The roles of the conserved pyrimidine bases in hammerhead ribozyme catalysis: evidence for a magnesium ion-binding site

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We report details of the synthesis and characterization of oligoribonucleotides containing 4-thiouridine or 2-pyrimidinone ribonucleoside ($^4\text{H}^{\text{C}}$). We have used these probes to examine the roles of the conserved pyrimidines in the central core of the hammerhead ribozyme. The effects on catalysis of singly-substituted hammerhead ribozyme and substrate strands were quantified in multiple-turnover reactions. Various effects were observed on k_{cat} and K_{m} , with up to a 7-fold decrease and a 3-fold increase respectively. For substitutions with $^4\text{H}^{\text{C}}$ at positions 3 or 17, catalytic activity in single turnover reactions can be increased up to 8-fold equivalent to 40 % of wild-type activity, by increasing the concentration of the Mg^{2+} cofactor, implying that these substitutions had a deleterious effect on Mg^{2+} binding.

Calculations of the change in the apparent free energy of binding for variants at positions 3, 4 or 17 are each consistent with deletion of a single hydrogen-bond to an uncharged group in the ribozyme. The cytidine 5' to the scissile phosphate had not previously been thought to play a direct role in catalysis, however, removal of the exocyclic amino group decreased k_{cat} 4-fold. Recently, the crystal structures of a hammerhead ribozyme bound to either a non-cleavable 2'-deoxy substrate strand or a ribo-substrate strand have been reported. The kinetic properties of the variants described here are consistent with several key interactions seen in the crystals, in particular they provide experimental support for the assignment of the proposed catalytically active magnesium ion-binding site.

INTRODUCTION

A number of viroids and satellite RNAs of certain plant viruses contain a self-cleaving domain of approximately 40 nucleotides, from which a consensus sequence has been derived which has become known as the hammerhead ribozyme [1]. It is composed of three base-paired helices that meet at a central core of 11 nucleotides, which are usually presented as being single-stranded (Figure 1). The plant pathogenic RNAs are thought to replicate by a rolling circle mechanism [2], and experimental evidence suggests that the hammerhead motif plays a crucial role in this process [3]. In these RNAs the hammerhead acts in *cis*. However, it can also act in *trans*, as first shown by Uhlenbeck [4]. Indeed, it can also be assembled from three separate fragments [5]. Owing

to its relatively small size the hammerhead is a good model system in which to explore RNA cleavage.

Hammerhead cleavage has an absolute requirement for divalent metal ions as a cofactor [4,6]. Cleavage occurs through transesterification of the scissile phosphodiester by the 2'-hydroxyl, generating a terminal 5'-hydroxyl and a 2',3'-cyclic phosphodiester. The kinetics of complex assembly and product dissociation have been studied in detail [7–9]. An extensive mutagenesis study showed that only 11 nucleotides in the consensus sequence are strictly conserved (Figure 1); nine in the single-stranded region and the base-pair closing helix III [10].

With the advent of readily accessible chemically synthesized RNA [11], many more sophisticated experiments are possible using single atom changes within a given sequence. The most simple is the incorporation of 2'-deoxynucleotides which examine the role(s) of 2'-hydroxyl groups, a series of which have been reported [12–16]. The 2'-hydroxyls have also been substituted with 2'-*O*-allyl, 2'-*O*-methyl, 2'-fluoro and 2'-amino groups [15,17–20]. Modifications to the 2'-position are of particular interest as they stabilize RNA towards nuclease activity, which is important for possible therapeutic applications [21].

The role(s) of the phosphodiester groups have been explored by the incorporation, both enzymically and chemically, of phosphorothioates at specific sites [6,22–24]. Four phosphodiester in the core region are necessary for efficient catalysis. Of particular interest is the phosphodiester at the cleavage site, since there is experimental evidence that the *pro-R_p*-oxygen is directly involved in binding to a divalent metal ion.

The roles of base functional groups have been explored by the incorporation of modified bases. Inosine (I), 2-aminopurine ribonucleoside, 7-deazaguanosine (c7G) and 6-methoxyguanosine (6OMeG) have been used to probe the role(s) of the exocyclic amine, O^6 , N^7 and N^1 respectively. At positions

Figure 1 Sequence and secondary structure of the hammerhead ribozyme studied here

The numbering scheme is according to Hertel et al. [50]. The boxed nucleotides are strictly conserved. The bases modified in this study are in **bold**.

Abbreviations used: $^4\text{S}^{\text{U}}$, 4-thiouridine; $^4\text{H}^{\text{C}}$, 2-pyrimidinone ribonucleoside; $\Delta\Delta G_{\text{app}}$, change in apparent free energy of binding; I, inosine; c7G, 7-deazaguanosine; 6OMeG, 6-methoxyguanosine; P, purine ribonucleoside; $^6\text{S}^{\text{dU}}$, 2'-deoxy-6-thiouridine; $^4\text{S}^{\text{dU}}$, 2'-deoxy-4-thiouridine; TBAF, tetrabutylammonium fluoride; RP-HPLC, reverse-phase HPLC; $K_{\text{Mg}^{2+}}$, magnesium(II) dissociation constant.

