Published online December 1, 2004

6240–6250 *Nucleic Acids Research, 2004, Vol. 32, No. 21*
doi:10.1093/nar/gkh957

The role of phosphate groups in the VS ribozyme–substrate interaction

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Received September 22, 2004; Revised October 20, 2004; Accepted November 5, 2004

ABSTRACT

The VS ribozyme *trans*-cleavage substrate interacts with the catalytic RNA via tertiary interactions. To study the role of phosphate groups in the ribozyme–substrate interaction, 18 modified substrates were synthesized, where an epimeric phosphorothioate replaces one of the phosphate diester linkages. Sites in the stem–loop substrate where phosphorothioate substitution impaired reaction cluster in two regions. The first site is the scissile phosphate diester linkage and nucleotides downstream of this and the second site is within the loop region. The addition of manganese ions caused recovery of the rate of reaction for phosphorothioate substitutions between A621 and A622 and U631 and C632, suggesting that these two phosphate groups may serve as ligands for two metal ions. In contrast, significant manganese rescue was not observed for the scissile phosphate diester linkage implying that electrophilic catalysis by metal ions is unlikely to contribute to VS ribozyme catalysis. In addition, an increase in the reaction rate of the unmodified VS ribozyme was observed when a mixture of magnesium and manganese ions acted as the cofactor. One possible explanation for this effect is that the cleavage reaction of the VS ribozyme is rate limited by a metal dependent docking of the substrate on the ribozyme.

INTRODUCTION

The *Neurospora* VS ribozyme is the largest ribozyme that catalyses the endonucleolytic transesterification of RNA molecules, to generate products terminating in a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (1). Chemical modification and mutagenesis experiments have established the secondary structure of the minimal 164 nucleotide VS ribozyme (2). The ribozyme forms a distinctive H structure consisting of six helical regions labelled I–VI, in some cases interrupted by internal loops and bulges, and contains two three-way junctions. In contrast to other ribozymes that recognize their substrates by Watson–Crick base pairing, the minimal substrate for *trans*-reactions of the VS ribozyme is a stem–loop (3), which is recognized by the ribozyme through tertiary interactions (Figure 1). Thus, the VS ribozyme offers an excellent paradigm for the study of RNA–RNA interactions.

Chemical mapping and mutagenesis experiments have identified a three base pair kissing interaction as a long-range contact between ribozyme and substrate. This kissing interaction is formed by Watson–Crick base pairing of residues in the hairpin loops of the substrate (G630, U631, C632) and stem V (C699, A698, G697) (Figure 1) (4). Based on covariation analysis, it has been proposed that docking of the substrate with the hairpin loop of stem V results in a rearrangement of the base pairing of the substrate (Figure 1) (5). In the rearranged form of the substrate, C634 is proposed to be bulged out of the duplex and new base pairs are formed between G625–C635, G624–C636 and G623–C637 (5) (Figure 1b). Molecular rearrangement of the substrate is thought to occur as a result of the formation of the kissing interaction and the presence of magnesium ions (6). Three NMR structures of the VS ribozyme substrate in isolation have been reported (7–9), including one study of a stabilized form of the proposed rearranged shifted structure (7). A combination of footprinting, nucleotide analogue interference mapping (NAIM), mutagenesis and cross-linking data, and studies of the folding of the ribozyme junctions, have allowed global models of ribozyme tertiary structure to be proposed and have led to the suggestion that the active site of the ribozyme is located in the A730 loop of stem VI (10–14). It has been suggested that A756 may play a direct role in VS ribozyme catalysis (15,16).

