Critical Nature of a Specific Uridine O^2-Carbonyl for Cleavage by the Hammerhead Ribozyme†

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Received December 17, 1998; Revised Manuscript Received April 15, 1999

ABSTRACT: Three modified hammerhead ribozyme/substrate complexes have been prepared in which individual uridine O^2-carbonyls have been eliminated. The modified complexes were chemically synthesized with the substitution of a single 2-pyridone (2P) base analogue for residues U_4, U_7, and $U_{16.1}$. Steady-state kinetic analyses indicate that the cleavage efficiencies for the U_7 and $U_{16.1}$ complexes were not significantly reduced relative to the native complex as measured by k_{cat}/K_M. The cleavage efficiency for the $2P_4$ complex, with the analogue present within the uridine loop, was reduced by greater than 2 orders of magnitude. This significant reduction in catalytic efficiency was due primarily to a decrease in k_{cat}. The pH vs cleavage rate profile suggests that the O^2-carbonyl of the U_4 residue of the hammerhead complex is critical for transition state stabilization and efficient cleavage activity. The results of a Mg^{2+} rescue assay do not implicate the O^2-carbonyl of U_4 in an interaction with a divalent metal ion. In addition, the results of a ribozyme folding assay suggest that the presence of the $2P_4$ within the uridine loop does not alter the folding pathway (relative to the native sequence) both in the absence and in the presence of Mg^{2+}. The O^2-carbonyl of U_4 appears oriented toward the interior of the catalytic pocket where it may be involved in a critical hydrogen bonding interaction necessary for transition state stabilization.

The hammerhead ribozyme represents one member of a small class of catalytically active nucleic acid complexes that appear to be important in RNA processing, particularly with respect to self-cleavage of multimeric RNA precursors *(1, 2)*. The hammerhead RNA is one of the smallest structures *(3, 4)* able to accelerate specific transesterification reactions; it consists of 3 helical arms and 11 consensus nucleotides with a requirement for a divalent metal cofactor (Mg^{2+} or Mn^{2+}) (see Figure 1a) *(5)*. The two products of the transesterification reaction contain either a terminal 5′-hydroxyl or a 2′,3′-cyclic phosphodiester *(3, 4)*. Complexes formed *in vivo* result from the folding of a single RNA molecule *(1, 6, 7)*, but *in vitro* complexes can be composed of two or even three fragments *(4, 8−11)*.

At least one metal cofactor and possibly more is required for catalytic activity, but the role of the cofactor(s) remains unclear *(12−16)*. Nucleoside analogues have been used for the identification of specific functional groups that are critical for catalytic efficiency *(11, 17−34)*. These important functional groups, located on individual bases as well as selected internucleotide phosphates and carbohydrates, may be involved in specific hydrogen bonding interactions or metal coordination (see ref *18*), or they may be involved in general acid/base chemistry (see refs *35* and *36*) facilitating the observed catalysis.

X-ray crystallographic analysis and NMR studies have provided information about the ground-state structure of the ribozyme *(32, 33, 37, 38)*. Conformational analyses based upon anomalous migration in polyacrylamide gels or FRET studies suggest that the solution conformation is very similar to the reported crystal structures *(39−41)*. This structure results from the coaxial stacking of helices II and III with helix I positioned at an angle, depending upon the Mg^{2+} concentration (see Figure 1b).

Substitution of some uridine analogues into the hammerhead ribozyme has been previously reported. Replacement of $U_{16.1}$ by deoxyuridine resulted in a decrease in catalytic efficiency by more than 2 orders of magnitude relative to the native complex *(42, 43)*. Other deoxyribonucleotides have been substituted into the stems and other sites in the core of the hammerhead ribozyme complex, resulting in decreases in k_{cat}/K_M from 2-fold to greater than 3 orders of magnitude *(27, 42−44)*. Nucleoside analogue substitutions at the U_7 position, in some cases, have resulted in ribozymes that are more active than the native sequence. For example, the substitution of the base analogue pyridin-4-one for U_7 resulted in a ribozyme that was 10-fold more active *(45)*, and replacement of U_7 by N^3-methyluracil resulted in a 2-fold increase in cleavage activity. Other analogues such as pyridin-2-one, pseudouridine, 6-methyluridine, and 6-azauridine decreased the activity of the hammerhead ribozyme *(45)*. The 2-pyrimidinone derivative has been substituted for C_3, U_4, U_7 and $U_{16.1}$, and C_{17} *(46)*. All substitutions resulted in decreased catalytic activity. A significant reduction in cleavage was observed (18-fold) with the analogue at the position 4, while at position 16.1 no detectable cleavage activity was observed. 4-Thiouridine was also substituted for U_4 and $U_{16.1}$, and these analogue complexes exhibited only slight decreases in activity *(46)*.

The present work involves the role of the O^2-carbonyl of the conserved U residues and its importance in the ribozyme cleavage reaction. We have synthesized and then incorpo-

† This work was supported by a grant from the NIH (GM47666).

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10.1021/bi982977a CCC: $18.00
Published on Web 05/26/1999

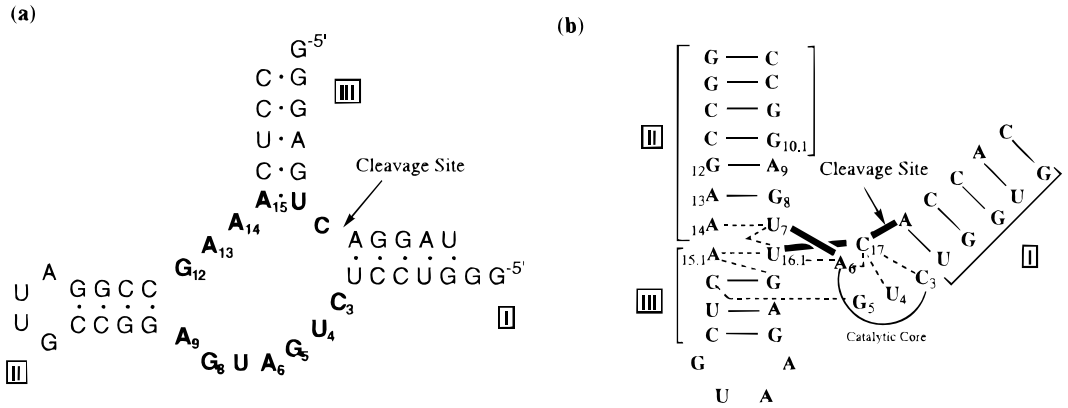


FIGURE 1: (a) Consensus sequence of the hammerhead ribzyme; (b) suggested conformation of the hammerhead ribozyme in the presence of divalent metal ions (*32*, *33*).