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Figure 2 Structure and numbering scheme for (a) 2-pyrimidinone ribonucleoside and (b) 4-thiouridine

guanosine-5 (G_5) and G_{12} (Figure 1) deletion of O^6 or the exocyclic amino group resulted in at least 20-fold reductions in catalytic activity [25–27]. At G_8 deletion of O^6 produced a 100-fold decrease in catalysis, whereas deletion of the exocyclic amino group produced only modest changes [25–28]. At G_5 , G_8 and G_{12} conversion of N^1 to N^1H by substitution with 6OMeG resulted in at least 50-fold reductions in catalysis [29], whereas deletion of N^7 by substitution with c7G had little effect [26]. The functional groups of adenosine have also been studied, using purine ribonucleoside (P) and 7-deazaadenosine, which probe the role(s) of the exocyclic amine and N^7 respectively. Deletion of N^7 from A_8 resulted in a 30-fold reduction in catalysis, whereas at A_9 and $A_{13-15.1}$ the N^7 has little effect on catalysis [30,31]. Deletion of the exocyclic amino group from A_8 or A_9 has little effect on catalysis [27]. Substitution of A_{13} , A_{14} or $A_{15.1}$ with P produces reductions in cleavage activity of up to 16-, 19- and 50-fold, respectively [26–28,30,31].

Although much attention has been focused on the role(s) of the core nucleotides, helix II also plays an important role in the function of the ribozyme [11,32]. It has been shown that 2 bp is the minimum length of helix II, when closed with a tetra-loop, to maintain efficient catalysis. Additionally, the base-pair closing the helix must be $G_{10.1}$ – $C_{11.1}$. Tertiary interactions within the hammerhead have been investigated by UV cross-linking experiments using 2'-deoxy-6-thioinosine (^{68}dI) and 2'-deoxy-4-thiouridine (^{48}dU) as photo-reactive probes [33,34]. ^{48}dU was singly-substituted at $U_{16.1}$, C_{17} or $U_{1.1}$, each substitution giving rise to multiple cross-links which could not be rationalized by a unique three-dimensional model. In contrast, substitution of C_{17} with ^{68}dI produced a single cross-link to U_7 . These results strongly suggest that the hammerhead may exist in several conformers in solution.

The functions of the conserved purine bases have been studied extensively. In contrast, the roles of pyrimidine bases have been explored mainly through mutagenesis studies [10]. Previously, we reported the incorporation of 4-thiouridine (^{48}U) [35] and 2-pyrimidinone ribonucleoside (^{48}C) [36] into chemically synthesized oligoribonucleotides (Figure 2). Substitution with ^{48}C can be used to probe the role of the exocyclic amino group of cytidine as well as N^3H and O^4 of uridine. Substitution with ^{48}U probes the role of O^4 in uridine. Here we report the detailed synthesis and reactivity of the phosphoramidites of ^{48}C and ^{48}U , and the kinetic characterization of seven pyrimidine base-modified ribozymes. The data are discussed in terms of the three-dimensional crystal structures of the ribozyme bound to a 2'-deoxy substrate [37] and the recently published all RNA version [51].

MATERIALS AND METHODS

Materials

The 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl nucleoside-derivatized controlled-pore glass (CPG) solid supports were purchased from Millipore. The 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl-nucleoside 3'-*O*-(2-cyanoethyl)-*NN*-diisopropylphosphoramidites were purchased from Chemgenes, Inc. HPLC was carried out on a Merck Lichrosphere RP-18 250-4 column using either a Beckman or LKB HPLC system.

Nucleoside syntheses

Brief accounts of the syntheses of 2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl-4-thiouridine 3'-phosphoramidite [35] and 2-pyrimidinone-1- β -D-(2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl)ribonucleoside 3'-phosphoramidite [36], along with relevant spectroscopic data, have already been reported. Here we report the detailed syntheses of these compounds, including improvements on the earlier protocols. Solvents were dried as follows: tetrahydrofuran was refluxed over sodium and benzophenone until purple before distillation, CH_2Cl_2 over CaH_2 , *NN*-diisopropylethylamine over CaH_2 and pyridine over KOH. Flash chromatography was performed with Sorbsil C60 (40–60 μm) silica gel (Rhône Poulenc).

2',3'-*O*-Isopropylidene-5'-*O*-tetrahydropyranloxyuridine (**1**)

A solution of 2',3'-*O*-isopropylideneuridine [38] (17.0 g; 59.8 mmol), 3,4-dihydro-2*H*-pyran (7.6 g; 8.2 ml; 90 mmol) and pyridinium *p*-toluenesulphonate (1.5 g; 6.0 mmol) in CH_2Cl_2 (150 ml) was stirred at ambient temperature for 1.5 h. The solution was washed with 5% (w/v) $NaHCO_3$ (2 $\times$ 50 ml) and saturated sodium chloride (50 ml), dried ($MgSO_4$) and the solvent removed. The residue was purified by flash chromatography, elution with diethyl ether/petroleum ether (40–60 $^\circ C$) (1–2:1) followed by diethyl ether, which afforded **1** (16.7 g; 76%).

4-*O*-Triisopropylbenzenesulphonyl-2',3'-*O*-isopropylidene-5'-*O*-tetrahydropyranloxyuridine (**2**)

A solution of **1** (16 g; 43 mmol), tri-isopropylbenzenesulphonyl chloride (20 g; 66 mmol), *NN*-dimethylaminopyridine (1.3 g; 11 mmol) and anhydrous *NN*-diisopropylethylamine (11 g; 15 ml; 87 mmol) in anhydrous CH_2Cl_2 (50 ml) was stirred at ambient temperature for 1.5 h. The solvent was removed and the residue purified by flash chromatography. Excess tri-isopropylbenzenesulphonyl chloride was eluted with petroleum ether (40–60 $^\circ C$)/ CH_2Cl_2 (1:1), while elution with diethyl ether/petroleum ether (40–60 $^\circ C$) (3:1) afforded **2** (22 g; 79%).

