8.15 (s, 1 H, H8), 7.62 (s, broad, 1 H, exchangeable with CD_3OD, N^2H), 7.55–7.52 (m, 2 H, *phenyl*), 7.43–7.39 (m, 4 H, 4-MeO-*phenyl*, H_{ortho}), 7.29–7.22 (m, 3 H, *phenyl*), 6.82–6.78 (m, 4 H, 4-MeO-*phenyl*, H_{meta}), 5.97 (d, 1 H, $J_{HH2'}$ = 4.4 Hz, H1′), 5.20 (m, 1 H, H2′), 4.41 (m, 1 H, H3′), 4.29 (m, 1 H, H4′), 3.79 (s, 3 H, 4-MeO-phenyl, OCH_3), 3.78 (s, 3 H, 4-MeO-phenyl, OCH_3), 3.58 (m, 1 H, H5′B), 3.27 (m, 1 H, H5′A), 2.78 (d, 1 H, J = 2.5 Hz, exchangeable with CD_3OD, 3′-OH), 1.27 (m, 1H, $CH(CH_3)_2$), 1.02 (d, 3 H, J = 6.8 Hz, $CH(CH_3)_2$), 0.90 (d, 3 H, J = 6.8 Hz, $CH(CH_3)_2$), 0.83 (s, 9 H, $SiC(CH_3)_3$), 0.00 (s, 3 H, $SiCH_3$), –0.23 (s, 3 H, $SiCH_3$) ppm.

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^2-isobutyryl-2-aminopurine Ribonucleoside 3′-N,N-Diisopropyl(cyanoethyl)phosphoramidite (**5**). To **4** (1.3 g, 1.7 mmol), dissolved in 20 mL of dry CH_2Cl_2, was added *N*-ethyldiisopropylamine (1.2 mL, 6.8 mmol), *N*-methylimidazole (70 μL, 0.85 mmol) and β-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.65 mL, 2.8 mmol). The reaction mixture was stirred under an argon atmosphere for 1.5 h at room temperature. The extent of product formation was monitored by TLC (S4; R_f(**4**) = R_f(unoxidized **5**) 0.56; R_f(oxidized **5**) 0.0) after iodine oxidation of reaction solution aliquots. After completion, the reaction was quenched by the addition of 2 mL of MeOH, and the mixture was poured into 50 mL of 1 M triethylammonium bicarbonate (pH 7.5). The product was extracted with 40 mL of CH_2Cl_2, and the organic phase was dried over anhydrous Na_2SO_4. The solvent was evaporated, and the product was further purified by flash chromatography on silica gel employing a stepwise gradient of hexane/CH_2Cl_2/NEt_3 from 15/4/1 to 12/7/1 (v/v). The product-containing fractions were combined, and the solvent was removed. The resulting white foam was further vacuum dried at 40 °C overnight: yield 1.5 g (92 %); ^{31}P NMR ($CDCl_3$), 2 diastereomers (a and b), δ = 152.2 (64%), 149.5 (34%) ppm; ^{1}H NMR ($CDCl_3$), 2 diastereomers, δ = 9.07, 9.06 (2 s, 1 H, H6), 8.18, 8.17 (2 s, 1 H, H8), 7.76 (s, broad, 1 H, exchangeable with CD_3OD, N^2H), 7.55–7.52 (m, 2 H, *phenyl*), 7.43–7.39 (m, 3 H, *phenyl*), 7.26–7.21 (m, 4 H, 4-MeO-*phenyl*, H_{ortho}), 6.82–6.78 (m, 4 H, 4-MeO-*phenyl*, H_{meta}), 6.04 (d, 1 H, $J_{HH2'}$ = 7.6 Hz, H1′), 5.12 (m, 1 H, H2′), 4.41 (m, 1 H, H3′), 4.28 (m, 1 H, H4′), 4.05 (m, 1 H,

POCH$_2$–), 3.95 (m, 1 H, POCH$_2$–), 3.78, 3.77, 3.76 (3 s, 6 H, 4-MeO-phenyl, OCH$_3$), 3.59–3.54 (m, 3 H, H5′B and NCH(CH$_3$)$_2$, 2 H), 3.20 (m, 1 H, H5′A), 2.73 (m, 2 H, –CH$_2$-CN), 1.28–0.82 (several m, COCH(CH$_3$)$_2$, 1 H, NCH(CH$_3$)$_2$, 12 H, COCH(CH$_3$)$_2$, 6 H), 0.74, 0.73 (2 s, 9 H, SiC(CH$_3$)$_3$), –0.01, –0.05 (2 s, 3 H, SiCH$_3$), –0.29 (2 s, 3 H, SiCH$_3$) ppm.

5′-O-(4,4′-Dimethoxytrityl)inosine (**6**) was prepared according to the procedure of Green et al. (1991). The product was purified by flash chromatography on silica gel using CH_2Cl_2 with a 0–10% MeOH gradient in the presence of 1% NEt_3. O^6-(*p*-Nitrophenyl)ethyl-5′-*O*-(4,4′-dimethoxytrityl)inosine (**7**) was prepared by following the transient protection protocol of Gao et al. (1986) on a 17-mmol scale. The product was isolated by flash chromatography on silica gel. The elution solvent polarity was slowly raised from 0% to 2% MeOH in CH_2Cl_2/hexane (3/1, v/v). The product fractions were combined, and the solvent was evaporated to yield **7** (35%). The synthesis of O^6-(*p*-nitrophenyl)ethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)inosine (**8**) was carried out as described for **4**. The progress of the reaction was monitored by TLC (S5; R_f(**7**) 0.05; R_f(**8**) 0.73; R_f(3′-*O*-silylated product) 0.61), and the 2′-*O*-silylated product was isolated by silica gel flash chromatography by gradually varying the solvent system from hexane/CH_2Cl_2 (1/1, v/v) to hexane/CH_2Cl_2/ethyl acetate (5/15/2, v/v) to yield 30% of the 2′-*O*-silylated isomer after solvent evaporation. The synthesis of O^6-(*p*-nitrophenyl)ethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tert-butyldimethylsilyl)inosine 3′-*N*,*N*-diisopropyl(cyanoethyl)phosphoramidite (**9**) was carried out on a 1.7-mmol scale as described for **5**. The reaction was followed by TLC (S6; R_f(**8**) 0.40; R_f(**9**, 2 diastereomers) 0.46, 0.53). The phosphoramidite **9** was isolated by flash chromatograpy on silica gel employing a stepwise gradient of hexane/CH_2Cl_2/NEt_3 from 19/0/1 to 14/5/1 (v/v). After solvent evaporation and vacuum drying at 40 °C, the yield was 91%: ^{31}P NMR ($CDCl_3$), 2 diastereomers, δ = 151.8 (38%), 149.6 (62%) ppm.