rated single residues of a 2-pyridone (2P) derivative at the U_4, U_7, and $U_{16.1}$ positions of the hammerhead ribozyme. With this analogue, the O^2-carbonyl is replaced by hydrogen, thus eliminating a potential hydrogen bond acceptor or a metal binding interaction. We report here the synthesis of the protected 2P phosphoramidite derivative, the construction of the "deletion-modified" ribozymes, and the kinetic characterization as well as the global folding of these ribozyme complexes each lacking a single O^2-carbonyl.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized using ribonucleoside phosphoramidites and solid supports obtained from Biogenex (San Ramon, CA) on an Applied Biosystems 381A DNA synthesizer. Fast protein liquid chromatography was performed with a Pharmacia FPLC system using a Dionex (Sunnyvale, CA) NucleoPac PA-100 (9 × 250 mm) ion exchange column at 60−65 °C. Acrylamide and bisacrylamide were obtained from ICN (Costa Mesa, CA). A Schleicher & Schuell (Keene, NH) Elutrap electro-separation system was used to elute RNA from polyacrylamide gels. Desalting was done with an EconoPac 10 DG column obtained from BioRad (Hercules, CA) or by Seppak columns from Millipore (Milford, MA). Enzymes were obtained from United States Biochemical Corp. (Cleveland, OH) or Boehringer Mannheim (Germany). 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. Absorption spectra were recorded by a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. [γ-^{32}P]ATP was obtained from NEN/Dupont (Wilmington, DE) or ICN (Costa Mesa, CA). Radioactivity was quantified with a Molecular Dynamics Phosphorimager 425. TLC analyses were performed in methanol/dichloromethane/triethylamine (5/95/trace).

Methods

*2-Hydroxy-5-iodopyridine (**3**).* A mixture of 2-aminopyridine (**1**) (3.8 g, 0.4 mmol), periodic acid dihydrate (1.83 g, 8 mmol), and iodine (4.08 g, 4 mmol) was heated in a solution of acetic acid (24 mL), water (4.8 mL), and sulfuric acid (0.79 mL) at 80 °C for 4 h. The reaction mixture was then poured into 10% aqueous $Na_2S_2O_3$ and subsequently extracted with diethyl ether. The extract was washed with 10% aqueous sodium hydroxide and water, dried (Na_2SO_4), and concentrated *in vacuo*. The residue was purified by column chromatography on silca gel (5% methanol/chloroform) to give 5.19 g of **2** (59%). This material was sufficiently pure to be carried onto the next step: $R_f = 0.38$; ^{1}H NMR (CD_3OD) δ 6.34 (d, 1H), 7.57 (d, 1H) 8.03 (s, 1H); HRMS (FAB) calcd for $C_5H_4NOI + H^+$ 221.9416, found 221.9416.

To 2-amino-5-iodopyridine, **2** (2.36 g, 10.7 mmol), dissolved in concentrated sulfuric acid (72 mL), with stirring in an ice bath, was added sodium nitrite (0.30 g, 10.7 mmol). The reaction mixture was stirred at 60 °C for 30 min and cooled to room temperature, boric acid (0.53 g, 21.4 mmol) was added to the reaction mixture, and the mixture was heated rapidly to 100 °C and cooled to ambient temperature. The mixture was neutralized by the addition of ammonium hydroxide with cooling in an ice bath. After rotary evaporation of the solvent in vacuo, the product was extracted by hot methanol. The pure product **3** (2.21 g, 94%) was obtained by column chromatography on silica gel (5% methanol/chloroform): $R_f = 0.25$; ^{1}H NMR (DMSO-d_6) δ 6.22 (d, 1H), 7.57 (d, 1H), 7.65 (s, 1H); HRMS (FAB) calcd for $C_5H_4NOI + H^+$ 221.9416, found 221.9416.

*5-Iodo-2-(4-nitrophenethoxy)pyridine (**4**).* Compound **3** (0.55 g, 2.49 mmol) was dissolved in anhydrous 1,4-dioxane under argon gas. Triphenylphosphine (2.42 g, 9.21 mmol), 4-nitrophenethyl alcohol (1.54 g, 9.21 mmol), and diethyl azodicarboxylate (1.45 mL, 9.21 mmol) were added, and the reaction mixture was stirred at ambient temperature for 3 h. The solvent was evaporated, and the residue was dissolved in dichloromethane. The solution was washed with water, dried, and reduced in vacuo. The residue was chromatographed on silica gel (chloroform) to give a colorless solid of compound **4** (0.46 g, 50%): $R_f = 0.85$; ^{1}H NMR ($CDCl_3$) δ 3.18 (t, 2H), 4.53 (t, 2H), 6.55 (d, 1H), 7.43 (d, 2H), 7.78 (d, 1H), 8.16 (d, 2H), 8.30 (s, 1H); HRMS (FAB) calcd for $C_{13}H_{11}N_2O_3I + H^+$ 370.9893, found 370.9893.

*5-{[3′-(1,1-Dimethylethyl)diphenylsilyoxy]-β-D-glycero-pentofuran-3′-ulos-1′-yl}-2-(4-nitrophenethoxy)pyridine (**5**).* A mixture of bis(dibenzylideneacetone)palladium(*0*) (0.26 g, 0.45 mmol) and 1,3-bis(diphenylphosphino)propane (0.19 g, 0.45 mmol) in anhydrous acetonitrile (30 mL) was stirred under argon gas at room temperature for 1 h. This mixture was then transferred by syringe to a solution of compound

4 (2.5 g, 6.77 mmol), Daves' sugar *(47)* (1.6 g, 4.51 mmol), and tri-*n*-butylamine (3.2 mL, 13.5 mmol) in dry acetonitrile (70 mL). The resulting solution was stirred under argon at 80 °C for 8 h. The reaction mixture was then filtered through Celite, and the volatiles were removed by rotary evaporation. The residue was purified by column chromatography (20% ethyl acetate/hexanes) to yield 1.04 g (40%) of compound **5** as a yellow oil. This material could not be completely purified since it coeluted during chromatographic procedures with small quantities of the unreacted glycal: $R_f = 0.57$; ^{1}H NMR ($CDCl_3$) δ 1.10 (s, 9H), 3.15 (t, 2H), 3.85 (m, 2H), 4.26 (m, 1H), 4.52 (t, 2H), 4.74 (m, 1H), 5.53 (d, 1H), 6.53 (d, 1H), 7.20 (dd, 1H), 7.26−7.81 (m, 12H), 7.89 (s, 1H), 8.15 (d, 2H).

