Purine Functional Groups in Essential Residues of the Hairpin Ribozyme Required for Catalytic Cleavage of RNA

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ABSTRACT: Synthetic chemistry techniques have been used to study the functional group requirements of the essential purine residues in hairpin ribozyme cleavage. Three-stranded ribozymes were prepared that had functional group deletions or alterations at single purine sites within loops A and B of the hairpin, and the kinetics of cleavage were compared to those of the unmodified ribozyme. Adenosine analogues used were purine riboside and N^7-deazaadenosine, and guanosine analogues used were inosine, N^7-deazaguanosine, and O^6-methylguanosine. In many cases, introduction of one of these analogues caused substantial loss of ribozyme cleavage activity. Most of the impairments of activity were found to be due to changes in k_{cat} rather than in K_M. The losses corresponded in magnitude to loss of at least one hydrogen bond, and the results were rationalized in terms of removal of potential cross-strand hydrogen bonds as well as potential hydrogen bonds between loops A and B. A new secondary structure model for loop B was proposed. Finally, the magnesium ion dependence of cleavage was studied for the modified ribozymes and compared to that of the unmodified ribozyme. It is proposed that magnesium binds in the ground state to the N^7-positions of G_{+1} and A_{43} and in the transition state to the N^7-position at A_9. The results provide further evidence for the folding of the two arms of the hairpin so that in the active conformation loops A and B approach closely to form a specific three-dimensional structure with a magnesium ion (or ions) placed between the loops, making contacts in the ground state and in the transition state.

There is considerable interest in the structures and mechanisms of catalytic cleavage afforded by small RNA ribozymes, such as the hammerhead, hairpin, and hepatitis delta ribozymes [reviewed in Symons (1992)]. In each case there is a similar reaction pathway that involves attack of a 2′-hydroxyl group on its neighboring 3′-phosphodiester to form two oligonucleotides, one containing a 2′,3′-cyclic phosphate and the other, a free 5′-hydroxyl group. The reaction requires a divalent metal ion, usually magnesium. However, the RNA primary and secondary structures of the three ribozyme types are quite distinct.

The hairpin ribozyme is the catalytic domain derived from the negative strand of the satellite RNA from tobacco ringspot virus (Feldstein *et al.*, 1989; Hampel & Tritz, 1989; Haseloff & Gerlach, 1989), the secondary structure of which has been established through mutation and *in vitro* selection procedures (Hampel *et al.*, 1990; Chowrira & Burke, 1991a; Berzal-Herranz *et al.*, 1992, 1993; Joseph *et al.*, 1993; Anderson *et al.*, 1994). There are four regions of Watson−Crick duplex and two internal loops (A and B). Most of the essential nucleotides lie within these loop regions (Figure 1). G_{+1} is the only completely immutable residue in the substrate strand, whereas in the catalytic strand there are three regions of essential nucleotide sequence (G_8−G_{11}, G_{21}−C_{25}, and A_{38}−C_{44}) (Berzal-Herranz *et al.*, 1993; Anderson *et al.*, 1994).

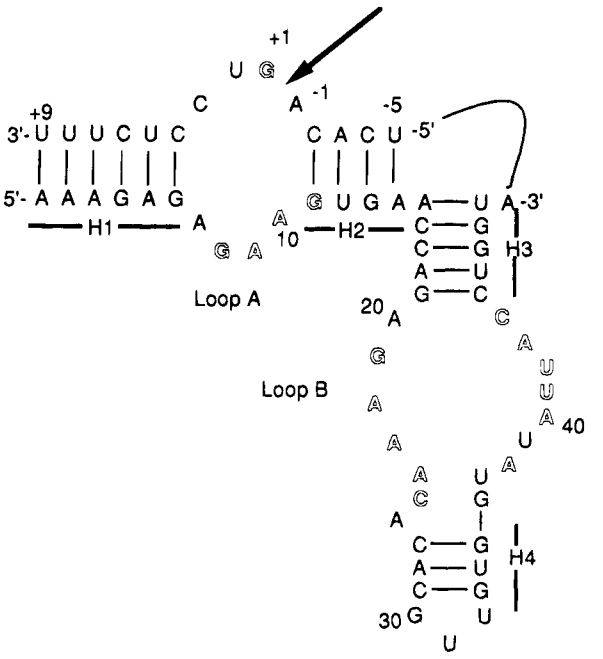

FIGURE 1: Secondary structure of the hairpin ribozyme showing the four regions of proposed Watson−Crick helix and the two internal loops A and B. The cleavage site is shown by an arrow, and the residues thought to be essential for cleavage are outlined (Berzal-Herranz *et al.*, 1993; Anderson *et al.*, 1994). Note that the evidence for Watson−Crick A_{24}·U_{37} and C_{25}·G_{36} base pairs is equivocal.

The conformations of nucleotides within the loop regions are unknown, but from photo-cross-linking studies it has been proposed that loop B may form a defined duplex structure containing a number of non-Watson−Crick base pairs and a motif identical to that of a number of other functionally

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distinct RNAs (Butcher & Burke, 1994a). In addition, loop B appears to fold independently of binding to both the magnesium ion (or ions) and the substrate strand (Butcher & Burke, 1994a). By contrast, loop A has been suggested to be conformationally flexible (Vitorino Dos Santos *et al.*, 1993). From a study of the ionic requirements of hairpin cleavage, it has been proposed that two metal ion binding sites are most consistent with the data (Chowrira *et al.*, 1993a).

Some functional group requirements of hairpin ribozyme cleavage have been determined. There are modest effects of thiophosphate substitution of phosphodiesters at positions 5′ to A_7, A_9, and A_{10} but not elsewhere (Chowrira & Burke, 1992). By contrast, there are drastic effects on catalysis of substitution of A_{10}, G_{11}, A_{24}, or C_{25} by 2′-deoxy- or 2′-*O*-methylnucleosides (Chowrira *et al.*, 1993b). In all cases the loss of activity is reflected in k_{cat} values rather than in K_M, suggesting that substrate binding is not affected by any of these substitutions. In two cases (G_{11} and C_{25}), the loss of catalytic efficiency can be restored by an increase in the Mg^{2+} ion concentration, and it has been proposed that these hydroxyl groups may be important sites for magnesium chelation (Chowrira *et al.*, 1993b).

The essential guanosine residue at +1 in the substrate strand has been substituted by 2-aminopurine and by inosine (Chowrira & Burke, 1991b). Whereas the former had no effect on catalysis, the loss of the 2-amino group of guanosine at this position resulted in undetectable ribozyme cleavage. It was therefore proposed that the 2-amino group of G_{+1} is directly involved in the catalytic cleavage mechanism. By contrast, there have been no studies of the functional group requirements of the essential residues of the catalytic strand of the hairpin ribozyme. We now report a detailed study of the essential purine residues of the hairpin ribozyme and show that modification or removal of numerous functional groups in most of the essential sequences causes drastic losses in catalytic cleavage activity.