5′-O-(4,4′-Dimethoxytrityl)xanthosine (**10**). Xanthosine dihydrate (5 g, 15.6 mmol) was dried by three successive evaporations with pyridine and dissolved in 250 mL of dry DMF/pyridine (8:2, v/v). DMTCl (6.33 g, 18.8 mmol) was added in three equal portions over 3 h. The reaction mixture was stirred at room temperature overnight. TLC (S7; R_f (starting material) 0.0; R_f(**10**) 0.56) still showed some starting material, and an additional 0.5 equiv (2.6 g, 7.8 mmol) of 4,4′-dimethoxytrityl chloride was added. After the mixture was stirred for an additional 2 h, the reaction was quenched by addition of imidazole (2.1 g, 30 mmol), and the solution was evaporated to dryness. The product was purified by silica gel chromatography using CH_2Cl_2 with a 5–50% MeOH gradient containing 0.5% triethylamine. Product-containing-fractions were pooled and evaported to dryness: yield 3.6 g (34%).

The synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)xanthosine (**11**) was carried out as described for **4**. The reaction required 2 equiv of the silylating reagent and was monitored by TLC (S7; R_f(**10**) 0.56; R_f(**11**) 0.65). The 2′-*O*-silylated product was isolated by silica gel flash chromatography with an increasing content of MeOH (0–30%) in CH_2Cl_2 containing 0.01% triethylamine to yield 20% of the 2′-*O*-silylated isomer after solvent evaporation. The synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(tert-butyldimethylsilyl)xanthosine 3′-*N*,*N*-diisopropyl(cyanoethyl)-phosphoramidite (**12**) was carried out on a 0.65-mmol scale as described for **5**. The reaction was followed by TLC (S7; R_f(**11**) 0.65; R_f(**12**) 0.75). The phosphoramidite **12** was isolated by flash chromatograpy on silica gel with an increasing content of MeOH (0–4%) in CH_2Cl_2 containing 2% triethylamine. After solvent evaporation and vacuum drying at 40 °C, the yield was 77%: ^{31}P NMR (DMSO-d_6), 2 diastereomers, δ = 151.9 (20%), 149.4 (80%) ppm.

Isoguanosine (**13**) was prepared according to Chern et al. (1991)with the exception that the oily residue obtained after reaction with dicyclohexyldiimide was extracted with boiling toluene instead of by partitioning between a mixture of water and chloroform as described in the literature.

5′-O-(4,4′-Dimethoxytrityl)-N^6-[(dimethylamino)methylene]isoguanosine (**14**). Isoguanosine (**13**; 5.7 g, 20.34 mmol) was suspended in DMF (250 mL). *N*,*N*-Dimethylformamide dimethyl acetal (203 mmol, 27 mL) was added over 20 min at room temperature. The reaction mixture was stirred at room temperature for 6 h, during which a clear solution was obtained, and TLC (S8; R_f(**13**) 0.03; R_f(amidine) 0.24) showed a single reaction product. The solution was reduced in volume and coevaporated twice with toluene to give a colorless residue, which was dried overnight *in vacuo*. The product was then redissolved in pyridine (200 mL), and DMTCl (7.58 g, 22.4 mmol) was added portionwise over 20 h to the stirred solution. Progress of the reaction was monitored by TLC (S7; R_f (amidine) 0.13; R_f(**14**) 0.46). After 20 h, methanol (5 mL) was added, and the solution was stirred for a further 5 min and evaporated to give a residue, which was redissolved in dichloromethane (200 mL). This was washed with 5% aqueous $NaHCO_3$ followed by water and dried over Na_2SO_4. The filtrate was evaporated in the presence of toluene to give a pale yellow product. The crude product was purified by flash column silica gel chromatography. The CH_2Cl_2/MeOH/Et_3N solvent polarity was increased from 100/0.1/0.1 to 100/1.5/0.1 (v/v). Product-containing fractions were pooled and evaporated: yield 4.7g (36%); λ_{max}(MeOH) 346, 229; λ_{min} 292; ^{1}H NMR (DMSO-d_6) δ = 11.09 (s, broad, 1 H, exchangeable with CD_3OD, N^{3}H), 9.14 (s, 1 H, NC*H*NMe$_2$), 7.97 (s, 1 H, H8), 7.37–7.18, 6.86–6.82 (m, 13 H, 4-MeO-*phenyl*), 5.74 (d, 1 H, J = 3.6 Hz, H1′), 5.58 (d, 1 H, J = 5.2 Hz, exchangeable with CD_3OD, OH), 5.17 (d, J = 6.0 Hz, exchangeable with CD_3OD, OH), 4.49 (dd, 1 H, H2′), 4.20 (dd, 1 H, H3′), 4.00 (dd, 1 H, H$_{4'}$), 3.72 (d, 6 H, 4-MeO-phenyl, OCH$_3$), 3.18 (s, 3 H, CHN*Me*$_2$), 3.17 (m, 2 H, H5′, H5″), 3.10 (s, 3 H, CHN*Me*$_2$).

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N^6-[(dimethylamino)methylene]isoguanosine(**15**). Compound **14** (1.72 g, 2.69 mmol) was dissolved in pyridine (20 mL); $AgNO_3$ (3.23 mmol, 0.55 g) was added, and the solution was stirred at room temperature for 15 min. TBDMSCl (2.69 mmol, 446 mg) dissolved in THF was then added, and the suspension was stirred in the dark at room temperature for 12 h. It was necessary to add an additional 1 equiv each of TBDMSCl and $AgNO_3$ over the following 6-h period, after which analysis by TLC (S7; R_f(**14**) 0.49; R_f(**15**) 0.57) showed the reaction to be complete. The mixture was then filtered into brine solution, extracted with CH_2Cl_2, and dried over Na_2SO_4, and the filtrate was evaporated to dryness in the presence of toluene. The crude product was purified by flash column chromatography using silica gel and a stepwise gradient of ethyl acetate/acetone from 99/1 to 94/6 (v/v): yield 0.97 g (48%); ^{1}H NMR (DMSO-d_6) δ = 11.08 (s, broad, 1 H, exchangeable with CD_3OD, N^{3}H), 9.14 (s, 1 H, NC*H*NMe$_2$), 7.92 (s, 1 H, H8), 7.39–7.19 , 6.88–6.84 (m, 13 H, 4-MeO-*phenyl*), 5.75 (d, 1 H, J = 4.7 Hz, H1′), 5.06 (d, 1 H, J = 6.4 Hz, exchangeable with CD_3OD, 3′-OH), 4.62 (triplet, 1

H, H2′), 4.15 (triplet, 1 H, H3′), 4.02 (triplet, 1 H, H4′), 3.72 (d, 6 H, 4-MeO-phenyl, OCH_3), 3.22 (m, 2 H, H5′, H5″), 3.20 (s, 3 H, $CHNMe_2$), 3.10 (s, 3 H, $CHNMe_2$), 0.80 (s, 9 H, $SiC(CH_3)_3$), 0.01 (s, 3 H, $SiCH_3$), −0.05 (s, 3 H, $SiCH_3$).