To a solution of compound **5** (0.92 g, 1.54 mmol) in anhydrous THF (28 mL) at 0 °C was added acetic acid (0.35 mL, 6.16 mmol), followed by a 1 M solution of tetra-*n*-butylammonium fluoride in THF (3.1 mL, 3.08 mmol). The desilylation reaction was complete in 10 min on the basis of TLC analysis. The volatiles were removed by rotary evaporation, and the residue was purified by column chromatography on silica gel (5% methanol/chloroform) to give compound **6** (0.40 g, 73%): $R_f = 0.38$; ^{1}H NMR ($CDCl_3$) δ 2.51 (dd, 1H), 2.85 (dd, 1H), 3.19 (t, 2H), 3.93 (m, 2H), 4.04 (t, 1H), 4.58 (t, 2H), 5.17(q, 1H), 6.75 (d, 1H), 7.45 (d, 2H), 7.72 (d, 1H), 8.14−8.19 (m, 3H); HRMS (FAB) calcd for $C_{18}H_{18}N_2O_6 + H^+$ 359.1243, found 359.1242.

5-(2′-Deoxy-β-D-ribofuranosyl)-2-(4-nitrophenethoxy)pyridine (7). To a solution of compound **6** (0.4 g, 1.54 mmol) in acetonitrile (37 mL) and acetic acid (37 mL) was added sodium triacetoxyborohydride (0.54 g, 3.08 mmol) at 0 °C. The reaction was complete within 10 min on the basis of TLC analysis. Volatiles were then removed under reduced pressure. The residue was purified by column chromatography (10% methanol/chloroform) to afford compound **7** (0.40 g, 99%): $R_f = 0.12$; ^{1}H NMR (DMSO-d_6) δ 1.60−2.10 (m, 2H), 3.16 (t, 2H), 3.37 (m, 2H), 3.72 (m, 1H), 4.17 (m, 1H), 4.47 (m, 1H), 4.76 (br m, 1H), 4.98 (q, 1H), 5.05 (br s, 1H), 6.74 (d, 1H), 7.55 (d, 2H), 7.66 (d, 1H), 8.13 (s, 1H), 8.18 (d, 1H); HRMS (FAB) calcd for $C_{18}H_{20}N_2O_6 + H^+$ 361.1399, found 361.1401.

5-[2′-Deoxy-5′-O-(4,4-dimethoxytrityl)-β-D-ribofuranosyl]-2-(4-nitrophenethoxy)pyridine (8). Compound **7** (0.24 g, 0.66 mmol) was dissolved in anhydrous pyridine (10 mL) at 0 °C under argon. Then 4,4-dimethoxytrityl chloride (0.45 g, 1.33 mmol), triethylamine (0.19 mL, 1.33 mmol), and 4-(dimethylamino)pyridine (0.08 g, 0.66 mmol) were added. The reaction mixture was stirred at room temperature for 48 h and concentrated under reduced pressure. The residue was dissolved in dichloromethane and washed with saturated sodium bicarbonate solution. The organic portion was concentrated in vacuo and the residue purified by column chromatography on silica gel (5% methanol/chloroform) to give compound **8** (0.31 g, 70%): $R_f = 0.48$; ^{1}H NMR ($CDCl_3$) δ 2.04 (m, 1H), 2.20 (dd, 1H), 3.18 (t, 2H), 3.29 (m, 2H), 3.76 (s, 6H), 4.04 (m, 1H), 4.45 (m, 1H), 4.56 (t, 2H), 5.12 (q, 1H), 6.66 (d, 1H), 6.79−6.85 (d, 4H), 7.18−7.47 (m, 11H), 7.61 (d, 1H), 8.10 (s, 1H), 8.16 (d, 1H); HRMS (FAB) calcd for $C_{39}H_{38}N_2O_8 + H^+$ 663.2706, found 663.2710.

5-(3′-O-(2-Cyanoethyl-N,N-diisopropylphosphino)-5′-O-(4,4-dimethoxytrityl)-β-D-ribofuranosyl)-2-(4-nitrophenethoxy)pyridine (9). Compound **8** (0.27 g, 0.41 mmol) was dissolved in anhydrous dichloromethane (25 mL) at 0 °C under argon, and then diisopropylethylamine (0.42 mL, 2.44 mmol) was added by syringe followed by the addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.27 mL, 1.22 mmol). The mixture was stirred at room temperature for 2 h, and then a small amount of methanol was added and stirring was continued for 20 min. Volatiles were removed by rotary evaporation, and the residue was dissolved in dichloromethane. Then hexane was added dropwise to precipitate the product, and the solvent was filtered to afford compound **9** (0.27 g, 77%): $R_f = 0.85$; ^{31}P NMR ($CDCl_3$) δ 148.2, 148.3; HRMS (FAB) calcd for $C_{48}H_{55}N_4O_9 + H^+$ 863.3784, found 863.3774.

Oligonucleotide Synthesis. The oligonucleotides were synthesized from 1 μmol of bound nucleoside on 500 Å silica supports using phosphoramidite chemistry *(48−50)* and an Applied Biosystems 381A DNA synthesizer. After assembly of each sequence, the glass beads were suspended in 2 mL of 2 M ammonia in methanol for 16 h at 55 °C. The glass beads were removed, and the ammonia solution was evaporated to dryness. To the residue was added 2 mL of 1.0 M tetrabutylammonium fluoride in tetrahydrofuran, and the reaction was protected from light and shaken for 18 h at ambient temperature. To the crude mixture of oligonucleotides was added 1 mL of 0.9 M sodium acetate (pH 6.0), and the mixture was extracted twice with ethyl acetate. The aqueous phase was desalted with an EconoPac 10 DG column. The 34-mer ribozymes were purified by gel electrophoresis in 20% polyacrylamide/1% bis(acrylamide)/7 M urea gels. The product band was visualized by UV shadowing, excised, and extracted by electroelution in 0.0045 M Tris−borate and 0.001 M EDTA (pH 8.0). The eluted oligonucleotide solution was desalted as above. The 12-mer substrates were purified by FPLC in a buffer gradient of 80 mM $LiClO_4$ and 20 mM NaOAc to 160 mM $LiClO_4$ and 20 mM NaOAc in 40 min. The eluted oligonucleotide solutions were desalted as above.