5'-Cyanoethyl-2',3'-*O*-isopropylidene-5'-*O*-tetrahydropyranloxy-4-thiouridine (**3**)

A solution of **2** (47.6 g; 75 mmol), $HSCH_2CH_2CN$ [39] (7.6 g; 87 mmol) and *N*-methylpyrrolidine (9.9 g; 12 ml; 120 mmol) in anhydrous CH_2Cl_2 (150 ml) was stirred at ambient temperature for 30 min. The solvent was removed and the residue purified by flash chromatography. Elution with CH_2Cl_2 was followed by 2–3% (v/v) MeOH in CH_2Cl_2 , which eluted **3** (31 g; 94%).

5'-Cyanoethyl-4-thiouridine (**4**)

A solution of **3** (1.0 g; 2.3 mmol) in CH_2Cl_2 (5 ml) was cooled to 0 $^\circ C$. After addition of trifluoroacetic acid (4.5 ml) and H_2O

(0.1 ml) the solution was removed from the ice-bath and stirred for 45 min. The solvents were removed and the residue purified by flash chromatography. Elution with CH_2Cl_2 and 2% (v/v) MeOH in CH_2Cl_2 yielded minor products, elution with 5–6% (v/v) MeOH in CH_2Cl_2 afforded **4** (0.62 g; 86%).

*S*⁴-Cyanoethyl-5'-*O*-dimethoxytrityl-4-thiouridine (**5**)

Nucleoside **4** (0.50 g; 1.6 mmol) was dried by co-evaporation with pyridine (2 × 15 ml), dissolved in anhydrous pyridine (20 ml) and cooled to 0 °C. 4,4'-Dimethoxytrityl chloride (0.61 g; 1.8 mmol) was added in eight aliquots over 8 h. After a further 1 h MeOH (5 ml) was added followed by removal of the solvents. The residue was purified by flash chromatography; elution with CH_2Cl_2 and 2% (v/v) MeOH in CH_2Cl_2 produced minor products, while elution with 3–4% (v/v) MeOH in CH_2Cl_2 afforded **5** (0.67 g; 68%).

2'-*O*-*t*-Butyldimethylsilyl-*S*⁴-cyanoethyl-5'-*O*-dimethoxytrityl-4-thiouridine (**6**)

A solution of **5** (2.0 g; 3.2 mmol), *t*-butyldimethylsilyl chloride (0.68 g; 4.5 mmol) and imidazole (0.61 g; 9.0 mmol) in anhydrous pyridine (20 ml) was stirred at ambient temperature for 20 h. After the addition of MeOH (5 ml) the solvents were removed and the residue dissolved in CH_2Cl_2 (100 ml). This solution was extracted with 5% (w/v) NaHCO_3 (2 × 30 ml) and saturated sodium chloride (30 ml), dried (MgSO_4) and the solvent removed. The residue was purified by flash chromatography, elution with diethyl ether/petroleum ether (40–60 °C) (2:1) afforded minor products, while with diethyl ether/petroleum ether (40–60 °C) (3:1) **6** (1.4 g; 60%) was eluted. Elution with ether afforded the corresponding 3'-isomer (0.6 g; 26%). This method is essentially the same as that reported by Fu et al. [26] and represents an improvement in yield, reaction time and amount of *t*-butyldimethylsilyl chloride used, over the method we reported earlier [35].

2'-*O*-*t*-Butyldimethylsilyl-*S*⁴-cyanoethyl-5'-*O*-dimethoxytrityl-4-thiouridine 3'-*O*-[(2-cyanoethyl)diisopropylamino]phosphoramidite (**7**)

A solution of **6** (0.44 g; 6.0 mmol), *N*-methylimidazole (28 μg; 25 μl; 2.1 mmol), anhydrous *NN*-di-isopropylethylamine (0.37 g; 0.5 ml; 48 mmol) and 2-cyanoethyl-*NN*-di-isopropylchlorophosphoramidite (2.0 ml; 9.2 mmol) in anhydrous CH_2Cl_2 (20 ml) was stirred at ambient temperature for 1.5 h. After the addition of MeOH (5 ml), the solution was extracted with 5% (w/v) NaHCO_3 (2 × 10 ml) and H_2O (10 ml), dried (MgSO_4) and the solvent removed. The residue was purified by flash chromatography. Elution with CH_2Cl_2 was followed by 10% (v/v) ethyl acetate in CH_2Cl_2 , which afforded the phosphoramidite, **7** (0.41 g; 74%). The compound could not be crystallized from hexane but yielded a white powder when pumped down to dryness.

2-Pyrimidinone-1-β-D-(2',3',5'-tribenzoyl)ribonucleoside (**8**)

This compound was made according to a general one-pot glycosylation procedure described by Vorbrüggen and Bennua [40]. A solution of SnCl_4 (6.3 g; 2.8 ml; 24 mmol) in acetonitrile (50 ml) was added to β-D-ribofuranose 1-acetate 2,3,5-tribenzoate (9.1 g; 18 mmol), 2-hydroxypyrimidine hydrochloride (2.7 g; 20 mmol), hexamethyldisilazane (2.4 g; 3.2 ml; 15 mmol) and trimethylsilyl chloride (1.6 g; 1.9 ml; 15 mmol) in acetonitrile (150 ml). After 3 h at ambient temperature the solvent volume was reduced to ≈ 20 ml and 5% (w/v) NaHCO_3

(60 ml) was added. After extraction with CH_2Cl_2 (3 × 50 ml) the combined extracts were washed with H_2O (2 × 50 ml), dried (MgSO_4) and the solvent removed. The residue was purified by flash chromatography, the unreacted sugar eluted with CH_2Cl_2 and **8** (8.3 g; 85%) eluted with 5% (v/v) MeOH in CH_2Cl_2 .