5′-O-(4,4′-Dimethoxytrityl)-2′-O-(tert-butyldimethylsilyl)-N⁶-[(dimethylamino)methylene]isoguanosine 3′-N,N′-Diisopropyl(cyanoethyl)phosphoramidite (**16**). Compound **15** (0.4 g, 0.52 mmol) was dissolved in dry CH_2Cl_2, and *N*-methylimidazole (20 μL, 0.25 mmol), *N,N*-diisopropylethylamine (380 μL, 2.23 mmol), and β-cyanoethyl *N,N*-diisopropylphosphoramidite (236 μL, 1.04 mmol) were added under argon. The clear solution was stirred under argon at room temperature for 3 h. TLC (S7; R_f(**16**) 0.56; R_f(**15**) 0.47) established completion of the reaction. Methanol (150 μL) was then added, and the solution was extracted between CH_2Cl_2 and 1 M triethylammonium bicarbonate (pH 7.5). The organic phase was dried over Na_2SO_4, and the filtrate was coevaporated in the presence of toluene to give a yellow oil, which was redissolved in CH_2Cl_2 and precipitated into *n*-hexane. The white precipitate was collected by filtration and dried overnight *in vacuo* at 40 °C: yield 468 mg (94%); ^{31}P NMR (CD_3CN) 151.30 (s), 150.15 (s).

Determination of Ribozyme Steady-State Parameters. Kinetic constants, k_{cat} and K_m, were determined from Eadie–Hofstee plots obtained from initial velocities under multiple turnover conditions with 5′-^{32}P-labeled substrate. Stock solutions of 25–200 nM ribozyme and 1 μM substrate RNA were prepared in 50 mM Tris-HCl (pH 7.5), preheated separately at 90 °C for 1 min, and cooled to 25 °C for 15 min. After $MgCl_2$ was added to a final concentration of 10 mM, the stock solutions were incubated for another 15 min at 25 °C. Ribozyme concentrations of 2.5–20 nM and substrate concentrations of 50–350 nM were used for the reactions in the presence of 10 mM $MgCl_2$ in 50 mM Tris-HCl (pH 7.5) at 25 °C. The reactions were initiated by the addition of 5 μL of ribozyme stock solution to 45 μL of the substrate solution. Initial rates were determined by transferring 8-μL aliquots into 16 μL of urea stop mix (3.5 M urea, 25 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol) at appropriate time intervals between 1 and 270 min of reaction time. Rates were linear even at high ribozyme concentrations for several turnovers.

First-order rate constants, k^s_{obs}, were measured at 500 nM saturating substrate concentration with 5–40 nM ribozyme concentrations at 10 and 50 mM $MgCl_2$ with essentially the same protocol as described above.

Product and substrate were separated by denaturing 20% polyacrylamide (8 M urea) gel electrophoresis. The degree of product formation was determined by radioanalytic scanning on a Fuji BAS2000 Bio-Imaging analyzer. For any given ribozyme, k_{cat}, k^s_{obs}, K_m, and k_{cat}/K_m were found to vary approximately by a factor of 2 from experiment to experiment.

RESULTS

The hammerhead chosen for these studies (Figure 1) was that introduced by Fedor and Uhlenbeck (1990) and has been used by us in previous studies (Olsen et al., 1991; Pieken et al., 1991; Tuschl & Eckstein, 1993; Williams et al., 1991). It is characterized by a high turnover number, reflecting the rate of chemical cleavage and not of product release (Fedor & Uhlenbeck, 1992). Ribozymes with a single-base substitution at each of the three central core guanosines, G^5, G^8, and G^{12} [for the numbering system, see Hertel et al. (1992)], with inosine, 2-aminopurine ribonucleoside, and xanthosine were synthesized by standard automated synthesis. The presence of the modified nucleosides in the oligoribonucleotides was confirmed by combined nuclease and phosphatase digestion and subsequent HPLC nucleoside analysis.

2-Aminopurine ribonucleoside has been previously incorporated into synthetic oligoribonucleotides by the phosphoramidite method using N^2-benzoyl- and 2′-*O*-TBDMS-protected (Doudna et al., 1990) as well as N^2-isobutyryl- and 2′-*O*-tetrahydropyranyl-protected 2-aminopurine ribonucleoside phosphoramidite (SantaLucia et al., 1991). We have combined the advantages of the more readily removable N^2-isobutyryl groups with that of the acid-insensitive 2′-*O*-TBDMS group. The latter allows the synthesis of longer oligoribonucleotides (Usman et al., 1987). Inosine was incorporated into the synthetic RNA following the approach of Green et al. (1991) using the O^6-*p*-nitrophenylethyl protecting group to enhance the solubility of the inosine phosphoramidite in acetonitrile, the solvent required for automated synthesis. For the N^6-protection of deoxyisoguanosine the *N,N*-dimethylamidine group was chosen (Seela et al., 1992).

N-Ethyldiisopropylamine, often used in the phosphitylation reaction, has been described to cause rapid isomerization of the 2′-*O*-silylated nucleoside with or without an acylating catalyst such as 4-dimethylaminopyridine in anhydrous THF (Milecki et al., 1989; Scaringe et al., 1990; Wu & Ogilvie, 1990). Scaringe et al. (1990) have used collidine together with catalytic amounts of *N*-methylimidazole. As we encountered difficulties in the removal of collidine, we have chosen to use *N,N*-diisopropylethylamine together with *N*-methylimidazole in anhydrous dichloromethane, conditions where we could not detect the formation of any 2′-*O*-phosphitylated products.

Oligoribonucleotide syntheses with the modified nucleotides proceeded essentially as those with the unmodified nucleotides, with the exception of syntheses with isoguanosine. Although isoguanosine phosphoramidite could be readily incorporated into the donor oligoribonucleotides (Figure 4), the elongation of these by addition of the next nucleotide was unsuccessful. We therefore attempted enzymatic ligation of the oligoribonucleotides with a 5′-terminal isoguanosine with the other half of the ribozyme. The procedure of Moore and Sharp (1992) using T4 DNA ligase, in the presence of a DNA splint, was adopted. In order to avoid the incorporation of isocytidine into the DNA template, we made use of the ability of the ligase to tolerate mismatches such as G/T and T/T at the 5′-ligation junction (Harada & Orgel, 1993). Isoguanosine can base pair not only with isocytidine but also with uridine (Switzer et al., 1989; Tor & Dervan, 1993), so we decided to use thymidine opposite the isoguanosine on the DNA splint. We thus report the use of T4 DNA ligase, in the presence of a DNA template, to efficiently ligate a modified ribonucleotide with an isoG/T mispair at the 5′-ligation junction, thereby extending the scope of possible ligations by T4 DNA ligase to base-modified nucleotides (Figures 4 and 5).