Nucleoside Analyses. Nucleoside composition was determined after S1 nuclease and calf intestinal alkaline phosphatase hydrolysis. A 10 μL reaction mixture containing 0.5 A_{260} unit of oligomer in 200 mM sodium chloride/5 mM $MgCl_2$/0.1 mM $ZnSO_4$/25 mM sodium acetate, pH 5.5, was incubated for 5 min at ambient temperature with 267 units of S1 nuclease. To the 10 μL reaction mixture were added 5 μL of 0.1 M Tris-HCl, pH 8.0, and 1 unit of calf intestinal alkaline phosphatase. After overnight incubation, a 5 μL aliquot was analyzed by HPLC using a 0.46 × 25 cm column of ODS Hypersil in 20 mM potassium phosphate, pH 5.5, and a gradient of 0−35% methanol (60 min). The retention times for the nucleosides were 5.9 (C), 7.4 (U), 7.4 (2P), 7.4 (dU), 10.8 (G), and 15.0 (A) min.

Radioisotopic Labeling. Each 12-mer was 5′-end-labeled with [γ-^{32}P]ATP as follows: a 25 μL reaction mixture containing 0.5 A_{260} unit of 12-mer, 40 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.02 mM EDTA, 450 μCi of [γ-^{32}P]ATP, and 120 units of T4 polynucleotide kinase was incubated for 60 min at 37 °C. The product was purified by electrophoresis in a 20% polyacrylamide/7 M urea gel. The product band was excised, extracted with 0.3 M sodium acetate, pH 6.0, and desalted with a Waters Sep-Pak C18 cartridge.

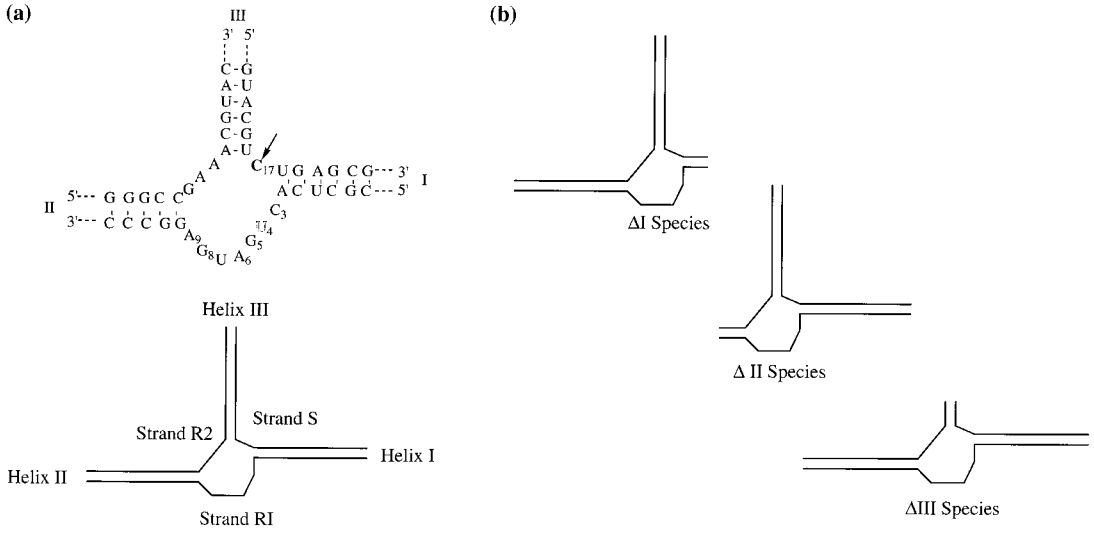


FIGURE 2: Complexes used in the comparative electrophoretic analysis of the conformation of the hammerhead ribozyme. (a) The RNA base sequence of the hammerhead core (above). The position of cleavage is indicated by an arrow. 2'-Deoxycytidine was substituted at position C17 to prevent self-cleavage and is indicated in boldface. 2P was substituted for U4 and is indicated in outline. Stylized illustration of the complex (below). (b) The hammerhead complexes used in this assay were constructed from three oligonucleotide strands: S, R1, and R2. The core of the ribozyme and 5 bp from each stem were synthesized with ribonucleotides. The extensions of the arms were synthesized from 2′-deoxynucleotides. Three variations of the full hammerhead species were synthesized in which each stem was systematically shortened.

Single-Turnover Cleavage Analysis. Two 25 μL solutions containing either 0.6 μM ribozyme or a trace of radiolabeled substrate in 50 mM Tris-HCl (pH 7.5) with 10 mM $MgCl_2$ were each heated to 95 °C for 1 min and cooled at 37 °C for 15 min. The reaction was initiated by mixing the two solutions. Aliquots of 8 μL were withdrawn, and the reaction was quenched by the addition of an equal volume of 50 mM Na_2EDTA/7 M urea/10% glycerol/0.05% xylene cyanol/0.05% bromophenol blue. The extents of cleavage were analyzed by electrophoresis in 20% polyacrylamide/1% bis(acrylamide)/7 M urea gels (14 × 16 cm) in 90 mM Tris−borate buffer and 2 mM Na_2EDTA, pH 8.0. The radioactivity of the substrate and product bands was quantified. The logarithm of the unreacted fraction was plotted against time, and the data points were fitted to a straight line. The cleavage half-lives ($t_{1/2}$) were used to obtain first-order rate constants ($k = 0.693/t_{1/2}$).

pH Studies. The same procedure for stoichiometric cleavage analysis was used with the exception that 50 mM of each of the following buffers was used in place of Tris-HCl: MES (pH 6.0), PIPES (pH 6.5), HEPES (pH 7.0 and 7.5), TAPS (pH 8.0, 8.5, and 9.0), and CHES (pH 9.5).

Magnesium Studies. The same procedure for stoichiometric cleavage analysis was used with the exception that the Mg^{2+} concentration was varied from 1 to 1000 mM.