MATERIALS AND METHODS

Preparation of Modified Phosphoramidites. 5′-*O*-(Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-O^6-[(*p*-nitrophenyl)ethyl]inosine 3′-*O*-(2-cyanoethyl)(*N,N*-diisopropylamino)phosphite, 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)purine riboside 3′-*O*-(2-cyanoethyl)(*N,N*-diisopropylamino)phosphite, and 5′-*O*-(dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-N^2-acetyl-O^6-methylguanosine 3′-*O*-(2-cyanoethyl)(*N,N*-diisopropylamino)phosphite were synthesized according to published methods (Green *et al.*, 1991; Fu & McLaughlin, 1992b; Slim & Gait, 1992; Grasby *et al.*, 1993a; Tuschl *et al.*, 1993).

2-[[(Dimethylamino)methylidene]amino]-7-[5-O-(monomethoxytrityl)-2-O-(triisopropylsilyl)-β-D-ribofuranosyl]-4-(7H)-pyrrolo[2,3-d]pyrimidinone 3-O-(2-Cyanoethyl)-(N,N-diisopropylamino)phosphite. To a flask containing dry tetrahydrofuran (10 mL) was added under argon via a syringe diisopropylethylamine (920 μL, 5.3 mmol) and (2-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphite (447 μL, 2.0 mmol). Then a solution of 2-{(dimethylamino)methylidene}-amino-7-(5-*O*-monomethoxytrityl-2-*O*-triisopropylsilyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-one (Seela *et al.*, 1993) (766 mg, 1 mmol) in dry tetrahydrofuran (10 mL) was added, and the reaction mixture was stirred overnight at room temperature. TLC (ethyl acetate/triethylamine, 9:1) indicated complete reaction. The reaction mixture was diluted with ethyl acetate (100 mL) and then washed with 5% aqueous $NaHCO_3$ solution (2 × 100 mL) followed by saturated brine (100 mL). The organic layer was dried over Na_2SO_4, filtered, evaporated, and coevaporated with diethyl ether, and the remaining oil was chromatographed on silica gel 60 (5 × 5 cm, ethyl acetate/triethylamine, 8:2). Product-containing fractions were evaporated, and the residue was dissolved in diethyl ether (10 mL), cooled in an ice bath, and precipitated with *n*-hexane (200 mL). The precipitate was dissolved in diethyl ether and evaporated to yield a white foam: yield of product (N^7-deaza-G amidite), 720 mg (75%); ^{31}P-NMR (d_6DMSO) δ 149.5, 152.4; TLC (ethyl actetate/triethylamine, 9:1) R_f 0.6.

4-[[(Dimethylamino)methylidene]amino]-7-[5-O-(monomethoxytrityl)-2-O-(triisopropylsilyl)-β-D-ribofuranosyl]-(7H)pyrrolo[2,3-d]pyrimidine 3-O-(2-Cyanoethyl)(N,N-diisopropylamino)phosphite. This was prepared as described for the N^7-deaza-G amidite using diisopropylethylamine (460 μL, 2.7 mmol), (2-cyanoethyl)(*N,N*-diisopropylamino)chlorophosphite (224 μL, 1 mmol) in tetrahydrofuran (5 mL) and 4-[[(dimethylamino)methylidene]amino]-7-[5-*O*-(monomethoxytrityl)-2-*O*-(triisopropylsilyl)-β-D-ribofuranosyl]-(7*H*)pyrrolo[2,3-*d*]pyrimidine (375 mg, 0.5 mmol) (Seela & Mersmann, 1993) in 5 mL of tetrahydrofuran. After chromatography on silica gel 60 (10 × 5 cm, ethyl acetate/dichloromethane/triethylamine, 85:15:5) and precipitation from *n*-hexane, a white foam was obtained: yield of product (N^7-deaza-A amidite), 380 mg (82%); ^{31}P-NMR (d_6-DMSO) δ 149.6, 152.5; TLC (ethyl acetate/dichloromethane/triethylamine, 85:10:5) R_f 0.45.

Preparation of Oligoribonucleotides. Oligoribonucleotides were synthesized on a 1-μmol scale using 2′-*O*-(*tert*-butyldimethylsilyl)nucleoside 3′-*O*-(2-cyanoethyl)(*N,N*-diisopropyl)phosphoroamidite monomers having phenoxyacetyl amino group protection for A and G and benzoyl protection for C (Glen Research via Cambio). The syntheses were undertaken using standard RNA synthesis procedures as previously described (Gait *et al.*, 1991) including modified phosphoramidites where appropriate. Oligoribonucleotides were deprotected by suspending the controlled-pore glass in methanolic ammonia overnight, decanting, and evaporating the resultant solution to dryness. Treatment with tetrabutylammonium fluoride to remove silyl groups and desalting by Sephadex NAP10 (Pharmacia) filtration were carried out as described previously (Gait *et al.*, 1991). Oligoribonucleotides containing O^6-methylguanosine residues were deprotected by suspension of the controlled-pore glass in 10% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dry methanol for 4 days (Grasby *et al.*, 1993). The DBU was neutralized by passing the solution over a small column of pyridinium form Dowex 50 ion-exchange resin or by the careful addition of dilute hydrochloric acid. The deprotection was then continued as described above.

The ribozyme substrate strand was purified by strong anion-exchange chromatography on a semipreparative Partisil 10-SAX column (Whatman/HiChrom) under denaturing conditions using gradients of potassium phosphate (pH 6.3) in 60% formamide (Gait *et al.*, 1991). Desalting was achieved via extensive dialysis against water. Ribozyme strands were purified by polyacrylamide gel electrophoresis (PAGE) on a 15% gel in the presence of 7 M urea and

Table 1: Kinetic Data for Cleavage of the Hairpin Ribozyme Containing Analogues in Loop A

position	substitution	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (rel)	$\Delta\Delta G^{\ddagger}_{app}$ (kJ mol^{-1})
	none	0.08(±0.02)	0.21(±0.02)	1.0	
S G_{+1}	I	nm[b]	<0.0002		
	O^6Me-G	nm	<0.0002		
	^{7}cG	0.08(±0.03)	0.0026(±0.0003)	0.012	11.4
RzA G_8	I	nm	0.0043(±0.0001)	0.023[a]	9.7
	O^6Me-G	nm	0.0028(±0.0002)	0.016[a]	10.7
	^{7}cG	0.44(±0.10)	0.015(±0.002)	0.012	11.3
RzA A_9	P	0.11(±0.07)	0.026(±0.004)	0.086	6.3
	^{7}cA	0.10(±0.02)	0.010(±0.007)	0.036	8.6
RzA A_{10}	P	0.22(±0.03)	0.013(±0.001)	0.021	10.0
	^{7}cA	0.22(±0.10)	0.027(±0.007)	0.045	8.0
RzA G_{11}	I	0.08(±0.03)	0.015(±0.002)	0.066	7.0
	O^6Me-G	0.08(±0.04)	0.0045(±0.0004)	0.021	9.9
	^{7}cG	0.21(±0.06)	0.017(±0.003)	0.030	9.0

[a] k_{cat}/K_M determined under saturating substrate conditions. [b] nm = not measureable (the cleavage rate was too slow to determine K_M).

visualized by UV shadowing; the product band was excised and eluted using gel elution buffer (0.5 M ammonium acetate, 1 mM EDTA, and 0.5% sodium dodecyl sulfate). The resultant oligonucleotides were concentrated and desalted by butanol extraction followed by gel filtration on a Sephadex NAP10 column (Pharmacia) as previously described (Gait *et al.*, 1991). The purity of oligoribonucleotides was assayed by electrophoresis on a 15% gel in the presence of 7 M urea followed by staining with toluidine blue (0.5%). The substrate oligomer was 5′-^{32}P-labeled (Slim & Gait, 1991) and subsequently purified by PAGE on a 20% gel as described above.