The effects of the single inosine or 2-aminopurine substitutions at positions 5, 8, and 12 of the invariant region of the ribozyme on the kinetic parameters of RNA cleavage are summarized in Table I. Whereas the kinetic parameters for the inosine- or 2-aminopurine-modified ribozymes were obtained with variable substrate concentrations, for the xanthosine- and isoguanosine-modified ribozymes only the cleavage velocities under substrate saturation were measured. This seemed to be justifiable, as the K_m values for the modified ribozymes were little affected. Substrate saturation of modified and unmodified ribozymes occurred above 400 nM

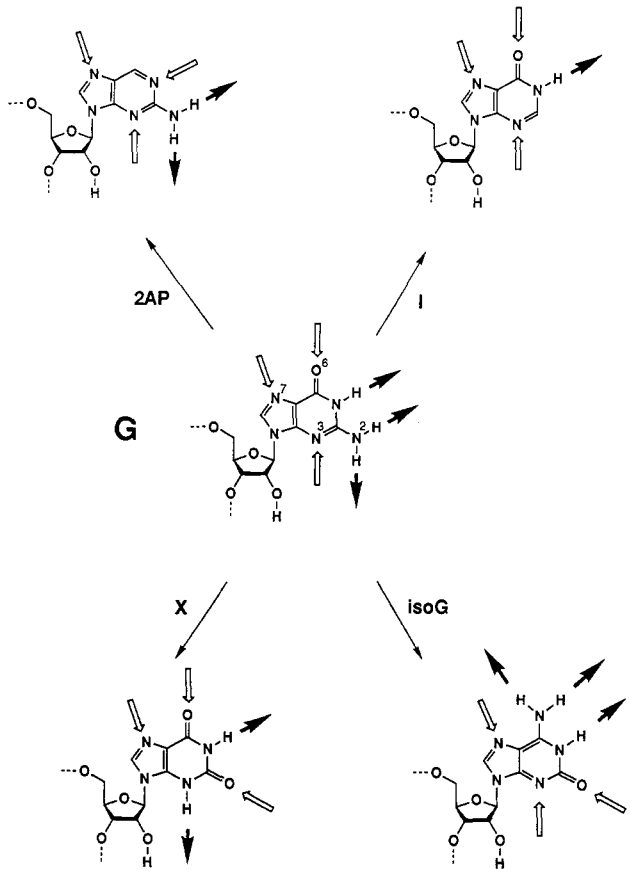

FIGURE 2: Structure and hydrogen-bonding capabilities of guanosine (G) and the guanosine analogues inosine (I), 2-aminopurine ribonucleoside (2AP), isoguanosine (isoG), and xanthosine (X). Arrows indicate hydrogen bond donor (solid arrows) and hydrogen bond acceptor (open arrows) abilities of the purine functional groups.

concentration, and initial rates remained constant up to 1500 nM substrate concentration. Thus 500 nM substrate concentration was chosen for measuring the maximum observed rate constant k_{obs}^{s} for all base-modified ribozymes (Table II). To compare k_{obs}^{s} and k_{cat} values obtained by Eadie–Hofstee plots, values were also determined and included in Table II for the inosine- or 2-aminopurine-modified ribozymes. There is good agreement between these values for ribozymes E_{unmod} and E-2AP[8], which both have low K_m values. For the others the agreement is within a factor of 2 except for E-I[12], where k_{cat} is 4 times higher than k_{obs}. Attempts to increase k_{obs} by using higher than 500 nM substrate were unsuccessful. Although it cannot be excluded that for some modified ribozymes saturation has not been reached with 500 nM substrate, the observed discrepancy between k_{obs}^{s} and k_{cat} values could also be due to the difficulty in accurately determining Michaelis–Menten parameters for the poorly active ribozymes.

Guanosine modifications at position 5 resulted in a 1000-fold reduced catalytic efficiency for E-I[5] and E-2AP[5] due to a dramatic decrease in k_{cat} and a slight increase in K_m (Table I). The turnover number of E-X[5] was similar to that of E-2AP[5] or E-I[5], but that of E-isoG[5] was too low to be accurately determined.

In contrast to G[5], replacement of G[8] by inosine results in very little loss of activity. The 5-fold reduction in efficiency is a result of a decrease in k_{cat} as well as an increase in K_m. Substitution of G[8] by 2AP and dG is not tolerated, with cleavage efficiencies decreased by a factor of 100 and 300, respectively. The reduced efficiencies of E-2AP[8] and E-dG[8] (Williams et al., 1991) are pure k_{cat} effects, while the K_m values remain unaffected. E-X[8] turns over with a 400-fold reduced rate, 150 times slower than E-I[8], and E-isoG[8] is the least acitve.

G[12] is probably the most critical position for base modification. E-2AP[12] has such a low catalytic efficiency that its Michaelis–Menten parameters could not be determined accurately. However, the cleavage rate under substrate saturation is reduced 3000-fold, a value which is also found for E-isoG[12]. E-I[12] has a 400-fold reduced catalytic efficiency, mainly caused by a decrease in k_{cat}. The rate constant of E-X[12] is a factor of 2 lower than that of E-I[12] but a factor of 5 higher than that of E-2AP[12].

In order to determine whether higher Mg^{2+} concentrations might compensate for any loss in activity of the modified ribozymes, activities were determined not only at 10 mM but also at 50 mM metal ion concentration. The unmodified ribozyme showed only a moderate increase in activity at the higher Mg^{2+} concentration. This is in agreement with results from Dahm and Uhlenbeck (1991) and Uhlenbeck (1987), who found that the cleavage rate increased steadily with Mg^{2+} concentration until a maximum was reached at 20 mM. All modified ribozymes, except the xanthosine-modified ribozymes, showed cleavage rates increased by a factor of 1.4–3.5 (Table II). The xanthosine ribozyme activities were lowered by a factor of 0.7 or 0.8. Thus, the Mg^{2+} concentration effect is minimal.

DISCUSSION

The nucleosides in the central core of the hammerhead ribozyme play a crucial role in its catalytic competence, with replacement by other natural nucleosides resulting in a loss of catalytic activity (Ruffner et al., 1990; Symons, 1992). Three highly conserved guanosines, G[5], G[8], and G[12], are located in the bulge region of the ribozyme where no simple base pairing is apparent. It is thus of interest to identify the roles played by these guanosines. One approach is to systematically replace these guanines by other purine bases containing alternative exocyclic base functionalities. The resultant effect on the rate of substrate cleavage should then assist in assigning the contributions of the individual groups to the hydrogen-bonding systems present in the ribozyme. A previous report describes the replacement of G[12] by inosine, resulting in a ribozyme with no detectable activity (Slim & Gait, 1992). Guanosines 5 and 8 have also been substituted by inosine, with position 5 substitution causing a drastic decrease in activity and position 8 substitution having only a minor effect (Fu & McLaughlin, 1992a). We have extended these studies to all three guanosines, using analogues such as 2-aminopurine ribonucleoside, xanthosine, and isoguanosine. We have also determined the rates for inosine and deoxyguanosine substitutions so as to obtain comparable data.