Multiple-Turnover Cleavage Analysis. A 425 μL solution of the ribozyme and 6−8 50 μL solutions of the radiolabeled substrate RNAs in 10 mM $MgCl_2$/50 mM Tris-HCl (pH 8.0) were each heated separately to 95 °C for 1 min and cooled at 37 °C for 15 min. The reactions were initiated by adding 50 μL of the ribozyme solution to each substrate solution. The final ribozyme concentration in these reactions was 2 nM. Six to eight substrate concentrations were used varying in final concentration from 30 to 1500 nM depending on the individual sequence. Aliquots of 15 μL were taken from the reaction mixture at various times and stopped in an equal volume of quenching buffer as described above. Values up to 20% cleavage were used in the calculation of the kinetic parameters. K_M and V_{max} values were obtained primarily from linear Eadie−Hofstee plots.

Gel Electrophoretic Analysis of the Global Conformation of the Hammerhead Ribozyme. The ion-induced folding of the hammerhead ribozyme was determined by comparative gel electrophoresis *(39, 40)*. Hammerhead complexes utilized in this assay were made up of an RNA core extending 5 bp in each arm followed by 34 bp DNA sequences making up the outer section of each arm (Figure 2). One full-length strand was synthesized for S, R1, and R2. Two shorter versions of each strand were also synthesized. Each was lacking 34 nucleotides from either the 5′ or the 3′ end of the strand. The three substrate strands were synthesized with deoxyribose substituted at C_{17} to prevent self-cleavage in the presence of magnesium. The DNA/RNA sequences were synthesized on an Applied Biosystems DNA synthesizer and deprotected as described previously.

Sequences for the full-length strands of the hammerhead were the following:

S strand, 5′-CGCAAGCGACAGGAACCTCGAGAAGCTTCCGGTAGUACCUCUGAGCGGTGGTTGAATTCCTCGAGGTTCCTGTCGCTTGCG-3′; R1 strand, 5′-CGCAAGCGACAGGAACCTCGAGGAATTCAACCACCGCUCACUGAUGAGGCCCACTGCAGTCTAGACTCGAGGTTCCTGTCGCTTGCG-3′; R2 strand, 5′-CGCAAGCGACAGGAACCTCGAGTCTAGACTGCAGTGGGCCGAAACGUACTACCGGAAGCTTCTCGAGGTTCCTGTCGCTTGCG-3′.

The RNA portion is underlined. The site of 2P substitution is indicated in boldface. The three substrate strands were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. They were purified by denaturing electrophoresis in 15% polyacrylamide. The bands were excised and electoeluted into 0.5 × TBE and recovered by ethanol precipitation. The radiolabeled substrate was combined with a 10-fold excess each of the corresponding R1 and R2 strands in the presence of 50 mM Tris (pH 7.5) and either 2.5 mM EDTA or 10 mM $MgCl_2$. Comparative electrophoresis was performed

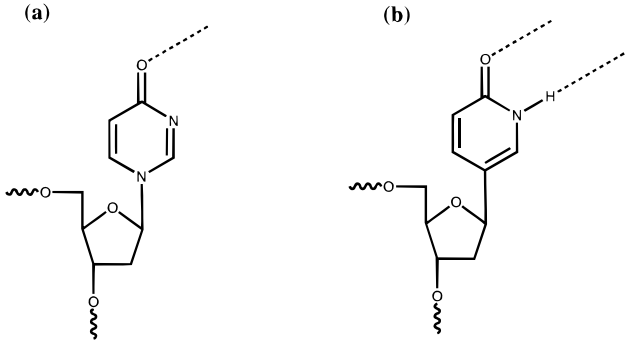


FIGURE 3: (a) Structure of the 4-pyrimidinone nucleotide, a possible uridine analogue in which the O^2-carbonyl has been deleted. (b) Structure of the 2-pyridone C-nucleoside as an alternative uridine analogue from which the O^2-carbonyl has been deleted. In both structures, dotted lines are the conserved Watson−Crick hydrogen bonding sites.

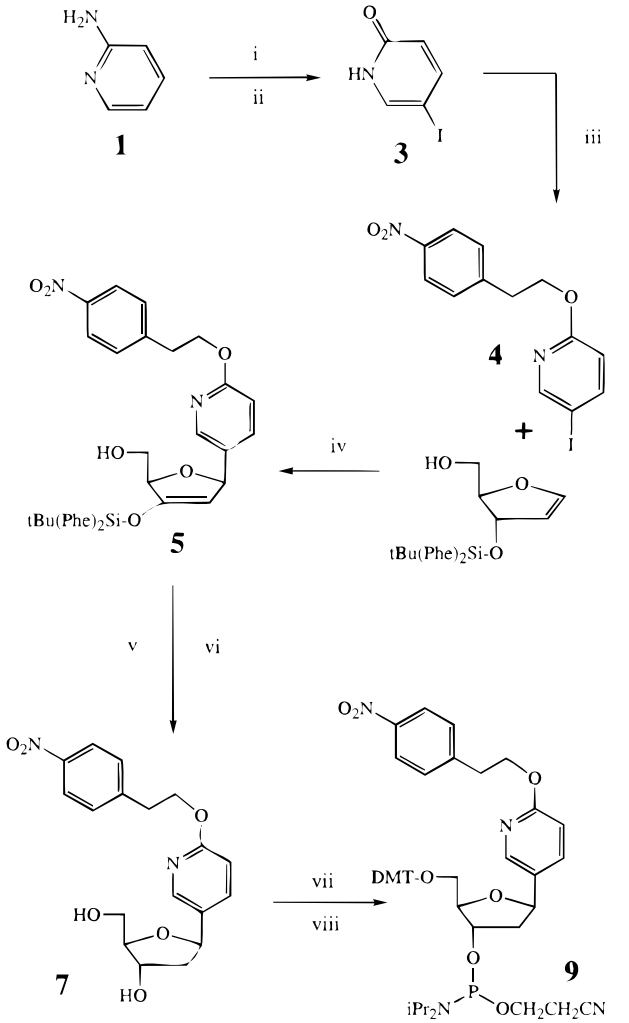
without any pre-isolation of the complex, using a nondenaturing (29:1 acrylamide:bisacrylamide) gel. The buffer for electrophoresis was recirculated at 25 °C and contained 90 mM Tris−borate, pH 7.5, with either 2.5 mM EDTA or 10 mM $MgCl_2$.