Characterization of Modified Oligonucleotides. To a solution of modified oligonucleotide (ca. 0.4 A_{260} unit) in 80 μL of water were added 1 μL of snake venom phosphodiesterase (Boehringer, 100 μg mL^{-1}) and 100 μl of 2× digestion buffer (20 mM $MgCl_2$, 200 mM NaCl, and 100 mM Tris·HCl, pH 7.4), and the mixture was incubated at 37 °C for 3 h. Calf alkaline phosphatase (Boehringer, 1 μL, 2 units) was added, and the mixture was incubated at 37 °C for a further 5 min. The products of enzymatic digestion were immediately analyzed by reversed-phase HPLC on a μ-Bondapak C18 column (Millipore) using buffer A (100 mM triethylammonium acetate, pH 6.3) and buffer B (100 mM triethylammonium acetate, pH 6.3, and 50% CH_3CN) as follows. (a) Purine riboside- and inosine-containing oligonucleotides: flow rate, 1.0 mL min^{-1}; 0% B, 5 min; 0−30% B, 25 min; 35−100% B, 30 min. Retention times: C, 6.0 min; U, 7.5 min; I, 11.1 min; G, 12.5 min; P, 15.2 min; A, 16.7 min. (b) 7-Deaza-G-containing oligonucleotides: flow rate, 0.8 mL min^{-1}; isocratic conditions of 10% B. Elution times: C, 5.7 min; U, 6.2 min; G, 8.2 min; ^{7}cG, 9.0 min; A, 13.6 min. (c) 7-Deaza-A-containing oligonucleotides: flow rate, 1.0 mL min^{-1}; isocratic conditions of 10% B. Retention times: C, 4.1 min; U, 4.6 min; G, 6.3 min; A, 11.2 min; ^{7}cA, 17.3 min. (d) O^6Me-G-containing oligoribonucleotides (Grasby *et al.*, 1993): flow rate, 1 mL min^{-1}; 0% B, 5 min; 0−35% B, 25 min; 35−100% B, 10 min. Retention times: C, 7.0 min; U, 9.5 min; G, 18.0 min; A, 21.9 min; O^6Me-G, 26.0 min; (absence of N^2-acetyl-O^6Me-G, 28.6 min).

Determination of Ribozyme Steady-State Parameters. A stock solution equimolar in ribozyme strands RzA and RzB (100 nM for unmodified ribozyme or for modified ribozymes with reasonable cleavage rates or 400 nM for very slowly cleaving ribozymes) was prepared as well as a stock solution of ^{32}P-labeled substrate strand (500 nM or 1 μM), each in 40 mM Tris·HCl (pH 7.5), and the two solutions were incubated separately at 90 °C for 1 min followed by cooling to 37 °C for 15 min. The ribozyme stock solution was adjusted to 10 mM $MgCl_2$ and incubated at 37 °C for 15 min. The cleavage reactions were carried out at 37 °C by adding appropriate volumes of substrate strand solution to a reaction mixture containing the ribozyme strands (final volume, 100 μL; 10 mM $MgCl_2$ and 40 mM Tris·HCl, pH 7.5), followed by brief vortexing. Final concentrations were 2−20 nM ribozyme strands and 10−300 nM substrate strand (for unmodified ribozyme or faster cleaving modified ribozymes) or 4−80 nM ribozyme strands and 20−600 nM substrate strand (slower cleaving modified ribozymes). Ten-microliter aliquots of the reaction mixture were removed at six suitable time intervals, and the reactions were quenched by addition to 20 μL of stop mix (7 M urea, 50 mM EDTA, 0.04% xylene cyanol, and 0.04% bromophenol blue). The samples were analyzed by PAGE on 20% denaturing gels, and the resultant autoradiographs were subjected to scanning densitometry (Smith & Thomas, 1990).

Kinetic parameters were determined from non-linear regression fitting to the Michaelis−Menten equation. Thus,

$$\frac{v}{[E]} = \frac{k_{cat}[S]}{K_M + [S]}$$

where v = initial rate, [S] = substrate concentration, and [E] = total enzyme concentration. The values of k_{cat} and K_M are shown in Tables 1 and 2.

Change of apparent binding energy was calculated from the equation (Fersht, 1988)

$$\Delta\Delta G^{\ddagger}_{app} = -RT \ln\left(\frac{k_{cat}/K_M(\text{modified})}{k_{cat}/K_M(\text{unmodified})}\right)$$

Determination of Initial Rates of Reaction at Saturating Substrate Conditions. In some cases where the rates of reaction were very slow, it was impossible to determine the Michaelis−Menten parameters of the modified ribozymes. The initial rates of reaction of these modified ribozymes were determined under saturating substrate conditions at a concentration of 500 nM substrate as follows. Stock solutions of 500 nM combined ribozyme strands and 2 μM substrate strand were prepared in 40 mM Tris·HCl (pH 7.5), preincubated separately at 90 °C for 1 min, and cooled to

Table 2: Kinetic Data for Cleavage of the Hairpin Ribozyme Containing Analogues in Loop B

position	substitution	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (rel)	$\Delta\Delta G^{\ddagger}_{app}$ (kJ mol^{-1})
	none	0.08(±0.02)	0.21(±0.02)	1.0	
RzA G_{21}	I	0.09(±0.03)	0.0029(±0.0006)	0.012	11.5
	O^6Me-G	0.08(±0.02)	0.0025(±0.0002)	0.012	11.5
	^{7}cG	0.46(±0.18)	0.021(±0.006)	0.017	10.5
RzA A_{22}	P	0.13(±0.03)	0.026(±0.002)	0.080	6.5
	^{7}cA	nm[b]	0.0004(±0.0001)	0.002[a]	15.8
RzA A_{23}	P	0.08(±0.02)	0.018(±0.002)	0.087	6.5
	^{7}cA	0.14(±0.03)	0.026(±0.014)	0.106	5.8
RzA A_{24}	P	nm	0.0021(±0.0002)	0.012[a]	11.5
	^{7}cA	0.09(±0.03)	0.22(±0.03)	0.90	0.3
RzB A_{38}	P	nm	0.0007(±0.0001)	0.004[a]	14.4
	^{7}cA	0.11(±0.03)	0.013(±0.001)	0.046	8.0
RzB A_{40}	P	0.03(±0.03)	0.047(±0.005)	0.66	1.1
	^{7}cA	0.17(±0.06)	0.26(±0.04)	0.55	1.5
RzB A_{43}	P	0.05(±0.02)	0.062(±0.01)	0.47	1.9
	^{7}cA	0.22(±0.05)	0.0012(±0.0002)	0.002	16.0

[a] k_{cat}/K_M determined under saturating substrate conditions. [b] nm = not measureable (the cleavage rate was too slow to determine K_M).