Inosine and 2-aminopurine ribonucleoside are considered deletion modifications of guanosine. In inosine the 2-amino group of guanosine is replaced by a hydrogen atom. In 2-aminopurine the *O*6-carbonyl group is replaced by hydrogen and the *N*1-imino hydrogen is removed. Xanthosine is derived from guanosine by replacement of its 2-amino group by a 2-carbonyl group and protonation of N3. In isoguanosine, which is predominantly in the N1–H form in aqueous solution (Sepiol et al., 1976), the guanosine exocyclic functional groups are reversed. Thus, the *O*6-carbonyl group is replaced by an amino group, and the 6-amino group is replaced by a carbonyl group. The modified hydrogen-bonding patterns of these analogues are illustrated in Figure 2. These modifications

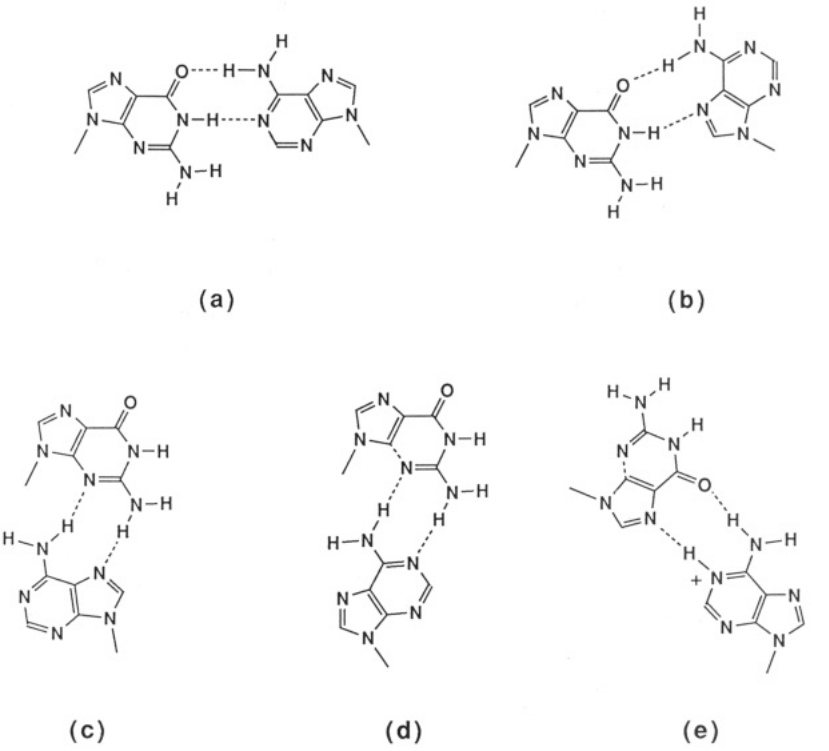

FIGURE 3: Base pairing of possible G/A mismatches (Li et al., 1991a,b): base pairing with hydrogen-bonded imino protons [(a) Watson–Crick-type G(anti)/A(anti); (b) Hoogsteen G(anti)/A(syn)], base pairing that involves the guanosine 2-amino group [(c) reversed Hoogsteen G(anti)/A(anti); (d) G(anti)/A(syn)], and base pairing under acidic conditions [(e) G(syn)/AH$^+$(anti)].

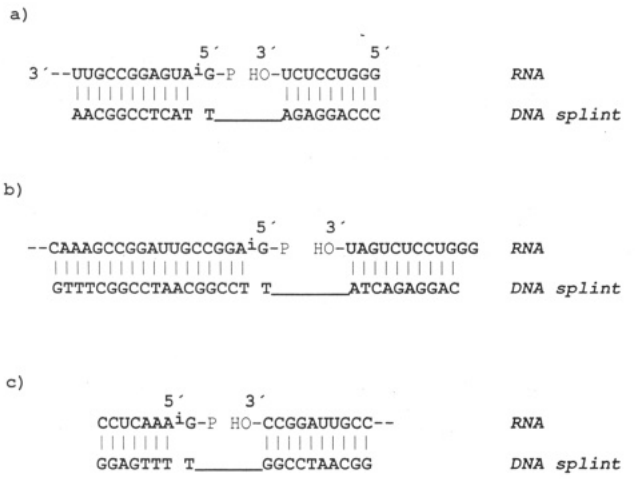

FIGURE 4: Schematic representation of synthesis of isoguanosine-containing ribozymes using T4 DNA ligase: (a) isoguanosine (^{i}G) at ribozyme position 5; (b) ^{i}G at position 8; (c) ^{i}G at position 12. Dots on the RNA strand denote the rest of the ribozyme sequence.

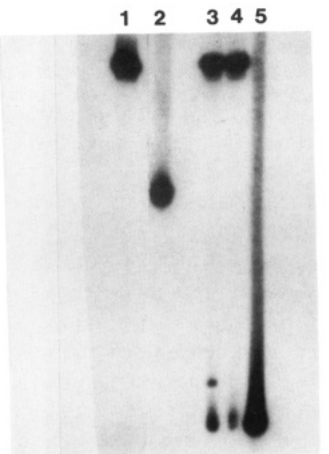

FIGURE 5: Gel electrophoretic analysis of ligation reaction c of Figure 4: lane 1, 34-nucleotide oligoribonucleotide marker; lane 2, 22-nucleotide marker; lanes 3 and 4, ligation reaction products; lane 5, 5′-^{32}P-labeled donor.

should permit probing of the hydrogen bonding role not only of the exocyclic groups of guanine but also of N1 and N3.

The ribozyme–substrate construct we have chosen for this study forms five base pairs within each of the stems I and III. This unmodified ribozyme turns over with a catalytic rate constant, k_{cat}, similar to the elemental rate of the cleavage step (Fedor & Uhlenbeck, 1992), and k_{cat} is not determined by product release. Thus, the measured rate constants for interconversion of substrate at the saturation concentration of substrate under multiple turnover conditions, k^s_{obs}, reflect the rates of the chemical cleavage and are identical to the k_{cat} values obtained from Eadie–Hofstee plots.

The large reduction in k^s_{obs} or k_{cat} values of approximately 250–3000-fold for ribozymes containing any of the modified bases at position 5 and 12 suggests that the G^5 and G^{12} bases participate with all their exocyclic functional groups in transition state stabilization (Table I). The 5–6-fold-increased K_m values of E-I^5 and E-2AP^5 could indicate that the

Table I: Kinetic Parameters for Unmodified, Inosine-Containing, and 2-Aminopurine-Containing Hammerhead Ribozymes[a]

ribozyme	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (μM^{-1} min^{-1})
E_{unmod}	2.8	78	36
E-I^5	0.017	457	0.038
E-2AP^5	0.012	394	0.031
E-I^8	1.9	247	7.6
E-2AP^8	0.02	63	0.32
E-I^{12}	0.033	323	0.1
E-2AP^{12}	nd[b]	nd	<0.03

[a] Values were determined as explained in Materials and Methods at 10 mM $MgCl_2$. [b] nd, not detectable; although a small amount of cleavage was detectable, no reliable Eadie–Hofstee plots could be obtained.