RESULTS AND DISCUSSION

While the O^2-carbonyl of uridine has not typically been observed to be involved in Watson−Crick hydrogen bonding interactions, it has been implicated in the G:U wobble base pair, such as that observed in yeast $tRNA^{Phe}$ *(51)*. The O^2- as well as O^4-carbonyls of T and U may take part in the coordination of metal ions directly or through water-mediated hydrogen bonds. In the crystallographic analysis of an ApU dimer, sodium ions were observed to be coordinated to the O^2-carbonyl of U *(52)*. Additionally, pancreatic RNase recognizes U at the pyrimidine binding site through interactions between the peptide NH of Thr_{45} and the O^2-carbonyl *(53, 54)*. It has also been demonstrated that DNA minor groove binding agents such as netropsin employ amide NH to pyrimidine O^2 interactions in complex formation *(55)*. The O^2-carbonyl of U_7 is involved in a ground-state interaction with the N^6 of A_{14} based upon the crystal structure of the hammerhead ribozyme *(33)*. Additionally, the O^2-carbonyl of U_4 has been implicated in a 3 Å aromatic n to π stacking interaction with C_{17} *(5, 33)*.

Nucleoside Synthesis. Probing the importance of the O^2-carbonyl of uridine residues present in the hammerhead ribozyme required the preparation of a modified nucleoside analogue in which the O^2-carbonyl was absent. Simple deletion of this residue from a pyrimidine ring system results in a tautomeric shift in which the proton on N^3 is lost (Figure 3a) *(56)*. To avoid such undesirable changes, we opted to prepare the 2-pyridone C-nucleoside (2P). 2-Pyridones tend to favor the keto rather than the enol tautomeric form, and such a derivative can result in "normal" U-like bidentate base pairing with A (Figure 3b). The 2-pyridone nucleoside was prepared in its fully protected form **9** (Scheme 1) for solid-phase-based DNA synthesis. In addition to the necessity of protecting the 2-pyridone carbonyl, the critical step in the synthetic route was the palladium-mediated coupling to generate the carbon−carbon bond between the heterocycle and the sugar. Owing to the need to use the 2-deoxyglycal

Scheme 1[a]



[a] (i) $HIO_4/I_2/CH_3COOH$; (ii) $H_2SO_4/NaNO_2$; (iii) *p*-nitrophenylethanol/Ph_3P/DEAD; (iv) $(dba)_2Pd^o/nBu_3N/Ph_2PCH_2CH_2CH_2PPh_2$; (v) $nBu_4N^+F^-$; (vi) $Na(OAc)_3BH/CH_3CHOOH$; (vii) DMT-Cl/DMAP/pyridine; (viii) Cl-PN(iPr)$_2$OCH$_2$CH$_2$CN/DIEA.

as one of the components of the coupling reaction, we prepared the 2P analogue as the 2′-deoxynucleoside derivative (Scheme 1).

Oligonucleotide Synthesis. The native ribozyme/substrate complex used in this work was similar to that described by Uhlenbeck *(57)* and others (see ref *58* and references cited therein). Each modified complex lacked a single O^2-carbonyl at a specific site, but because the 2P analogue was prepared as a 2′-deoxy C-nucleoside, the corresponding dU-containing control complexes were prepared. These particular ribozyme/substrate complexes were formed using a 12-mer substrate which was cleaved into 7-mer and 5-mer fragments by a 34-mer ribozyme (see Figure 4). After assembly, deprotection required the use of DBU/pyridine to remove the *p*-nitrophenylethyl protecting group. After PAGE or FPLC purification of each 34-mer ribozyme and 12-mer substrate, a small portion of each was completely hydrolyzed by S1 nuclease and alkaline phosphatase to confirm the absence of any unidentified species—the 2P residue was observed to coelute with U. In each digestion, the integration of the eluted peaks indicated the presence of one additional residue in the peak corresponding to U. Partial pancreatic RNase digestion of the dU- or 2P-containing sequences indicated that the site

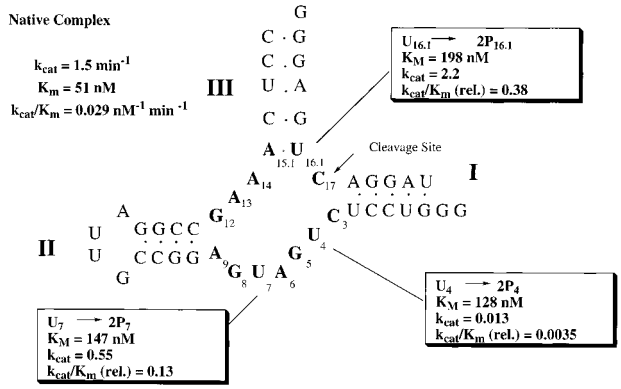


FIGURE 4: Proposed secondary structure for the hammerhead ribozyme/substrate complex with relative k_{cat}/K_M values indicated for the various analogue complexes.

containing the analogue was refractory to cleavage, indicative of the presence of a 2′-deoxynucleoside.

Importance of the O^2-Carbonyl for Catalytic Activity. All five analogue complexes (2P_4, 2P_7, 2$P_{16.1}$, dU_4, and d$U_{16.1}$) exhibited RNA cleavage activity although the catalytic efficiencies of the complexes varied significantly. The K_M values were 2.5−5.3 times higher than that observed for the native complex (Table 1). These results suggest that the absence of the O^2-carbonyl at the various sites did not dramatically affect the affinity between ribozyme and substrate. This result is not surprising since the affinity of the ribozyme for the substrate is most likely to result largely from interstrand Watson−Crick hydrogen bonding between the two molecules and the O^2-carbonyls of the U residues do not participate in these interactions.

With the present complex, the chemical cleavage event, that of the transesterification of the scissile phosphodiester bond, is considered to be the rate-limiting step near neutral pH *(57)*. Reductions in k_{cat}, therefore, can be correlated with less effective stabilization of the transition state (see ref *58*). In this study, the dU_4, 2P_7, d$U_{16.1}$, and 2$P_{16.1}$ analogue complexes exhibited kinetic parameters that were very similar to those of the native complex. For these three complexes, k_{cat}/K_M values were reduced by less than 10-fold (Figure 4). These experiments suggest that the O^2-carbonyl (and the 2′-OH), at each of these positions, does not contribute significantly to substrate binding or to transition-state stabilization.