37 °C for 15 min. The ribozyme stock solution was adjusted to 10 mM $MgCl_2$ and incubated at 37 °C for 15 min. The cleavage reactions were carried out at 37 °C in a volume of 100 μL of 10 mM $MgCl_2$ and 40 mM Tris·HCl using ribozyme concentrations of 100 nM and initiated by addition of the ^{32}P-labeled substrate strand to give a final concentration of 500 nM, followed by brief vortexing. Reaction mixtures were covered with mineral oil to prevent evaporation during the long incubation times. Ten-microliter aliquots of the reaction mixture were removed at seven suitable time intervals, and the reactions were quenched and analyzed as described above.

Change of apparent binding energy was calculated from the equation

$$\Delta\Delta G^{\ddagger}_{app} = -RT \ln\left(\frac{v(\text{modified})/E}{v(\text{unmodified})/E}\right)$$

where v(unmodified)/E at 500 nM substrate is calculated from the steady-state parameters as 0.18 min^{-1}.

Magnesium Ion Titrations. The effect of increasing magnesium ion concentration on the kinetic parameters of the cleavage reaction was determined using a substrate concentration of 200 nM and an enzyme concentration of 10 nM (for fast-cleaving ribozymes) or 25 nM (for slow-cleaving ribozymes) at 37 °C and with magnesium chloride concentrations ranging from 5 to 150 mM. The ionic strength was kept constant by adjustment with Tris·HCl (pH 7.5) such that the minimum Tris concentration was 40 mM. Rate constants for cleavage were calculated by measuring the initial cleavage rates at varying magnesium ion concentrations (Table 3 and Figure 4). The apparent magnesium dissociation constant $K_{Mg^{2+}}$ was calculated graphically from the magnesium ion concentration at which the observed cleavage rate was half-maximal.

RESULTS

A three-stranded hairpin ribozyme, assembled from a substrate strand and two catalytic strands, was chosen for the nucleotide substitution studies (Figure 2). In this way each strand is sufficiently short to be prepared by RNA solid-phase chemical synthesis. The sequences chosen for the three strands are identical to those previously described such that helix 4 is extended to six contiguous base pairs

Table 3: Magnesium Ion Dependence of Cleavage of Hairpin Ribozymes Containing Analogues

position	substitution	increase in rate constant between 5 and 150 mM Mg^{2+} (x-fold)	$K_{Mg^{2+}}$(app) (mM)
	none	7	15
S G_{+1}	^{7}cG	9	80
RzA G_8	I	30	20
	O^6Me-G	29	20
	^{7}cG	8	30
RzA A_9	P	10	30
	^{7}cA	110	25
RzA A_{10}	P	14	25
	^{7}cA	8	40
RzA G_{11}	I	28	50
	O^6Me-G	13	30
	^{7}cG	10	25
RzA G_{21}	I	7	30
	O^6Me-G	10	30
	^{7}cG	8	15
RzA A_{22}	P	6	25
	^{7}cA	11	25
RzA A_{23}	P	25	30
	^{7}cA	19	40
RzB A_{24}	P	9	20
RzB A_{38}	P	9	25
	^{7}cA	5	25
RzB A_{43}	^{7}cA	11	80

(Chowrira *et al.*, 1993b). This ribozyme is reported to have kinetic properties similar to those of the normal two-stranded hairpin ribozyme (Chowrira & Burke, 1992), and the chemical cleavage step is rate limiting (Chowrira *et al.*, 1993b). We have determined the Michaelis–Menten kinetic constants for the three-stranded hairpin ribozyme under previously reported conditions [10 mM $MgCl_2$ and 40 mM Tris·HCl (pH 7.5) at 37 °C] (Chowrira *et al.*, 1993b) to be $K_M = 0.08(\pm 0.02)$ μM and $k_{cat} = 0.22(\pm 0.01)$ min^{-1}. The K_M is approximately 2-fold higher, and the k_{cat} nearly 8-fold lower, than those reported previously (Chowrira *et al.*, 1993b). These experiments were repeated several times and with different batches of purified synthetic RNA.

We have no explanation for the discrepancy between our results and those previously reported (Chowrira *et al.*, 1993b). However, we note that our values are close to those reported by other workers for a two-stranded hairpin ribozyme (Feldstein *et al.*, 1989; Hampel & Tritz, 1989). In addition, we have measured the $K_{Mg^{2+}}$ for our hairpin

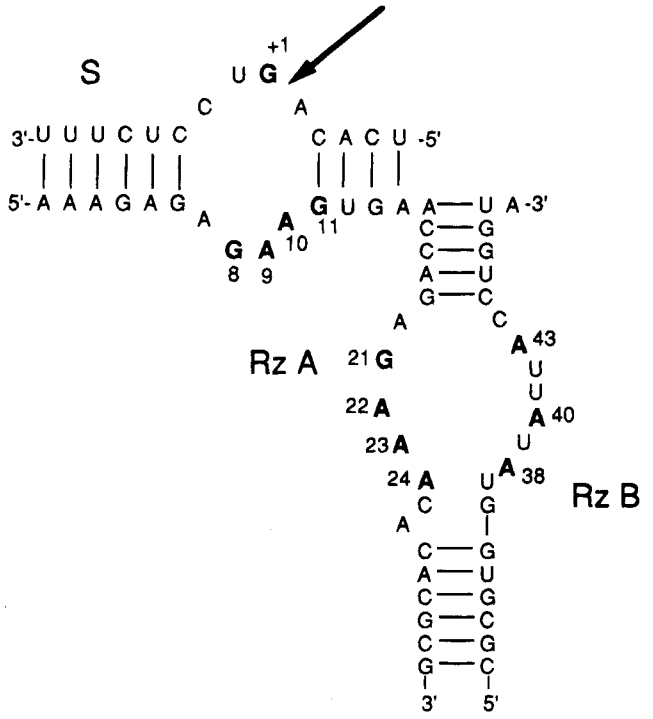

FIGURE 2: The three-stranded hairpin ribozyme (Chowrira *et al.*, 1993b) used in these functional group studies showing the substrate strand (S) and the two ribozyme strands (Rz A and Rz B). Residues studied in this work are in boldface type. The numbering system used is the same as for the natural hairpin ribozyme so that the results can be easily compared.