Table II: Rate Constants of Ribozyme Cleavage under Conditions of Substrate Saturation (500 nM) at Different $MgCl_2$ Concentrations[a]

ribozyme	k^s_{obs}, 10 mM $MgCl_2$ (min^{-1})	k^s_{obs}, 50 mM $MgCl_2$ (min^{-1})	Mg effect[b]
E_{unmod}	3.0	3.3	1.1
E-I^5	0.012	0.032	2.7
E-$2AP^5$	0.005	0.012	2.3
E-X^5	0.004	0.003	0.7
E-isoG^5	<0.001	<0.001	
E-2′dG^5	0.011	0.028	2.6
E-I^8	1.1	3.8	3.5
E-$2AP^8$	0.018	0.038	2.2
E-isoG^8	0.003	0.005	1.7
E-X^8	0.007	0.006	0.8
E-2′dG^8	0.025	0.038	1.5
E-I^{12}	0.008	0.023	2.9
E-$2AP^{12}$	0.001	0.002	2.0
E-isoG^{12}	0.001	0.002	2.0
E-X^{12}	0.005	0.004	0.8
E-2′dG^{12}	4.6	6.3	1.4

[a] Values were determined as described in Materials and Methods. [b] Mg effect is defined by the ratio k^s_{obs}(50 mM $MgCl_2$)/k^s_{obs}(10 mM $MgCl_2$).

6-carbonyl and the 2-amino group of G^5 might directly interact with the bound substrate in the transition state. If guanosine N3 were acting as a hydrogen bond acceptor, the inversion of its hydrogen-bonding character by xanthosine substitution should have decreased the turnover rate considerably more than with the I or the 2AP substitution alone.

The picture is different for position 8. Substitution with inosine is tolerated very well with no significant reduction in k_{cat}, indicating that the 2-amino group is not essential for activity. The 100-fold decrease in activity upon 2-aminopurine substitution points to the fact that the guanosine amide function must be involved in hydrogen bonding. The even more pronounced decrease with the introduction of isoguanosine is explained by additional repulsive interactions of the 2-amino group (Table II). Xanthosine, which has the 6-carbonyl in common with guanosine and inosine, is less active than guanosine by a factor of 400. The high activity of E-I^8 shows that no substituent is required at purine position 2. Thus the presence of the 2-carbonyl should not interfere with activity. However, the difference in protonation at xanthosine N3 could be responsible for this decrease in rate, or the 2-carbonyl might make an unfavorable interaction. The conclusion from these results is that G^8 contributes with the O6–N1 amide function and N3 to transition-state stabilization without involvement of the 2-amino group. The unaltered K_m value of E-$2AP^8$ suggests that the transition-state interactions of G^8 carbonyl functions does not affect substrate binding, whereas the slight effect of inosine substitution might be explained by the loss of a weak substrate contact to the guanosine 2-amino function.

Mg^{2+} Binding. It is possible that any of the three guanosines may be involved in the binding of Mg^{2+}. Fu and McLaughlin (1992b) have put forward such a model, where the 2-amino group of G^5 functions as a hydrogen bond donor in the coordination to a hydrated Mg^{2+}. If the 2-amino group of G^5 were indeed involved in the binding of the metal ion, a ribozyme containing inosine at this position should have a lower affinity for Mg^{2+}, resulting in lowered activity. It would then be expected that this low affinity could be overcome by increasing the Mg^{2+} concentration. We have therefore determined the cleavage rates not only at 10 mM but also at 50 mM Mg^{2+} to see whether any reduction in activity can be compensated by the higher Mg^{2+} concentration. This is not the case for any of the base-modified ribozymes.

The 2′-hydroxyl groups of $G^5$and G^8 have been shown to be essential for activity (Fu & McLaughlin, 1992a; Paolella et al., 1992; Perreault et al., 1990, 1991; Williams et al., 1991).[2] The 2′-hydroxyl group of G^5 has been suggested by Perreault to be a hydrogen bond donor to a hydrated Mg^{2+} (Perreault et al., 1991). However, a comparison of rates of ribozymes containing deoxyguanosines at positions G^5, G^8, and G^{12} at 10 and 50 mM Mg^{2+} again only showed a moderate increase at the higher metal ion concentration (Table II). This makes the involvement of these 2′-hydroxyl groups in the coordination of Mg^{2+} unlikely. We therefore conclude that none of these three guanosines in the core region are responsible for the binding of the catalytic metal ion cofactor by either the base functional groups or the 2′-hydroxyl groups.

G/A Mispairs. The question remains as to what interactions these guanosines are involved in. It has been postulated that the A9/G12 and G8/A13 residues, adjacent to stem II of the hammerhead, are capable of forming G/A double-mismatched base pairs (Li et al., 1991a; Slim & Gait, 1992). G/A mismatches are conformationally variable and can exist in four different structures, all of which involve normal base tautomers with guanosine in the anti conformation (Figure 3a–d) (Li et al., 1991a). Protonation of adenosine under acidic conditions allows additional G(syn)/A(anti) pairing (Figure 3e) (Gao & Patel, 1988; Leonard et al., 1990). Three of the unprotonated structures (Figure 3a–c) have been observed experimentally (Li et al., 1991). While single G/A pairs destabilize duplex structures, adjacent 5′-G/A-3′ mismatches can reach stabilities similar to fully Watson–Crick base-paired duplexes (Ebel et al., 1992; Li et al., 1991b; SantaLucia et al., 1990). Tandem G/A mismatches in the sequence context of d(5′-RG/AN-3′) adopt the imino pairing scheme (Figure 3a,b) and do not require the participation of the guanosine 2-amino group. G/A to I/A substitution in this sequence context is not duplex destabilizing (Ebel et al., 1992). In contrast, deoxyinosine substitution in the d(5′-YG/AR-3′) sequence produces relatively unstable tandem I/A mismatches, indicating that the 2-amino group of guanosine is crucial for stability in this stacking environment (Figure 3c,d) (Ebel et al., 1992; Li et al., 1991a; Lane et al., 1992). RNA duplexes with the G/A double mismatch in the sequence context of 5′-CA/GG-3′ and 5′-CG/AG-3′ displayed similar stability (SantaLucia et al., 1990), but the high stability of the reversed mismatch sequence 5′-A/G-3′ in RNA is unusual (Ebel et al., 1992; Li et al., 1991b). NMR and functional group modification studies on these RNA duplexes support two distinct types of base pairing, the A/G sequence that involves hydrogen-bonded imino protons (Figure 3a) and the G/A sequence that does not (SantaLucia et al., 1990, 1991). The substitution of adenine by purine reduces drastically the loop stability of the G/A as well as the A/G sequence, whereas the guanosine to inosine substitution has a slightly stabilizing effect.

While the above G/A double mismatches are embedded in the symmetrical environment of a double helix, potential G/A pairing in the hammerhead can only occur in the asymmetrical environment of the central core. The latter situation is related to G/A single mismatch pairing in ribosomal RNA, where it is found that G/A pairs (A at the 3′-end) often adjoin the ends of postulated double-helical regions. An A(syn)/G(anti) base pair proposed to fit at the end of an undistorted double helix would favor a break in the double helix, thus producing a nonlinear tertiary structure (Traub & Sussman, 1982). The

[2] There is some discrepancy for results obtained with dG^8. Williams et al. (1991) and Fu and McLaughlin (1992a) report a large decrease in rate for this modification, but Perreault et al. (1990) report none.

base pairs in double-helical regions adjacent to the G/A mispairs of rRNA show a strong preference for a guanosine. This is also found to be the case in the hammerhead ribozyme. The hammerhead $G^{10.1}/C^{11.1}$ base pair, at the end of stem II, is essential for efficient ribozyme cleavage. Although it can be replaced, with some loss in activity, by A/U and G/U pairs, none of the inverted purine/pyrimidine pairs are tolerated (Ruffner et al., 1990; Tuschl & Eckstein, 1993).