As the identity of few nucleobases within stem–loop I is critical for VS ribozyme activity (5), contacts between the ribozyme and substrate are likely to involve interactions with the backbone of the stem–loop RNA. The role of sugar hydroxyl groups in the VS ribozyme–substrate interaction has been studied previously by both NAIM and by replacement of individual ribonucleosides with their 2′-deoxynucleoside counterparts (17,18). The possibility of ribose zipper-type interactions between residues 634–637 of the substrate and the ribozyme, proposed on the basis of NAIM (17), appears unlikely given that these residues tolerate individual replacement with 2′-deoxynucleosides (18). A second proposal for an A minor motif ribose zipper type interaction, involving A621 and A622 in the rearranged substrate (7), is partially consistent with the individual replacement study, as

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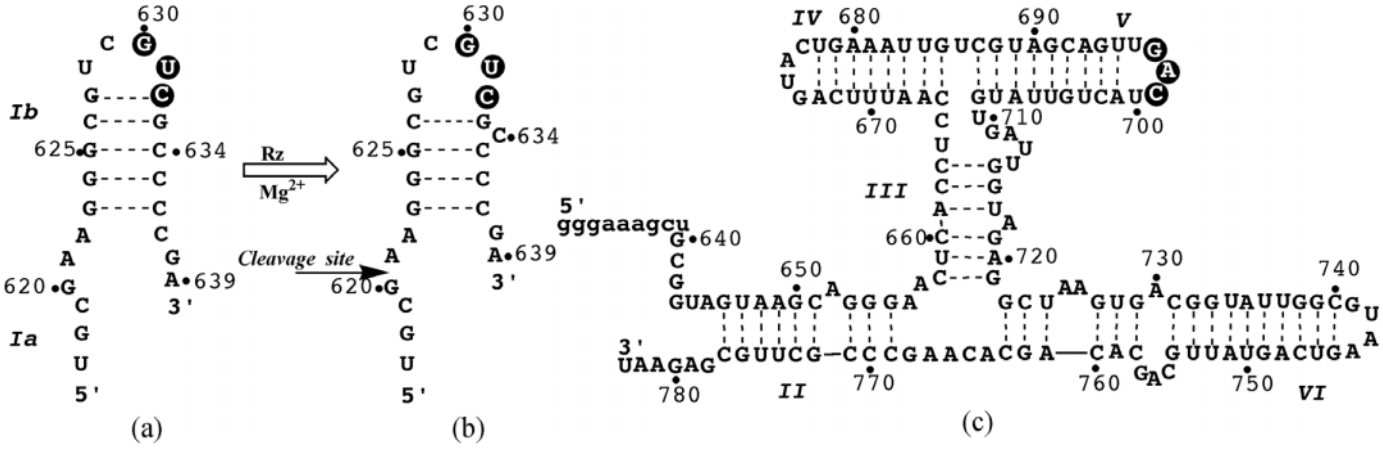

Figure 1. (**a**) The VS ribozyme intermolecular substrate. The residues involved in a kissing interaction with stem–loop V of the ribozyme are shown with black circles. (**b**) The rearranged or 'shifted' structure of the VS ribozyme substrate. (**c**) The *trans*-cleaving VS ribozyme used in this study. Lower case nucleotides represent nucleotides added to facilitate transcription. Helix Ia is formed by base pairing of residues C619, G618 and U617 of the *trans*-cleaving substrate with G640, C641 and G642 of the ribozyme.

deletion of the 2′-hydroxyl of A621, but not that of A622, is detrimental to reaction (18). NAIM has also been used to study the role of phosphate groups using an *R*p-phosphorothioate substitution, and identified two important phosphate residues within the substrate (19). Here, we report a systematic study of the role of phosphate groups within the VS ribozyme substrate, using chemically generated epimeric phosphorothioate replacements to probe the role of both oxygens of the phosphate diester linkages.

MATERIALS AND METHODS

Preparation and purification of ribozyme

Ribozyme (Figure 1) was transcribed, purified and characterized as described previously (18).

Synthesis, purification and characterization of the substrates and the substrate analogues

Ribozyme substrates were chemically synthesized on a 1 μmol scale using 2′-*O*-*tert*-butyldimethylsilyl nucleoside 3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers having a *tert*-butyl-phenoxyacetyl amino group protection for A, G and benzoyl protection for C (Proligo Biochemie Gmbh), and where appropriate 5′-fluorescein labelling (5′-fluorescein (FAM), Glen Research via Cambio). Phosphorothioate containing oligomers were synthesized using 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) as described (20). Standard deprotection procedures were used for the synthesis of all the oligonucleotides (20).