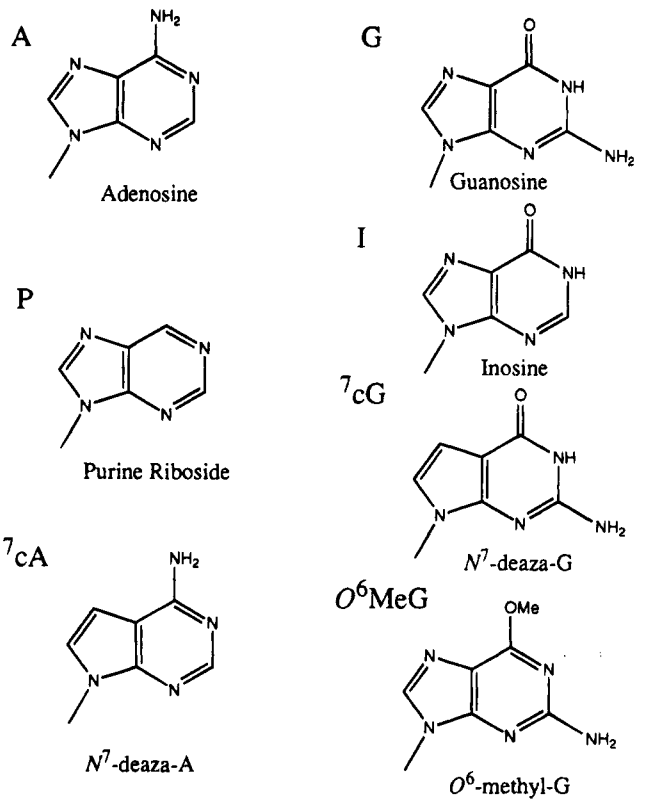

FIGURE 3: Structures of the base analogues used in this study compared to the normal A and G bases.

ribozyme and find that the value obtained (ca. 15 mM) is nearly 2-fold higher than the previously reported 8 mM value (Chowrira *et al.*, 1993b). We have been able to obtain kinetic parameters similar to the reported values by increasing the magnesium ion concentration to 50 mM (see below). However, since we are concerned in this study primarily with comparisons of modified and unmodified ribozymes, the absolute values of the kinetic constants are of less relevance as long as the conditions are identical in each case. We have therefore chosen the standard buffer conditions and a magnesium ion concentration of 10 mM.

The purine nucleoside analogues chosen for this study are those we and others have used for studies of the hammerhead ribozyme (Figure 3). Purine riboside (nebularine) and inosine are A and G analogues, respectively, where the exocyclic amino group is removed (Odai *et al.*, 1990; Fu & McLaughlin, 1992b; Slim & Gait, 1992; Tuschl *et al.*, 1993). N^7-Deaza-G (Fu *et al.*, 1993; Grasby *et al.*, 1993b) and N^7-deaza-A (Fu & McLaughlin, 1992a; Seela *et al.*, 1993) each have the N^7 replaced by a C−H moiety. In O^6-methyl-G (Grasby *et al.*, 1993a), the N^1 hydrogen atom is removed, while the maintenance of O^6 allows possible hydrogen bonding to this oxygen atom, despite some possible steric effects of the methyl group. This combination of analogues allows testing of the hydrogen-bonding requirements of functional groups in both the Watson−Crick and the Hoogsteen faces of each purine base.

Hairpin ribozymes were assembled from three strands of synthetic oligoribonucleotide. In each case two strands were unmodified and the third contained a single modified base at the appropriate position (Figure 2). Kinetic parameters for the modified and unmodified ribozymes were obtained from determination of the initial rate of reaction at concentrations of substrate around K_M (Tables 1 and 2). When modification resulted in slow cleavage, the concentration of enzyme was increased but was always at least 5 times lower than the substrate concentration. The reaction velocities, normalized to a fixed enzyme concentration, followed a conventional Michaelis−Menten curve. In a few cases of extremely slow cleavage, saturating substrate and high enzyme concentrations were required in order to obtain an initial rate of cleavage large enough to be measured with reasonable accuracy.

We first studied the essential purines in loop A (Figure 2 and Table 1). In the substrate strand, G_{+1} is the only nucleotide which cannot be replaced by any of the other three unmodified nucleotides (Berzal-Herranz *et al.*, 1993). Replacement by ^{7}cG resulted in a 80-fold reduction in k_{cat}, whereas K_M was unaffected. The kinetic parameters for the I and O^6MeG substitutions could not be determined because the cleavage rates were too slow to be measured (<0.0002 min^{-1}). Thus the rates of cleavage for these modified ribozymes are at least 3 orders of magnitude slower than for the unmodified ribozyme. In the case of the $G_{+1} \rightarrow$ I substitution, our result is consistent with that previously reported where no cleavage was seen even after prolonged incubation times (Chowrira & Burke, 1991b).

In the catalytic strand, all analogues in each of the four positions tested (G_8, A_9, A_{10}, and G_{11}) showed significant reductions in catalytic efficiency (11−100-fold). K_M was within 2.5-fold of the unmodified ribozyme except for the $G_8 \rightarrow {}^7$cG substitution, where K_M was increased 5-fold. By contrast, k_{cat} values were decreased 8−75-fold. From the reductions in cleavage rate we have calculated the apparent binding energy changes ($\Delta\Delta G^{\ddagger}_{app}$) for each modification (Table 1) (Fersht, 1988). The results show that, in all cases for which k_{cat} could be determined, values of $\Delta\Delta G^{\ddagger}_{app}$ are

between 6 and 11 kJ mol^{-1}. These values are sufficient to suggest in each case loss of a hydrogen bond to an uncharged partner, usually 2−8 kJ mol^{-1} (Fersht, 1988). The variability in the values is consistent with a comparison of the thermodynamic parameters of a 10-residue stem−loop RNA with the same RNA where an individual nucleoside in the loop was substituted by purine, inosine, or 2-aminopurine (SantaLucia *et al.*, 1992). These substitutions were designed to test the strengths of proposed non-Watson−Crick hydrogen bonds across the loop. The calculated values of $\Delta\Delta G°$ were found to vary between 1 and 3 kJ mol^{-1}, and these results indicate that the free energy changes upon deletion of a hydrogen bond in an RNA are likely to be context-dependent. Indeed SantaLucia *et al.* (1992) found that a pair of proposed hydrogen bonds was worth less in some cases than a single hydrogen bond in another context. Thus we have not attempted to correlate the values of $\Delta\Delta G^{\ddagger}_{app}$ we have obtained with a particular number of hydrogen bonds deleted.