2-Pyrimidinone-1-β-D-ribo-nucleoside (**9**)

Nucleoside **8** (8.0 g; 15 mmol) was treated with 30% (w/v) sodium methoxide in methanol (30 ml) for 45 min. The solvent was removed and the residue was purified by flash chromatography. Elution with CH_2Cl_2 was followed by 10% (v/v) MeOH in CH_2Cl_2 , which afforded **9** (2.8 g; 82%).

2-Pyrimidinone-1-β-D-(5'-*O*-dimethoxytrityl)ribonucleoside (**10**)

4,4'-Dimethoxytrityl chloride (3.0 g; 8.7 mmol) and **9** (1.9 g; 8.3 mmol) were reacted and worked up in a similar fashion to that described for **5**. Purification by flash chromatography involved elution with CH_2Cl_2 and 2% (v/v) MeOH in CH_2Cl_2 before 5% (v/v) MeOH in CH_2Cl_2 , which afforded **10** (3.2 g; 73%).

2-Pyrimidinone-1-β-D-(2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl)ribonucleoside (**11**)

Nucleoside **10** (3.0 g; 5.7 mmol) and silver nitrate (1.5 g; 8.7 mmol) were stirred in anhydrous pyridine (10 ml). After 10 min *t*-butyldimethylsilyl chloride (1.3 g; 7.0 mmol) in anhydrous tetrahydrofuran (50 ml) was added and the mixture stirred for 20 h. NaHCO_3 (5% w/v; 10 ml) was added, the mixture was filtered and the solvents removed. CH_2Cl_2 (50 ml) was added and the solution was extracted with H_2O (20 ml), dried (MgSO_4) and the solvent removed. The residue was purified by flash chromatography. Elution with diethyl ether/petroleum ether (40–60 °C) (3:1) followed by diethyl ether afforded **11** (2.1 g; 56%). Continued elution with diethyl ether gave the 3'-isomer (≈ 10%).

2-Pyrimidinone-1-β-D-{2'-*O*-*t*-butyldimethylsilyl-5'-*O*-dimethoxytrityl)ribonucleoside 3'-*O*-[(2-cyanoethyl)diisopropylamino]phosphoramidite} (**12**)

A solution of **11** (1.45 g; 2.25 mmol), *NN*-dimethylaminopyridine (55 mg; 6.7 mmol), anhydrous *NN*-di-isopropylethylamine (1.5 g; 2.0 ml; 11 mmol) and 2-cyanoethyl-*NN*-di-isopropylchlorophosphoramidite (0.69 g; 650 μl; 2.9 mmol) in anhydrous tetrahydrofuran (20 ml) was stirred at ambient temperature for 50 min. After the addition of MeOH (5 ml), the solvents were removed. The residue was taken up in ethyl acetate (40 ml), extracted with 5% (w/v) NaHCO_3 (20 ml) and H_2O (20 ml), dried (MgSO_4) and the solvent removed. The residue was purified by flash chromatography where the required phosphoramidite, **12** (1.52 g; 80%), eluted with 20–25% (v/v) ethyl acetate in CH_2Cl_2 . The compound was crystallized from hexane to form a white powder.

Oligonucleotide synthesis

Solid-phase synthesis of the substrate (24-mers) and ribozyme (19-mers) oligoribonucleotides was carried out on an ABI 391 PCR mate DNA synthesizer using the standard 1 μmol DNA assembly cycle, with the phosphoramidite coupling time extended to 15 min [41], and an additional 1 s acetonitrile delivery at 7.5 min. For incorporation of ⁴⁸S the coupling time was extended a further 7.5 min. The base-protecting groups were benzoyl for

adenine and cytosine, and isobutryl for guanine. All other synthesizer reagents were the same as used in standard DNA synthesis. ^{45}C coupled with a stepwise efficiency similar to that of the standard phosphoramidites ($> 97\%$). ^{45}U coupled with lower efficiency, ranging from 65–95%.

Before incubation with methanolic ammonia, oligoribonucleotides containing ^{45}U were first treated with 0.3 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile for 1 h [42]. Deprotection and purification of the oligoribonucleotides were performed as previously described [43]. The final yield of unmodified oligoribonucleotides was 1.3 and 1.8 mg for the 24- and 19-mer respectively. The modified 24- and 19-mer yields ranged from 0.8 to 1.1 mg, with the exception of the ^{45}U -containing 24-mer (0.4 mg).

Oligonucleotide characterization

Base composition analysis was performed using minor modifications to the procedure described by Eadie et al. [44]. Shrimp alkaline phosphatase was used in place of calf alkaline phosphatase and $\approx 0.1 A_{260}$ of RNA was incubated at 37 °C for 4 h in place of overnight incubation. The HPLC buffers used were 50 mM potassium phosphate (pH 7) (A) and 50% (v/v) methanol/buffer A (B). The gradient employed was 10 min at 1% B, 25 min to 15% B, 30 min to 45% B and 40 min to 100% B. The nucleoside peaks were identified by comparison with authentic standards. Retention times were as follows: C, 7.5 min; U, 11 min; ^{45}C , 11 min; G, 23 min; ^{45}U , 26 min, and A, 38 min. The modified nucleosides ^{45}C and ^{45}U were detected at 310 and 332 nm respectively.