The existence of any hydrogen-bonded G/A mismatch would require the participation of the adenosine amino group along with either N1 (Figure 3a,d,e) or N7 (Figure 3b,c). Surprisingly, the substitution of the hammerhead A^9 by 7-deazaadenosine (Fu & McLaughlin, 1992b) and purine riboside (nebularine) (Fu & McLaughlin, 1992a) affected the cleavage rate by less than a factor of 2. This makes the existence of G^{12}/A^9 hydrogen bonding, of the types previously discussed (Figure 3), highly improbable. The substitution of the opposite G^{12} by inosine reduces the cleavage rate by a factor of 370 and excludes G/A hydrogen bonding of the imino type (Figure 3a,b). 2-Aminopurine riboside substituted for G^{12}, which reduces cleavage by a factor of 2700, should maintain its potential amino base pairing (Figure 3c,d) but is also capable of forming a stable 2AP/A wobble base pair (SantaLucia et al., 1991) that could hinder transition-state formation. Indeed, that this 2AP/A alternative wobble base pairing might be present is indicated by the xanthosine modification, which, although the 2-amino group is removed and N3 is protonated, did not exhibit a much more pronounced rate reduction than the inosine modification. Thus, we conclude that no standard base pairing between G^{12} and A^9 (Figure 3) exists in the central core of the ribozyme.

The second of the supposedly stable tandem G/A mismatches,the adjacent A^{13}/G^8 pair, displayed completely different behavior from that of the G^{12}/A^9 structure upon functional group modification. The A^{13} N7-nitrogen is not required (Fu & McLaughlin, 1992b), but the amino group is now important (Slim & Gait, 1992). At the same time, inosine at the G^8-position is well tolerated, while 2-aminopurine is not. This would suggest G(anti)/A(anti) imino base pairing (Figure 3a). However, this is not in agreement with the aborted reactivity upon G^8 replacement with xanthosine, as the guanosine amino group should not be involved in pairing interaction and thus replacement with the smaller xanthosine carbonyl group should be tolerated. Thus, these modification results do not support the existence of a mispair for A^{13}/G^8. Taken together with the evidence described above, the results reported here are not in agreement with the existence of *GA* tandem mismatches for positions A^{13}/G^8 and G^{12}/A^9 in the hammerhead ribozyme.

Transition-State Stabilization. Even though it is at present impossible to identify the partners in the hydrogen-bonding network in which the functional groups of the guanosines in the central core are involved, it is possible to at least classify the type of partner by determining the apparent transition-state destabilizing energies of the modified ribozymes. This can be done by comparing the first-order rate constants of the modified and the unmodified ribozyme under conditions were cleavage is rate limiting (Fersht, 1988). As mentioned earlier, this condition is fulfilled by the ribozyme we have chosen for this study (Fedor & Uhlenbeck, 1992). It forms a total of 10 base pairs between the substrate and the ribozyme, and the free energy contributions of substrate binding via helix–coil transition have little effect on the rate of the cleavage step for this ribozyme. As long as the functional group modification is not drastically affecting the K_m, the relative cleavage rates of the modified ribozymes lead to an estimate of the incremental apparent free energies of transition-state stabilization, $\Delta\Delta G^{\ddagger}$, rather than of substrate binding. $\Delta\Delta G^{\ddagger}$ can then be measured with high precision because values are calculated from the ratio of first-order rate constants, $(k^s_{obs})_{rel} = (k^s_{obs})_{mod}/(k^s_{obs})_{unmod}$, for the interconversion of substrate under saturating conditions (Wells & Fersht, 1986). These rate constants are, therefore, independent of both the concentration of enzyme and the concentration of substrate and less sensitive to dispensing errors. According to simple transition-state theory, the relative cleavage rate of a modified ribozyme under substrate saturation, $(k^s_{obs})_{rel}$, is related to the difference in apparent transition-state free energy, $\Delta\Delta G^{\ddagger}$, occurring during substrate cleavage in reactions with modified versus unmodified ribozymes,

$$\Delta\Delta G^{\ddagger} = RT \ln(k^s_{obs})_{rel} \quad (1)$$

The apparent free energy changes in substrate binding and transition-state stabilization for protein enzymes (Fersht, 1987; Fersht et al., 1985; Lowe et al., 1987; Wells & Fersht, 1986) as well as for protein folding (Matouschek et al., 1989) and protein stability (Alber et al., 1987; Serrano & Fersht, 1989) have been determined. In the interpretation of apparent free energy changes it should be borne in mind that they are the result of a number of opposing effects. The contributions of the bond in question, of hydrophobic and electrostatic interactions, and of hydration can result in an overall small $\Delta\Delta G^{\ddagger}$ value, although the individual values can be quite large (Gao et al., 1989; Wolfenden & Kati, 1991; Johnson & Benkovic, 1990). Nonetheless, deleting a hydrogen bond to an uncharged hydrogen bond donor/acceptor by site-directed mutagenesis weakens apparent binding by 0.5–1.8 kcal mol^{-1} for nondisruptive substitution (Fersht, 1987), but disruption of a hydrogen-bonding network can weaken apparent binding and transition-state energy up to 4.5 kcal mol^{-1} (Lowe et al., 1987).

For RNA interactions, the apparent binding energies of the guanosine analogues inosine and 2-aminopurine ribonucleoside to the self-splicing intervening sequence of *Tetrahymena* RNA have been quantified to be 2.9 and 2.8 kcal mol^{-1}, respectively (Bass & Cech, 1984). The average free energy contribution per hydrogen bond is thus 1.4 kcal mol^{-1} if additional stacking interactions are excluded. Substitution of inosine for guanosine in the terminal G/C base pairs of GCCGGC and CGGCCG provides apparent hydrogen-bonding energies of –1.6 and –0.8 kcal mol^{-1}, respectively (Turner et al., 1987). Hydrogen bonding within internal loops containing G/A double mismatches contributes at least –1.4 kcal mol^{-1} to duplex stability (SantaLucia et al., 1991). Hydrogen bonds within the unusually stable GCAA hairpin loop all contribute less than 1 kcal mol^{-1} to hairpin stability (SantaLucia et al., 1992). The free energy change for the mutation of another extrastable hairpin with a UUCG loop to the less stable UUUG loop is 1.5 kcal mol^{-1} (Varani et al., 1991).