Kinetic parameters were also measured for analogue substitutions of essential purines in loop B (Figure 2 and Table 2). The $G_{21} \rightarrow {}^{7}cG$ substitution showed a 5.5-fold increase in K_M, but in all other cases K_M was within 2.7-fold of the unmodified ribozyme. By contrast most of the values for k_{cat} showed a susbtantial reduction. Overall catalytic efficiencies (k_{cat}/K_M) were substantially reduced (11−500-fold) in all but four cases ($A_{24} \rightarrow {}^{7}cA$, $A_{40} \rightarrow P$, $A_{40} \rightarrow {}^{7}cA$, and $A_{43} \rightarrow P$), which showed 47−90% activity. Calculated values of $\Delta\Delta G^{\ddagger}_{app}$ were 0.3−1.9 kJ mol^{-1} for these four analogue substitutions, which are probably too low to indicate any change in hydrogen-bonding status of these ribozymes. By contrast, the remainder were in the range 5.8−16.0 kJ mol^{-1}, suggesting that the effect of each of these modifications is in loss of hydrogen bonding. In three cases, $A_{22} \rightarrow {}^{7}cA$, $A_{38} \rightarrow P$, and $A_{43} \rightarrow {}^{7}cA$, the values are high enough to suggest that two hydrogen bonds are lost or that there is loss of a hydrogen bond to a charged partner (12−25 kJ mol^{-1}) (Fersht, 1988). It should be noted that breakage of one hydrogen bond may also lead to weakening or loss of other hydrogen bonds which are consequentially misaligned.

Apart from hydrogen-bonding interactions, base residues in the hairpin ribozyme might also be involved in binding the essential magnesium ion or ions. A reduction in cleavage rate observed upon removal of a functional group could be due to removal of a ligand interaction in the ground state, which would be manifested by a change in $K_{Mg^{2+}}$ or in the transition state, which might be inferred if an increase in magnesium ion concentration can compensate for the reduction in k_{cat}. We therefore carried out studies to compare the magnesium dependence of cleavage for the unmodified and modified ribozymes. For the unmodified hairpin ribozyme, it can be seen (Figure 4) that cleavage rates increase dramatically between 5 and 40 mM with full cleavage activity being obtained only above 50 mM. However, we have been unable to fit the cleavage data to a simple magnesium-binding isotherm. Instead, the curve shape is consistent with there being at least two magnesium-binding sites. This is in agreement with a previous proposal that there are precisely two magnesium-binding sites (Chowrira *et al.*, 1993a). We were therefore only able to obtain a value for the apparent $K_{Mg^{2+}}$ (15 mM for the unmodified ribozyme)

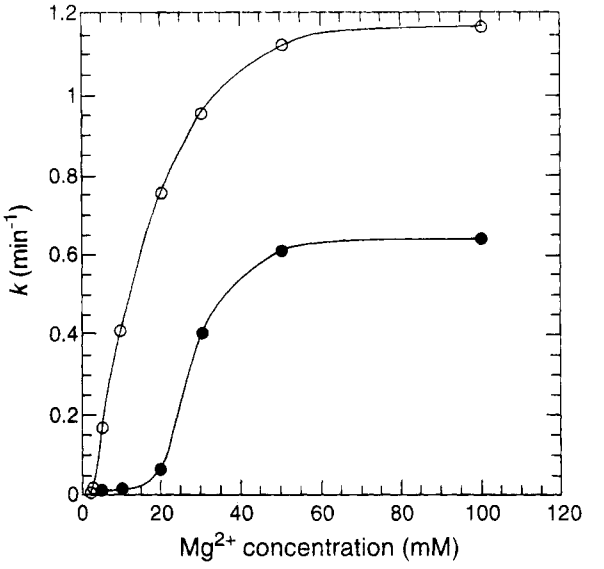

FIGURE 4: Magnesium dependence of cleavage rate constant of the unmodified wild-type hairpin ribozyme (○) and of the ribozyme containing N^7-deazaA at position A_9 (●). For full conditions of cleavage, see Materials and Methods.

by determining the magnesium concentration at which the cleavage rate is half-maximal. Only two analogue substitutions ($G_{+1} \rightarrow {}^{7}cG$ and $A_{43} \rightarrow {}^{7}cA$) were found to lead to significantly increased values (5-fold) of $K_{Mg^{2+}}$(app) (Table 3). In addition, for only one analogue substitution ($A_9 \rightarrow {}^{7}cA$) was the rate of cleavage restored to near the wild-type value when the magnesium ion concentration was raised to 150 mM (k_{cat} increased 110-fold to 0.65 min^{-1}, whereas that for the unmodified ribozyme increased only 7-fold to 1.2 min^{-1}) (Figure 4 and Table 3). Partial restorations of cleavage rate were observed for $G_8 \rightarrow I$, $G_8 \rightarrow O^6$Me-G, $G_{11} \rightarrow I$, and $A_{23} \rightarrow P$.

DISCUSSION

We wished by use of functional group alteration studies to shed light on which groups of the essential purine nucleotides are likely to be involved in cross-strand non-Watson−Crick base pairs, which groups might be involved in tertiary interactions (for example, between loops A and B), and which groups may be involved in binding to magnesium ion in either the ground state or the transition state. In contrast to findings for the hammerhead ribozyme, where in most cases removal of an exocyclic amino group or an N^7-nitrogen atom from a conserved purine residue did not have drastic effects on ribozyme cleavage (Fu & McLaughlin, 1992a,b; Slim & Gait, 1992; Fu *et al.*, 1993; Grasby *et al.*, 1993b; Seela *et al.*, 1993; Tuschl *et al.*, 1993), we have found that, for 11 out of the 12 essential purines in the hairpin ribozyme, functional group alteration considerably affected ribozyme cleavage (Tables 1 and 2). The decreases in cleavage activity we observed are reflected predominantly in changes in k_{cat}, except for N^7-deaza substitution at G_8 and G_{21}, where K_M was 5-fold increased. We therefore conclude that almost all the effects seen are due to alterations of interactions critical to the energy of the transition state of the cleavage reaction rather than to substrate−enzyme binding in the ground state.

The values of $\Delta\Delta G^{\ddagger}_{app}$ obtained in most cases (6−16 kJ mol^{-1}) are high enough to be consistent with the loss of at

least one hydrogen bond or its equivalent. We interpret such energy differences as primarily due to loss of internucleotide hydrogen-bonding interactions or to changes in metal ion binding. It should be noted, however, that in principle functional group alterations to the purine bases could also have effects on base-stacking interactions due either to an intrinsic difference in the stacking properties of the modified base or to repositioning effects as a result of concomitant changes to the sugar conformation. Some effects could also occur as a result of changes of the pK_a of the base. In the case of inosine and nebularine (purine riboside) substitution, Turner and colleagues have studied the thermodynamic parameters of duplex oligoribonucleotides containing unpaired inosine or nebularine as 3′-dangling ends and concluded that these modified bases contribute similar free energy and enthalpy increments compared to their unmodified counterparts, suggesting that stacking interactions are not substantially altered (Freir *et al.*, 1983; Turner *et al.*, 1987). Such studies have not been carried out for the N^7-deazanucleotides or for O^6MeG, but it is unlikely that stacking interaction changes alone would be sufficient to explain the magnitude of the effects we have observed. By contrast, we believe that the small values of $\Delta\Delta G^{\ddagger}_{app}$ obtained (0.3−1.9 kJ mol^{-1}) for both analogue substitutions at A_{40} and for one of the substitutions at each of A_{38} and A_{43} (Table 2) are much more likely to be a reflection of stacking interaction changes or repositioning effects in these cases.