Radiolabelling

RNA was radiolabelled at the 5' terminus in a 20 μl volume containing 50 mM Tris/HCl pH 8, 10 mM MgCl_2 , 5 mM dithiothreitol, 1 μM ATP, 0.2 μM RNA, 20–50 μCi [γ - ^{32}P]ATP (3000 $\mu\text{Ci}/\text{mmol}$) and 15 units of polynucleotide kinase. Reactions were incubated for 30 min at 37 °C. The reactions were quenched with an equal volume of RNA loading buffer (25 mM EDTA/8 M urea).

Single-turnover reactions

Standard conditions. Two solutions, one containing 2 μM ribozyme and the other 0.4 μM substrate, both in 50 mM Tris/HCl/10 mM MgCl_2 , pH 7.9, were heated at 90 °C for 1 min, allowed to cool to room temperature, centrifuged and

then incubated at 37 °C for 10 min. The substrate solutions contained a trace of radiolabelled substrate at 100 000 c.p.m. per 15 μl . The reactions were initiated by combining 15 μl of both ribozyme and substrate solutions. A minimum of six 2 μl aliquots were withdrawn and quenched with 5 μl of 50 mM EDTA in 8 M urea and then subjected to denaturing PAGE. The radiolabelled bands were visualized and quantified using a FUJIX BAS1000 Phosphorimager. Semi-log plots of the proportion of substrate cleaved versus time were used to determine the half-lives of the hammerhead variants. The linear correlation coefficients were in the range $r = 0.99$ – 0.98 .

Cleavage at higher magnesium ion concentrations

The reactions were performed as above except that the MgCl_2 concentration was adjusted accordingly. Also, the volume of quench was increased so that the final concentration of EDTA was at least twice that of Mg^{2+} , e.g. 2 μl of reaction mixture containing 100 mM Mg^{2+} was quenched with 10 μl of 50 mM EDTA in 8 M urea, resulting in final Mg^{2+} and EDTA concentrations of 17 and 42 mM respectively.

Steady-state kinetics

The reactions were performed as for the single-turnover reactions, but with a final ribozyme concentration of 0.1 μM and substrate concentrations ranging from 1 to 8 μM . Initial rates were determined from a minimum of six time-points using linear plots up to the first 10% of cleavage; linear correlation coefficients were in the range $r = 0.98$ – 0.94 . Rates were determined at five or six substrate concentrations. Values of k_{cat} and K_m were determined from both Eadie–Hofstee and Lineweaver–Burk plots with correlation coefficients in the range $r = 0.98$ – 0.91 and $r = 0.98$ – 0.95 respectively, and also from the statistically superior Cornish-Bowden plots. The values obtained from all three methods were in close agreement and those derived from both the Eadie–Hofstee and Cornish-Bowden plots are listed in Table 1.

RESULTS AND DISCUSSION

Synthesis and purification of variant oligoribonucleotides

The phosphoramidites were synthesized to be compatible with the well-established scheme for RNA chemical synthesis described by Usman et al. [11]. Synthesis of the ^{45}U -containing 24-mer was straightforward, however, deprotection and purification

Table 1 Kinetic data for complexes used in this study

ncd = no cleavage detected after 30 min; nd = not determined. $\Delta\Delta G_{\text{app}} = RT \ln(k_{\text{rel}})$, where $k_{\text{rel}} = (k_{\text{cat}}/K_m)^{\text{mod}}/(k_{\text{cat}}/K_m)^{\text{unmod}}$.

Complex	Eadie–Hofstee		Cornish–Bowden		k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mol}^{-1}$)	$\Delta\Delta G_{\text{app}}$ ($\text{kJ} \cdot \text{mol}^{-1}$)
	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)		
Wild type	0.29 ± 0.03	0.8 ± 0.2	0.32	1.1	0.36 ± 0.10	—
$^{45}\text{C}_3$	0.040 ± 0.002	0.5 ± 0.1	0.041	0.5	0.08 ± 0.02	4.0 ± 0.9
$^{45}\text{C}_4$	0.046 ± 0.003	2.5 ± 0.3	0.052	2.4	0.019 ± 0.003	7.6 ± 0.8
$^{45}\text{U}_4$	0.096 ± 0.004	0.7 ± 0.1	0.098	0.8	0.14 ± 0.02	2.4 ± 0.9
$^{45}\text{C}_7$	0.20 ± 0.03	0.7 ± 0.2	0.18	0.9	0.27 ± 0.07	1.0 ± 1.0
$^{45}\text{C}_{16,1}$	nd	nd	nd	nd	nd	nd
$^{45}\text{U}_{16,1}$	0.19 ± 0.01	1.0 ± 0.2	0.20	1.1	0.19 ± 0.04	1.5 ± 0.9
$^{45}\text{C}_{17}$	0.083 ± 0.006	2.3 ± 0.3	0.081	2.3	0.037 ± 0.006	5.8 ± 0.8

Figure 3 HPLC chromatograms of crude substrate and crude ribozyme containing ^{45}U and HPLC base composition analysis

(a) HPLC chromatogram of the crude substrate strand 5'-GGCCGAAACACCGUGXCUCGAGC-3', where X = ^{45}U . For peaks 1, 2 and 3, X = C, U and ^{45}U respectively. Peak 3 is the desired strand. Detection was at 280 nm. (b) HPLC base composition analysis of peak 3 from (a), the upper and lower traces were detected at 260 and 332 nm respectively. (c) HPLC chromatogram of the crude ribozyme strand 5'-GGCUCGAC ^{45}U GAUGAGGCGC-3'. Detection was at 280 nm.

presented some difficulty. First, insolubility in triethylamine trihydrogen fluoride ($\text{Et}_3\text{N}_3\text{HF}$) meant 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran was used for 2'-O-silyl deprotection. Secondly, the HPLC chromatogram showed three main peaks (Figure 3a), all of which migrated as 24-mers in