The thermodynamic contributions of some 2′-hydroxyl contacts in tRNA for the recognition by its cognate tRNA synthetase have been evaluated for the *Escherichia coli* $tRNA^{Ala}$ system. Single 2′-OH functions within the acceptor stem can stabilize the transition state for aminoacylation up to 1.8 kcal mol^{-1} (Mousier-Forsyth & Schimmel, 1992). The apparent free energy contributions of some 2′-hydroxyl groups to substrate binding in the transition-state stabilization of the *Tetrahymena* ribozyme were shown to be similar in value. The 2′-hydroxyl groups of cytidine at position –2, of uridine

Table III: Relative Kinetic Rate Constants and Apparent Transition-State Destabilization Energies of Modified Ribozymes[a]

ribozyme	$(k^s_{obs})_{rel}$	$-\Delta\Delta G^{\ddagger}$ [b] (kcal mol^{-1})	ribozyme	$(k^s_{obs})_{rel}$	$-\Delta\Delta G^{\ddagger}$ [b] (kcal mol^{-1})
E-I[5]	0.0040	3.3	E-isoG[8]	0.0017	3.8
E-2AP[5]	0.0018	3.8	E-2′dG[8]	0.0084	2.8
E-X[5]	0.0014	3.9	E-I[12]	0.0027	3.5
E-2′dG[5]	0.0037	3.3	E-2AP[12]	0.0004	4.7
E-I[8]	0.3700	0.59	E-X[12]	0.0017	3.8
E-2AP[8]	0.0061	3.0	E-isoG[12]	0.0004	4.7
E-X[8]	0.0024	3.6	E-2′dG[12]	1.5500	–0.26

[a] In the presence of 10 mM $MgCl_2$ under conditions of substrate saturation (500 nM). [b] $\Delta\Delta G^{\ddagger}$ was calculated at 298.15 K (25 °C) with the gas constant R = 1.98 cal K^{-1} mol^{-1}. Positive values of $-\Delta\Delta G^{\ddagger}$ represent slower reactions of modified ribozymes relative to the unmodified ribozyme.

at position –3, and of adenosine at position +1 of the substrate independently contribute 1–2 kcal mol^{-1} (Bevilacqua & Turner, 1991; Herschlag et al., 1993a; Pyle & Cech, 1991), with the 2′-OH of the uridine donating a hydrogen bond to the N1 of adenosine 302 in the ribozyme core (Pyle et al., 1992).

In contrast to these relatively small values for transition-state stabilization by single hydrogen bonds to uncharged groups, studies with proteins have shown that deletion of a hydrogen bond to a charged donor/acceptor weakens binding by some 3–6 kcal mol^{-1} when either one or both partners are charged (Fersht, 1987). This has also been found in the *Tetrahymena* ribozyme-catalyzed reaction, where a hydrogen bond from the 2′-hydroxyl group of the substrate to the neighboring 3′-oxygen anion, formed in the transition, contributes 4.8 kcal mol^{-1} (Herschlag et al., 1993a,b). Thus, binding is much more strongly affected by interruption of hydrogen bonds to charged than to uncharged residues.

The apparent transition-state destabilizations we have obtained in the hammerhead cleavage reactions of most deletion-modified ribozymes, except for inosine at position 8 and deoxyguanosine at position 12, for which the values are very low, vary between 2.8 and 4.7 kcal mol^{-1} (Table III). Given some uncertainty that not all k_{obs} might have been obtained under complete substrate saturation conditions, these $\Delta\Delta G^{\ddagger}$ values would be smaller by 0.4 kcal mol^{-1} if the difference in k_{obs} were a factor of 2 and by 0.8 kcal mol^{-1} if it were a factor of 4. These possible differences will not invalidate our conclusions. Figure 6 summarizes the individual contributions of all guanosine functional groups within the hammerhead central core. These values reflect apparent binding energies (Fersht, 1987, 1988) which are too high to be explained by a simple loss of one hydrogen bond. If one considers that inosine and 2-aminopurine riboside can delete two possible hydrogen bonds and thus that these $\Delta\Delta G^{\ddagger}$ values should be divided by two, $\Delta\Delta G^{\ddagger}$ spans a range of 1.5–3.3 kcal mol^{-1} for each individual hydrogen bond. These values are still too high for single hydrogen bonds to uncharged residues but at the same time are too low to be assigned to those to charged groups. The most likely interpretation for these results is that they might be indicative of the disruption of a hydrogen-bonding network, where loss of one hydrogen bond is associated with the loss of additional ones. Precedence for this can be found in proteins, as discussed above (Lowe et al., 1987). The very high apparent transition-state destabilization of xanthosine- and isoguanosine-modified ribozymes with $\Delta\Delta G^{\ddagger}$ values from 3.8 to more than 4.7 kcal mol^{-1} presumably could be a consequence of a number of effects, such as additionally introduced electrostatic interactions, steric effects, or van der Waals contacts.

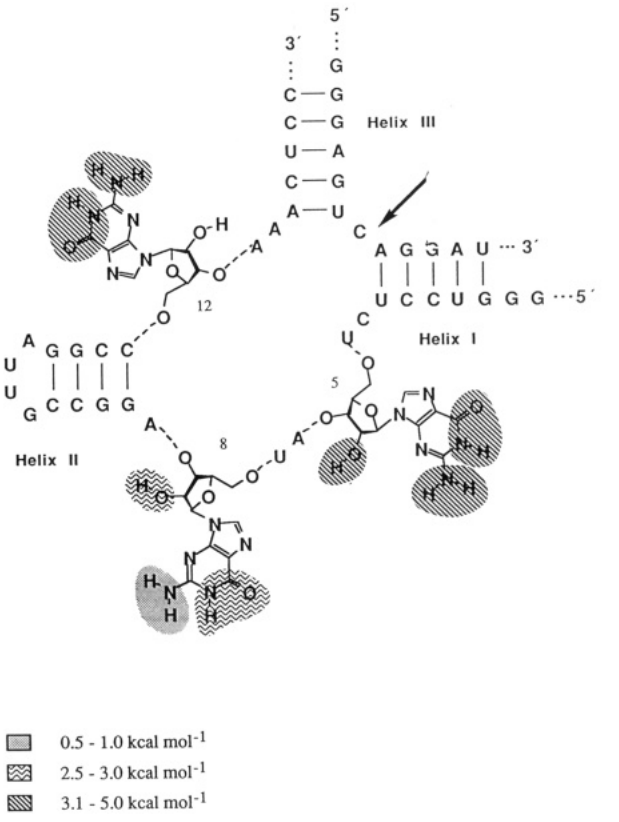

FIGURE 6: Apparent free energy contributions to transition-state formation of all conserved guanosine functinal groups within the hammerhead ribozyme. Values of $\Delta\Delta G^{\ddagger}$ are from Table III.

Conclusion. In summary, the results reported here do not support the existence of G/A mismatched base pairs in the central core and are not consistent with any of the guanosine base functional groups or 2′-hydroxyl groups being responsible for the binding of the catalytically active Mg^{2+}. They do suggest, however, that these groups are part of a hydrogen-bonding network for which the partners still have to be identified.

ACKNOWLEDGMENT

We thank O. Heidenreich and D. Williams for stimulating discussions, U. Kutzke for expert technical assistance, B. Seeger for recording the NMR spectra, and D. Herschlag, K. Nakamaye, and J. Thomson for critial reading of the manuscript.

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