The functional group studies of purines in loop A (Tables 1 and 3) have shown that each of the groups tested in the five essential purine residues is critical to ribozyme cleavage. For G_{11}, inosine or O^6MeG substitution would each be expected to give rise to loss of a Watson−Crick hydrogen bond between substrate and enzyme (Figures 1 and 2). It is interesting to note that the K_M of each of these mutant ribozymes was not affected at all, suggesting that even here the loss of a hydrogen bond was not sufficient to affect ground-state enzyme−substrate binding. The substantial drops in k_{cat} and the resultant values of 7−9 kJ m^{-1} in $\Delta\Delta G^{\ddagger}_{app}$ therefore reflect entirely transition-state destabilization and act as good calibration of the sorts of energy losses to be expected from hydrogen bond removal. The N^7-position of G_{11}, which is not involved in Watson−Crick base pairing, would therefore seem likely to be involved in a tertiary hydrogen-bonding interaction. It should also be noted that 2′-deoxy substitution at G_{11} has been reported to result in a drastic loss of ribozyme cleavage activity, but an increase in magnesium ion concentration restores the cleavage rate (Chowrira *et al.*, 1993b).

Ribozymes containing analogues in G_8, A_9, or A_{10} showed substantial losses of cleavage activity. Cross-strand hydrogen bonding could explain some of these results. However, since only G_{+1} is absolutely essential in the substrate strand, it is not possible to assign any particular cross-strand base pairs unequivocally. G_{+1} could be a possible base partner for either G_8, A_9, or A_{10}. However, since the rates of cleavage of ribozymes containing I or O^6MeG at G_{+1} were too low to be measured (at least 3 orders of magnitude drop in k_{cat}), the likely destabilization in energy would be too much to be accounted for by hydrogen bonding alone. Chowrira and Burke (1991b) have argued for a direct catalytic role of the exocyclic amino group of G_{+1}. Our results are consistent

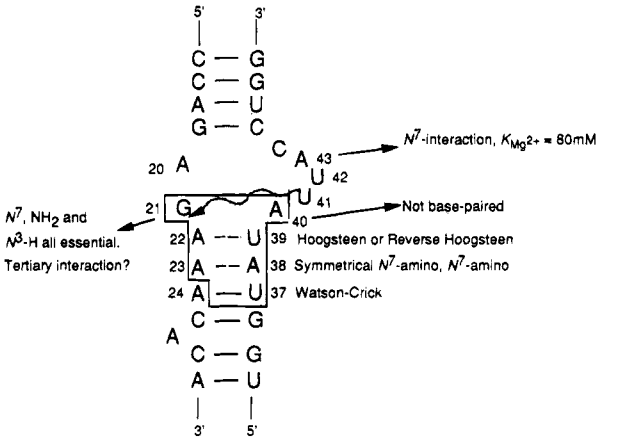

FIGURE 5: Proposed secondary structure model for the region around loop B of the hairpin ribozyme. The UV-induced cross-link is shown by a wavy arrow, and the boxed residues show the consensus region for UV-sensitive loops (Butcher & Burke, 1994a). The proposed types of secondary and tertiary interactions are also marked.

with this hypothesis. However, we have no satisfying explanation for why the substitution by O^6MeG at G_{+1} also caused a dramatic loss in activity, whereas Chowrira and Burke (1991b) found that 2-aminopurine substitution had very little effect. Both substitutions result in loss of N^1-H and removal or modification at O^6. However, we note that the N^7 of G_{+1} is one of only two essential purines for which the $K_{Mg^{2+}}$(app) is significantly increased. This nitrogen atom may therefore be involved in ground-state magnesium ion binding, and perhaps in the case of O^6MeG the extra proximal methyl group sterically prevents proper location of magnesium into its requisite binding site.

From cross-linking studies it has been proposed that loop A is very flexible (Vitorino Dos Santos *et al.*, 1993). This suggests that either there are only a few cross-strand hydrogen bonds in loop A or, because the substrate strand may vary in sequence somewhat, alternative base-pairing schemes may be allowed. Moreover, since every functional group tested on G_8, A_9, and A_{10} is critical to ribozyme cleavage, there seem to be too many functional groups on the enzyme strand to be accounted for by cross-strand hydrogen bonding alone. Our results suggest therefore that at least some of the functional groups on G_8, A_9, and A_{10} are involved in tertiary hydrogen-bonding interactions with loop B. This hypothesis is corroborated by the complementary results from modification of G_{21} in loop B (see below). However, the N^7-position on A_9 is not one of these sites of tertiary interaction, since the N^7-deaza analogue is the only one tested to have shown recovery of cleavage activity to nearly the wild-type value when the magnesium concentration was increased to 150 mM. We propose therefore that this nitrogen atom is likely to be involved in magnesium binding in the transition state of the reaction.

A number of important conclusions can be drawn from the results of loop B functional group studies (Tables 2 and 3). The data are consistent with a new model for cross-strand hydrogen bonding as shown in Figure 5. In this model, we propose that helix 4 is extended into loop B and contains two additional non-Watson−Crick base pairs (A_{22}•U_{39} and A_{23}•A_{38}). This model differs from the secondary structure proposed by Butcher and Burke (1994a) in which A_{22}•U_{41or42} and A_{23}•A_{40} pairs and an additional G_{21}•A_{43} pair

are suggested. By contrast, our results indicate that neither A_{43} nor A_{40} is cross-strand base paired.

However, there are some similarities to the Butcher and Burke model in that the consensus motif for UV-sensitive RNA loop domains is maintained (boxed residues in Figure 5). These authors found that U_{41} became specifically photo-cross-linked to either G_{21} or A_{22} upon UV irradiation and that there is significant homology of loop B with other UV-sensitive internal loops, such as is found in eukaryotic 5S rRNA and in the conserved C domain of viroids (Butcher & Burke, 1994a). In our model, U_{41} would be located opposite the G_{21} region but somewhat displaced from A_{22}. Since we propose that U_{41} is in a flexible single-stranded environment, as it approaches the opposite strand it could readily adopt a transient configuration where the pyrimidine base is located above G_{21} and within sufficient proximity to allow cross-linking. In the Butcher and Burke (1994a) model, by contrast, U_{41} approaches below G_{21} and opposite A_{22}, and their model is more strictly analogous in secondary structure to the other UV-sensitive RNA sites than is ours. A variation of our model, consistent with the possibility of U_{41} cross-linking to G_{21}, would be for A_{22} to pair with U_{41} and for U_{39} and A_{40} to be bulged below.