Figure 4 HPLC chromatograms of crude ribozyme containing ^{44}C and HPLC base composition analysis

(a) HPLC chromatograms of crude ribozyme strand 5'-GGCUCGACUGA ^{44}C GAGGCGC-3'. The upper and lower traces were after 36 and 12 h in methanolic ammonia at 30 °C respectively. Detection was at 260 nm. (b) HPLC base composition analysis of purified 5'-GGCCGAAACACCGUGU ^{44}C UCGAGC-3'; the upper and lower traces were detected at 260 and 310 nm respectively.

denaturing PAGE. The desired ^{45}U -containing oligoribonucleotide was identified as the late eluting peak (peak 3) by its distinct UV profile, with maxima at 260 and 335 nm, and by base composition analysis (Figure 3b). The longer retention time of this oligoribonucleotide in reverse-phase HPLC is consistent with the presence of the more hydrophobic sulphur atom. Peak 2 was similarly identified as wild-type 24-mer from base composition analysis. Its cleavage activity was also identical with that of an authentic sample. Hydrolysis of the 4-thiocarbonyl group to generate uridine is presumably caused by the presence of water ($\approx 5\%$ in commercial TBAF in tetrahydrofuran) during 2'-silyl deprotection. Recently, we have overcome this problem by using a modification of the $\text{Et}_3\text{N}_3\text{HF}$ deprotection procedure [44a]. Peak 1 exhibited slow cleavage activity, and base composition analysis suggested that ^{45}U had been converted into cytidine. Similar substitution of the 4-thiocarbonyl group with an amino group during aqueous ammonia deprotection has been reported in the preparation of 4-thiothymidine-containing oligodeoxyribonucleotides [45]. These results contrast with the ^{45}U -containing 19-mer which showed only one major peak in HPLC analysis (Figure 3c), which was identified as the desired product.

The ^{44}C -containing oligoribonucleotides were deprotected by

treatment with methanolic ammonia for 12–14 h. Longer treatment afforded a second earlier eluting peak, the amount of which increased with increasing exposure to methanolic ammonia (Figure 4a). The two species co-migrated on denaturing PAGE. A similar result was reported during the incorporation of 5-methyl-2-pyrimidinone-2'-deoxyribonucleoside into oligodeoxyribonucleotides [46]. The presence of ^{14}C in the major peak was confirmed by base composition analysis (Figure 4b) and UV (shoulder at 310 nm) and fluorescence (emission at 367 nm when excited at 295 nm) spectrophotometry. Although the ^{14}C -containing oligoribonucleotides were only treated with methanolic ammonia for 12–14 h, base composition analysis indicated that the purified oligoribonucleotides did not contain any incompletely deprotected nucleosides.

Kinetic analyses

The hammerhead motif shown in Figure 1 has been used previously [4,26,27,30] and has been shown to obey conditions that enable the Michaelis–Menten formalism to be applied to kinetic data collected under conditions approximating substrate saturation. It has been reported that at 25 °C, when 12 or more base-pairs undergo helix-coil transitions during hammerhead cleavage, product dissociation becomes rate limiting [8]. The complex used in this study potentially contains only 11 such pairs and in addition our experiments were carried out at 37 °C. The observed half-life for the cleavage of the wild-type complex was 2.3 min^{-1} , which is consistent with values obtained by other workers using the same complex and similar conditions [4,10,26,27,30]. The single- (0.31 min^{-1}) and multiple-turnover (0.29 min^{-1}) rates for our native complex also suggest that product release was as fast, or at least of the same order, as the catalytic step [8]. The minimal modifications introduced into the hammerhead here make it extremely unlikely that product dissociation was significantly different for the variant RNAs.

Steady-state cleavage analyses were performed with a ribozyme concentration of $0.1 \mu\text{M}$ for each variant studied and the substrate concentration was varied from 1 to $8 \mu\text{M}$, to give excesses of between 10 and 80-fold. The kinetic parameters (k_{cat} and K_m) and the calculated change in the apparent free energy of binding ($\Delta\Delta G_{\text{app}}$) for the variant ribozymes studied are listed in Table 1. The native complex had values for K_m of $0.8 \mu\text{M}$ and k_{cat} of 0.29 min^{-1} , which are also consistent with values reported for the identical complex using either chemically or enzymically synthesized oligoribonucleotides [4,26,27,30]. All of the modified complexes had K_m values that varied no more than 3-fold from the wild-type complex, suggesting that the modifications do not greatly affect substrate binding. The changes in k_{cat} therefore primarily reflect changes in the observed catalytic step.

Substitution of cytidine with ^{14}C at position 3 ($^{14}\text{C}_3$) resulted in a 4-fold reduction in k_{cat} , suggesting that the exocyclic amino group is involved in an important interaction affecting the catalytic step within the hammerhead. Substitution of uridine with ^{14}C at position 4 ($^{14}\text{C}_4$) produced a 6-fold reduction in k_{cat} . Substitution with ^{45}U at the same position ($^{45}\text{U}_4$) produced much smaller effects with a 2.5-fold reduction in k_{cat} . The ^{14}C substitution is more deleterious presumably because either O^4 has been removed, preventing any hydrogen-bond interaction at this position, or N^3H has been changed from a hydrogen-bond donor to an acceptor. Substitution of U_7 with ^{14}C produced a modest reduction in multiple-turnover activity, which is consistent with the reported non-conserved nature of this position, although incorporation of cytidine has been reported to reduce cleavage activity 5-fold [10].