The O^2-Carbonyl of $U_{16.1}$. The 2$P_{16.1}$ hammerhead complex exhibited a k_{cat} value increased by 1.6-fold and a K_M value increased by 3.9-fold, resulting in a k_{cat}/K_M that was decreased by only 2-fold relative to native parameters (Table 1). The ground-state crystal structure of the hammerhead complex suggests that the O^2-carbonyl of $U_{16.1}$ is involved in a hydrogen bond to the 2′-OH of A_6. The lack of any significant effect on k_{cat} or k_{cat}/K_M by substitution of 2P for $U_{16.1}$ suggests that the O^2-carbonyl does not take part in any critical transition-state stabilizing interactions. By comparison, the O^4-carbonyl of $U_{16.1}$ has been implicated in a single non-Watson−Crick hydrogen bond to N^6 of $A_{15.1}$ in the ground-state structure, and its elimination resulted in a ribozyme with no detectable cleavage activity, suggesting that the O^4-carbonyl of $U_{16.1}$ is involved in a critical transition-state interaction *(46)* (although it must be recognized that the analogue used in this study also altered the tautomeric state of the N^3-H nitrogen). The k_{cat}/K_M values for the d$U_{16.1}$ hammerhead complex are decreased by 13-fold relative to the native complex, and this effect is consistent with that observed previously after the substitution of T for $U_{16.1}$ (k_{cat}/K_M down 22-fold) *(41)*.

The results of the 2P substitution for $U_{16.1}$ are somewhat surprising; one would expect an activity at least 13-fold lower relative to the native complex since the 2′-OH is absent in this analogue, regardless of any alteration to the O^2-carbonyl. The O^2-carbonyl of $U_{16.1}$ does not appear to be involved in an important transition-state interaction, but its absence must compensate in some manner for the loss of the 2′-OH and transition-state stabilizing interactions. The 2P analogue, lacking the O^2-carbonyl, would be less sterically hindered to rotation about the glycosidic bond, and this movement might permit the O^4-carbonyl and/or N^3-H to form new interactions not observed in the crystal structure, and thus compensate for the transition-state energy lost with the absence of the 2′-OH.

The O^2-Carbonyl of U_7. The 2P_7 complex exhibited kinetic parameters that were not significantly different than native. The k_{cat} was reduced by 2.7-fold, the K_M was increased by 2.9-fold, and the k_{cat}/K_M was decreased by 7.3-fold (Table 1). These results suggest that the O^2-carbonyl of U_7 does not have a critical role in the catalytic cleavage reaction. U_7 is not a conserved residue in the hammerhead ribozyme core, and substitution of U_7 by T had previously been shown to exhibit native-like cleavage rates *(27)*. Therefore, kinetic analysis involving the replacement of U_7 by dU was not performed in this study. The O^2-carbonyl of U_7 is involved in an observable hydrogen bonding interaction in the ground-state hammerhead structure, based on crystallographic analyses *(5, 32, 33)*. The O^2-carbonyl at this location appears to play no role in the cleavage event as 2P substitution at this site has no significant effect on k_{cat} or K_M (Table 1).

The O^2-Carbonyl of U_4. The 2P_4 complex was found to be a significantly less efficient catalyst than the native complex with a k_{cat} value reduced more than 2 orders of

Table 1: Steady-State Kinetic Parameters for Cleavage of the dU- and 2P-Containing Ribozymes[a]

ribozyme	K_M[b] (nM)	k_{cat}[b] (min^{-1})	k_{cat}/K_M ($\times 10^{-3}$ nM^{-1} min^{-1})	k_{cat}/K_M(rel)
native	51	1.5	29	1.0
dU_4	209 ± 36	0.95 ± 0.052	4.5	0.16
2P_4	128 ± 5	0.013 ± 0.00070	0.096	0.0034
2P_7	147 ± 37	0.55 ± 0.008	4.0	0.13
d$U_{16.1}$	272 ± 33	0.59 ± 0.010	2.2	0.076
2$P_{16.1}$	198 ± 54	2.2 ± 0.29	11	0.38

[a] Reactions were conducted in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 2 nM ribozyme, and 10−1000 nM substrate at 25 °C. [b] The K_M and k_{cat} values given for each complex are the averages of at least two independent analyses and were determined from Eadie−Hofstee plots. The difference in K_M and k_{cat} values between two or more experiments for each complex was typically less than ±10%.

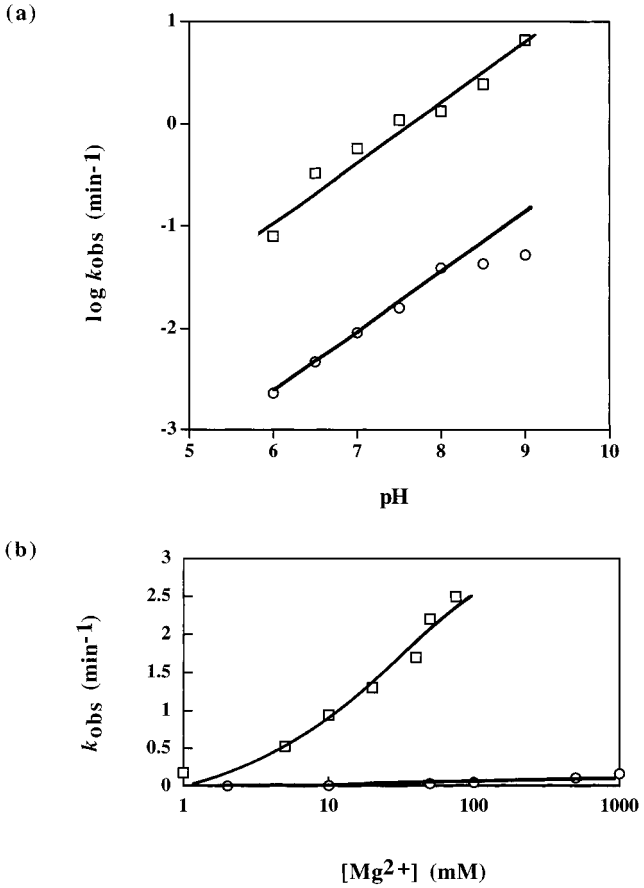


FIGURE 5: (a) Hammerhead ribozyme cleavage rates for the native complex (□) and $2P_4$ analogue complex (○) as a function of pH in the presence of 10 mM Mg^{2+}. (b) Hammerhead ribozyme cleavage rates for the native complex (□) and $2P_4$ analogue complex (○) as a function of Mg^{2+} concentration at pH 7.5.