Evidence for our model is as follows. First, the A_{24} functional group studies show that the exocyclic amino group is essential, but the N^7-position is not. This is consistent with a Watson−Crick pairing of A_{24} with U_{37}. However, *in vitro* mutagenesis studies on the hairpin ribozyme suggest that Watson−Crick pairing is not essential here since, although the mutant $A_{24} \rightarrow U$ is catalytically inactive and there is a strong preference for A at postion 24, the complementary mutation of $U_{37} \rightarrow A$ did not restore activity (Berzal-Herranz *et al.*, 1993). Therefore, either alternative base-pairing schemes here are acceptable or there is another tertiary hydrogen-bonding role for the amino group at A_{24}. Both exocyclic amino groups and both N^7-positions are essential in A_{23} and A_{38}. Assuming cross-strand pairing does take place here, the data is most consistent with a symmetrical N^7-amino−N^7-amino pairing scheme for the A_{23}•A_{38} pair. For A_{22}, modification of either the exocyclic amino group or the N^7-position leads to an impaired ribozyme. This data is consistent with either a Hoogsteen or a reverse Hoogsteen pair with U_{39}. It is of interest to note that the E loop of eukaryotic 5S rRNA in the analogous base pair shows a reverse Hoogsteen orientation (Wimberly *et al.*, 1993). Further, it has been found that $U_{39}C$ is a general up mutation (Joseph & Burke, 1993), and this mutation is consistent with our model if an A_{22}•C_{39} pair is formed with a reverse Hoogsteen configuration.

Our data also show that neither A_{40} nor A_{43} is cross-strand base-paired, since in each case removal of the exocyclic amino group causes only a very small change in cleavage rate. This data in particular is inconsistent with the previous pairing scheme of Butcher and Burke (1994b). In the case of A_{40}, modification of the N^7-position also has no effect. However, for A_{43}, removal of the N^7 has a drastic effect. This is also the only position tested in loop B which has a $K_{Mg^{2+}}$(app) which is significantly increased over that of the wild-type ribozyme. Therefore, A_{43} may be a site of interaction with magnesium in the ground state.

G_{21} is particularly interesting in that all three modifications (inosine, N^7-deazaG and O^6MeG) led to substantially impaired ribozymes. It is possible that there is some cross-strand hydrogen bonding here, for example, a wobble pair to U_{41} or U_{42}. However, it seems likely that at least one face of G_{21} must be involved in a hydrogen-bonding interaction other than cross-strand. For example, this could be to one of the essential purine residues in the substrate strand of loop A.

Feldstein and Bruening (1993) proposed that a catalytically active geometry is achieved when loops A and B closely approach each other. This was predicted following experiments where oligonucleotide linkers were inserted between U_{-5} and U_{49} to covalently bridge these positions. The rate of cleavage was reduced substantially when fewer than seven nucleotides were inserted. In a similar study where oligo-(propyl phosphate) linkers were inserted between U_{-5} and U_{49}, it was found that the rate of cleavage increased dramatically as the number of propyl phosphates was increased from 3 to 10 and plateaued thereafter (Komatsu *et al.*, 1994). Our functional group data is consistent with these proposals in that in the catalytically active molecule the hairpin bends at the junction of helixes 2 and 3 such that loops A and B approach each other. It is likely that one or more magnesium ions are involved with this folding process. This is in line with the proposal of Chowrira *et al.* (1993a) based on the ionic requirements of hairpin ribozyme cleavage. Our results suggest that A_{43} and G_{+1} may be involved in ground-state magnesium binding and that A_9 is one of three sites involved in magnesium binding in the transition state. The other two are the hydroxyl groups at G_{11} and C_{25} (Chowrira *et al.*, 1993b). Taken together, all these results point to the formation of a specific three-dimensional structure of the active hairpin ribozyme, which is held together by interloop hydrogen bonds as well as by interactions with a magnesium ion (or ions) placed between the two loop regions.

Since the submission of the original version of this paper, a paper has been published describing a complementary approach to structure mapping of the hairpin ribozyme which involves assessment of the accessibility of particular residues to a range of chemical modification agents in the presence and absence of magnesium ion and of substrate strand (Butcher & Burke, 1994b). In general, the results confirm the high degree of secondary and tertiary structure that is present in the hairpin RNA when magnesium is added. With regard to the accessibilities of essential purines in loops A and B, many of the results are consistent with the functional group studies we have described. For example, in loop A, Butcher and Burke's proposal that G_8 hydrogen bonds to the substrate strand is consistent with our results showing drastic loss of ribozyme acitivity when either the exocyclic amino group N^7 or N^1−H of G_8 is removed. In loop B the situation is less clear cut. For example, Butcher and Burke interpret a suppression of G_{21} modification upon magnesium addition and a lack of reactivity of N^7 at A_{43} to diethylpyrocarbonate as evidence for a G_{21}•A_{43} pair in line with their previous model (Butcher & Burke, 1994a). By contrast, we have proposed that G_{21} is a key potential residue for interaction with loop A and that A_{43} N^7 may interact with magnesium in the ground state. Such potential interactions would also be expected to result in reduced chemical modification susceptibility. Further, their dimethyl sulfate accessibility studies can be interpreted as justification for an A_{23}•A_{40} pair in their model and equally well for an A_{23}•A_{38} pair in our model. It is clear, therefore, that further

experimentation will be required to adequately distinguish between the alternative base-pairing schemes for loop B and to clarify potential interactions in loop A. For example, through the use of synthetic chemistry it should be possible to introduce site-specific base alterations into the essential pyrimidine bases as well as to those bases in loops A and B not previously thought to be essential.

The synthetic methods are particularly valuable to probe potential secondary, tertiary, and magnesium interactions in the cases of RNAs where little or no structural information is yet available through NMR or crystallography, which is currently the case with the hairpin ribozyme. However, it is interesting to note that in the first X-ray crystal structure of a hammerhead ribozyme, which has just been published (Pley *et al.*, 1994), the amount of cross-strand hydrogen bonding found was considerably more than had been predicted, and some of these interactions did not correlate well with previous functional group studies. This is perhaps not so surprising when one considers that crystal structures provide information about the ground state of the ribozyme, whereas functional group studies are particularly directed to the requirements for ribozyme cleavage in the transition state. However, we note that in the hammerhead crystal structure the N^7-position of a G residue was shown to interact with a metal ion (Pley *et al.*, 1994). Thus when the crystal structure of the hairpin ribozyme is eventually solved, it will be interesting to see whether evidence is found for our predictions of magnesium binding to the N^7-positions of A_{43} and G_{+1} in the ground state.

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