Figure 5 Structure and numbering scheme for (a) adenosine and (b) cytidine

Substitution of $\text{U}_{16.1}$ with ^{14}C produced the largest effect of the variants studied, with the single-turnover activity being completely inhibited. This is consistent with disruption of the conserved $\text{A}_{15.1}\text{--}\text{U}_{16.1}$ base-pair that closes helix III. Substitution with ^{45}U at this position led to a 3-fold reduction in k_{cat} , again consistent with a weakening of the base-pair hydrogen-bonding due to the reduced electronegativity of the sulphur atom. These observations are in agreement with previous studies involving modification to this base-pair [10,26,28].

It has been reported that position 17 is not strictly conserved [10], however, we observed reductions of 4-fold in k_{cat} upon ^{14}C substitution. Adenosine, uridine and guanosine have been substituted at position 17 [10] with reported activities of 70, 5 and $< 1\%$ respectively. The reduction in cleavage rates for the uridine and guanosine variants have been explained in terms of the formation of an extra base-pair at the base of helix III, between A_{14} and U_{17} , or at the base of helix I, between C_3 and G_{17} . The effect of ^{14}C incorporation at this position suggests an alternative explanation, namely that the exocyclic amino group is involved in a hydrogen-bond, which the adenosine can mimic (Figure 5). In both cases the exocyclic amino groups have a similar orientation, the main difference being a small lateral displacement. Guanosine and uridine are unable to duplicate this interaction.

Metal binding

The exocyclic amino groups of adenosine, cytidine and guanosine have not been reported to bind metal ions directly but can hydrogen-bond to ligands of the metal [47]. If the cytidine exocyclic amino groups at positions 3 and 17 of the hammerhead are involved in forming an Mg^{2+} -binding site, ^{14}C substitution should lower the affinity for the Mg^{2+} ion, resulting in lower activity. In principle, this effect could be overcome by increasing the Mg^{2+} concentration. To test this idea the single-turnover cleavage rates for the wild-type complex and variants $^{14}\text{C}_3$, $^{14}\text{C}_4$ and $^{14}\text{C}_{17}$ were determined at Mg^{2+} concentrations of up to 120 mM. The native complex showed a slight increase in cleavage rate above 10 mM, a result that has been observed previously [13,25,29]. At 90 mM Mg^{2+} , variants $^{14}\text{C}_3$, $^{14}\text{C}_4$ and $^{14}\text{C}_{17}$ also showed increased cleavage activity equivalent to 29, 2.6 and 41% of wild-type activity respectively, consistent with the hypothesis that positions 3 and 17 are at least involved in defining an Mg^{2+} -binding site. For the $^{14}\text{C}_3$ and $^{14}\text{C}_{17}$ variants the rates were then determined over a range of Mg^{2+} concentrations (Figure 6). From these plots a value of $K_{\text{Mg}^{2+}} = 9.0 \pm 1.1 \text{ mM}$ was calculated for the wild-type complex, which is consistent with values reported by other workers which range from 5.3 to 13 mM

Figure 6 Semi-log plots of single-turnover activity versus Mg^{2+} concentration

□, wild-type complex; △, $^4\text{H}\text{C}_3$ complex; and ○, $^4\text{H}\text{C}_{17}$ complex. First-order rate constants were calculated from the relationship $v = \ln 2 / t_{1/2}$, where v is the rate of product formation. The data were fitted to sigmoidal curves using regression analysis, the reduced chi-squared values were 2.7×10^{-4} , 0.5×10^{-4} and 1.4×10^{-4} respectively. $K_{\text{Mg}^{2+}}$ values were calculated from the $[\text{Mg}^{2+}]$ corresponding to $0.5 (v_{\text{max}} + v_{\text{min}})$.

Figure 7 Hammerhead motif in crystal structure from Pley et al. [37]

The boxed nucleotides are strictly conserved. The positions modified in this study are in **bold**.

[6,13,24,29]. For the $^4\text{H}\text{C}_3$ and $^4\text{H}\text{C}_{17}$ variants the values were $K_{\text{Mg}^{2+}} = 58 \pm 8$ and 80 ± 2 mM respectively. The rates decreased at concentrations above 90–100 mM Mg^{2+} , an effect that may be due to the increased concentration of $\text{Mg}(\text{OH})^+$ at high Mg^{2+} concentrations which has been suggested to inhibit the reaction [48]. This, however, does not alter the conclusions about the effect of the substitutions on metal binding.

Apparent binding energy

The effect on the apparent binding free energy ($\Delta\Delta G_{\text{app}}$) of chemical substitutions within the complex can be determined from the standard relationship $\Delta\Delta G_{\text{app}} = -RT \ln(k_{\text{eff}})$, where $k_{\text{eff}} = (k_{\text{cat}}/K_{\text{m}})^{\text{mod.}} / (k_{\text{cat}}/K_{\text{m}})^{\text{unmod.}}$ [49], where mod. and unmod. are the modified and unmodified complexes respectively. The calculated values of $\Delta\Delta G_{\text{app}}$ are listed in Table 1. Values for the changes in $\Delta\Delta G_{\text{app}}$ for substrate binding and transition-state

stabilization have been determined for protein enzymes, including those which bind RNA as a substrate. These data suggest that the deletion of a hydrogen-bond from an uncharged partner results in an energy penalty of 2–7.5 kJ mol⁻¹, whereas the deletion of a hydrogen-bond from a charged partner results in an energy penalty of 12–25 kJ mol⁻¹ [49]. The $\Delta\Delta G_{\text{app}}$ values for the variants $^4\text{H}\text{C}_3$, $^4\text{H}\text{C}_4$, $^4\text{H}\text{C}_{17}$ and $^4\text{S}\text{U}_4$ are consistent with the deletion of single hydrogen-bonds to an uncharged partner. The $\Delta\Delta G_{\text{app}}$ of the variant $^4\text{H}\text{C}_4$ showed one of the largest effects, the value of 7.6 kJ mol⁻¹ is at the upper limit for the deletion of a single hydrogen-bond to an uncharged partner. This may not only be due to the removal of O⁴ but also to the reversal of the hydrogen-bonding function at N¹.