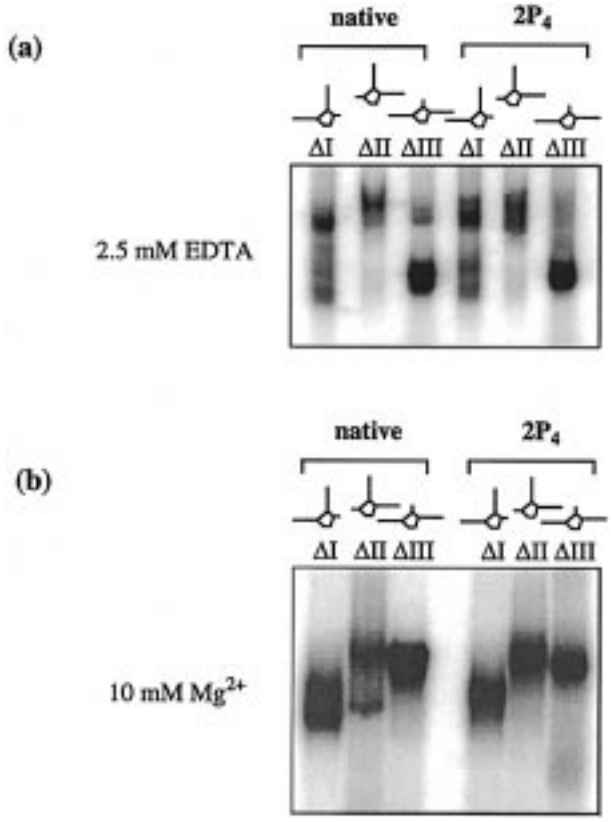


FIGURE 6: Comparative gel electrophoretic analysis of the native hammerhead ribozyme and $2P_4$ analogue complex. For each complex, three species (ΔI, ΔII, and ΔIII; see Figure 2), each with one shortened arm, were electrophoresed in nondenaturing conditions in Tris−borate buffer. (a) Electrophoresis in the presence of 2.5 mM EDTA. (b) Electrophoresis in the presence of 10 mM Mg^{2+}.

magnitude (down 300-fold) (Table 1). To verify that effects in the chemical cleavage step were the cause of the reductions in the cleavage activity observed for the $2P_4$ complex, we examined the cleavage event under first-order conditions over the pH range 6.0−9.5 (Figure 5a). Observation of a linear rate vs pH profile for the hammerhead cleavage reaction that is of the same slope as that for the native complex suggests that the chemical step, rather than a conformational change, is the rate-determining step *(12)*. We also examined the effects of increased Mg^{2+} on the cleavage rate (Figure 5b), but even at 1 M Mg^{2+} the cleavage activity of the $2P_4$ ribozyme was at least an order of magnitude below that observed for the native ribozyme at 75 mM Mg^{2+}.

The apparent binding free energy ($\Delta\Delta G_{app}$) was calculated for the $2P_4$ complex, and the 14.1 kJ mol^{-1} value suggests the loss of a hydrogen bond, possibly to a charged partner. It may be possible that the O^2-carbonyl of U_4 has a critical transition-state interaction with a nearby protonated residue or metal hydrate in the catalytic pocket, and the elimination of such a hydrogen bond by the elimination of the O^2-carbonyl destabilizes the transition state and lowers the catalytic efficiency of the ribozyme. But it would be possible that the reduced catalytic activity is the result of a change in the global conformation (and/or folding pathway) as a result of the elimination of the O^2-carbonyl of U_4.

Analysis of the Global Folding of the $2P_4$ Hammerhead Ribozyme. The U_4 residue is centrally positioned in the uridine turn present in the catalytic pocket (Figure 1b). Loss of the O^2-carbonyl should alter the syn/anti equilibrium for $2P_4$, possibly impacting the structural integrity of the uridine turn and altering the folding pathway of the ribozyme complex. To verify that the reduced cleavage activity of the $2P_4$ ribozyme was not due to a change in the global folding of the ribozyme, a comparative gel electrophoretic assay was performed. This assay assumes that the presence of metal ions is necessary in order for the complex to fold correctly and achieve the proper conformation for an in-line attack of the 2′-hydroxyl on the cleavage site phosphodiester *(39, 40)*. By extending the arms of the complex in an asymmetric manner, the "Y-shaped" conformation depicted in the crystal structures *(39, 40)* takes on a largely linear or dramatically bent shape. These changes in overall shape can be predicted based upon anomalous migration of the various asymmetric complexes in nondenaturing polyacrylamide gels *(39, 40)*.

We prepared the three asymmetric native complexes in which two of the three helical arms were elongated, and then prepared the same three molecules for the analogue 2P4 complex (ΔI, ΔII, ΔIII; see Figure 2). The $2P_4$ complexes, in the absence of Mg^{2+} (2.5 mM EDTA), all result in electrophoretic mobilities that are similar to the native complex—the ΔIII species has the fastest migration followed by ΔI and ΔII (Figure 6a). In the presence of 10 mM Mg^{2+}, the electrophoretic gel mobilities for the $2P_4$ species are again similar to those of the native complex (Figure 6b). The greatest changes in mobility for the native complex as well as the $2P_4$ species were observed with the ΔI and ΔIII

species, consistent with previously reported results *(39, 40)*. These similar electrophoretic patterns observed for the species of the native and $2P_4$ complexes, both in the absence and in the presence of Mg^{2+}, suggest that the elimination of the O^2-carbonyl of U_4 does not alter the global folding pathway for the analogue hammerhead ribozyme. The significant reduction in catalytic efficiency for the $2P_4$ ribozyme therefore is not likely to be the result of an altered conformation due to the elimination of the O^2-carbonyl and is therefore more probable to be the result of a loss of a critical interaction involving the O^2-carbonyl with attendant destabilization of the transition state.

CONCLUSIONS

The loss of one hydrogen bond acceptor from the 46 nucleotide hammerhead ribozyme/substrate complex, specifically, the O^2-carbonyl of U_4, results in a significant loss in catalytic efficiency. The O^2-carbonyl of U_4, along with functional groups identified in other studies as being important to transition-state stabilization, appears to be located largely in two regions of the ribozyme complex. The O^2-carbonyl of U_4 is located in the CUGA section of the ribozyme which makes up the uridine turn. Important functional groups have been identified on the C, G, and A residues of this uridine turn. Many of these groups do not appear to be involved in ground-state stabilization of the hammerhead ribozyme and yet do not seem to be critical for stabilization of the ground-state structure. The close proximity of many of these functional groups to each other and to the site of cleavage suggests that they are involved in a hydrogen bonding network which is not observed in the ground-state crystal structure but is critical to the stabilization of the transition state.

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BI982977A