Correlation with the crystal structure

Recently, the crystal structure of a hammerhead ribozyme complexed with a non-cleaving DNA substrate strand (Figure 7) has been determined to a resolution of 2.6 Å (1 Å = 0.1 nm) [37]. The complex is described as a wishbone with stems I and II (helices I and II in Figure 1) diverging from the conserved core at an acute angle, while stem III (helix III) points in the opposite direction. There are a large number of non-Watson–Crick or non-wobble base-pair interactions in the conserved core, which serve to stabilize the overall fold. Only one divalent metal-ion binding site (for Mn^{2+} or Cd^{2+}) was identified at the base of stem II. There was no evidence for a metal ion bound to the *pro-R* oxygen at the scissile phosphate group which is believed to play a crucial role in catalysis [6,23,34]. The structure does not reveal any obvious mechanism for the proposed in-line attack by the 2'-oxygen required for cleavage to occur. The authors proposed that in the presence of an RNA substrate strand the 2'-hydroxyl groups would favour a conformational adjustment which would allow such a reaction to take place. It is therefore important to confirm that the tertiary interactions observed in the crystal structure also form in catalytically active, fully RNA forms of the hammerhead. The data reported here for the pyrimidine variants provide striking evidence consistent with a number of these interactions.

In the crystal, N³ of C_3 is hydrogen-bonded to the exocyclic amino group C_{17} . Substitution of $^4\text{H}\text{C}$ for C_3 would be expected to reduce the strength of this interaction. This is caused by a reduction in the electron density at N³ upon substitution of the strong resonance-donating amino group with hydrogen. Similarly, $^4\text{H}\text{C}$ substitution for C_{17} eliminates the hydrogen-bonding donor group. The quantitative effects for each substitution are similar, consistent with the idea that the same interaction is affected in each case. Additionally, the $\Delta\Delta G_{\text{app}}$ values are consistent with the deletion of such a hydrogen-bond. The partial restoration of catalytic activity for the $^4\text{H}\text{C}_3$ and $^4\text{H}\text{C}_{17}$ variants at higher magnesium concentrations is also consistent with this interaction because C_{17} is adjacent to the scissile phosphate which is believed to bind a metal ion in the cleavage step. Indeed, these results would also be consistent with the exocyclic amino groups interacting with a hydrated magnesium ion. Very recently, we were made aware of the results of a crystal structure determination for an all-RNA hammerhead ribozyme–substrate complex, in which interactions between a hydrated magnesium, postulated to be the catalytically active metal, and exocyclic amino groups of C_3 and C_{17} , have been observed [51]. The new structure agrees very closely with the conformation of the ribozyme–DNA substrate complex [37], suggesting that both are good descriptions of the catalytically active form of the molecule. The all-RNA complex crystals were obtained at lower concentrations of monovalent counterion, which appears to enable

some of the magnesium-binding sites to be identified. The variant data reported here offer the first experimental support for the role of pyrimidine bases in the proposed catalytically active metal-binding site based on functional studies.

U₄ is located in the uridine turn, C₃U₄G₅A₆U₇, between stems I and II and appears to play a vital role in stabilizing tertiary interactions. The 2'-hydroxyl and N³ are hydrogen-bonded to the N⁷ of A₆ and *pro*-S_p phosphate oxygen of U₇, respectively. The ⁴H C₄ variant is one of the most deleterious, consistent with the loss of the interaction with the phosphodiester backbone due to reversal of the hydrogen-bonding properties at N³. The ⁴S U₄ variant had a more modest effect on the cleavage activity, consistent with the differences in pK_a values for ⁴S U and uridine (8.2 and 9.2 respectively), which would imply that there is approximately seven times more of the thio-enolate than the enolate form present at pH 7.9, the conditions used in the cleavage reactions. These forms would act as hydrogen-bond acceptors rather than donors, as seen in the crystal.

The activity of the ⁴H C₇ variant cannot be readily explained on the basis of the crystal structure, which shows two hydrogen-bonds to U₇, one from the amino group of A₁₄ to O² and one between N³ and O⁶ of G₈. Replacement with ⁴H C₇ would eliminate this latter interaction. However, the effect on catalysis was slight, indicating that the G₈-U₇ interaction is not vital to maintain an active conformation of the hammerhead. Indeed, both these residues are involved in forming other inter-base hydrogen-bond interactions and these may be sufficient to maintain activity.

In the deoxy-substrate crystal structure, position U_{16,1} is occupied by T, which forms a hydrogen-bond between O⁴ and the amino group of A_{15,1}. Elimination of this interaction by substitution of U_{16,1} with ⁴H C₇ completely inactivates the ribozyme, suggesting that it is vital. The ⁴S U_{16,1} variant has a milder effect on cleavage activity, consistent with the formation of a weaker hydrogen-bond with the less electronegative sulphur atom.

These results are consistent with four out of five key non-Watson-Crick pyrimidine base-pair interactions seen in the two crystal structures and provide good evidence that such interactions are present in the active RNA form of the hammerhead. These data illustrate the importance of coupling structural studies with functional analysis. Arguments about the nature of pyrimidine interactions based upon changes to the apparent free energy of binding for the variants also appear to be well founded.

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