All fluorescently labelled oligonucleotides were purified by preparative reverse phase high-performance liquid chromatography (HPLC) on a μ-Bondapak C18 column (Waters), 300 × 3.9 mm with a gradient of acetonitrile in triethylammonium acetate (pH 6.5) (t = 0 min, 10% B; t = 25 min, 75% B, where Buffer A is 100 mM triethylammonium acetate with 5% acetonitrile, pH 6.5 and Buffer B is 100 mM triethylammonium acetate with 65% acetonitrile, pH 6.5, flow rate = 1.0 ml/min). The retention time of oligomers was ∼20 min. The purified oligonucleotides were desalted by dialysis. Non-fluorescein labelled substrates were purified by preparative PAGE and were 5′-^{32}P-labelled (18). The concentration of all RNAs and modified oligonucleotides were determined by measurement of their absorbance at 260 nm.

The chemical homogeneity of the purified and desalted oligonucleotides was examined by MALDI-TOF mass-spectrometry (Brüker Reflex III MALDI-TOF instrument; positive mode), denaturing HPLC (dHPLC) on a DNA Sep™ Column (Transgenomic Inc.) and denaturing PAGE (20%). Observed molecular weights were within 0–3 D of the expected mass and all oligomers gave a single peak on dHPLC and a single band on denaturing gel. Mass spectra of phosphorothioate containing oligomers all indicated the absence of any unmodified material.

Determination of Michaelis–Menten parameters

Michaelis–Menten parameters of the radioactive substrates were determined as described previously with a final buffer concentration of 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0 (18). The equivalent experiments for fluorescently labelled substrates were carried out as described for the radioactive equivalent with the following modifications. The progress of reactions were followed by removing aliquots at ten appropriate time intervals and quenching by addition of 100 mM EDTA (50 μl). The substrate was separated from the products and the relative amounts quantified on dHPLC [DNA Sep™ Column (Transgenomic Inc.)] equipped with a fluorescence detector (t = 0 min, 20% B; t = 5 min, 50% B, t = 9 min, 60% B; t = 9.6 min, 100% B; where Buffer A is 2.5 mM tetrabutylammonium bromide, 0.1% acetonitrile and 2 mM EDTA, pH 7.5 and Buffer B is 2.5 mM tetrabutylammonium bromide, 70% acetonitrile and 2 mM EDTA, pH 7.5, flow rate = 0.9 ml/min). The retention times of the substrate and the product are in the range of 5.6–6.3 min (product) and 9.6–9.8 min (substrate), depending on their length.

For unmodified substrate 2, determination of the steady state catalytic parameters was also carried out in the presence

of manganese [final concentration 20 mM $MgCl_2$, 5 mM $MnCl_2$, 25 mM KCl and 40 mM Tris–HCl (pH 8.0)].

Kinetics under pseudo first-order conditions

Stock solutions of the ribozyme (15 μM in 40 mM Tris–HCl, pH 8.0) and FAM- or ^{32}P-labelled substrate (1 μM in 40 mM Tris–HCl, pH 8.0) were prepared. These were incubated separately at 90°C for 1 min, and were then allowed to cool to 37°C over 15 min. The concentrations of $MgCl_2$ and KCl were each adjusted to 25 mM and samples were further incubated at 37°C for 30 min. The reaction was initiated with the addition of the substrate solution (final volume of the reaction mixture, 300 μl; 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0) and brief vortex mixing. The final concentration of the substrate strand was 10 nM, and 75 nM for the ribozyme strand. The progress of the reactions was followed by taking aliquots (20 μl) at fifteen appropriate time intervals and quenching with 100 mM EDTA (50 μl). Individual samples were analysed as above, depending on the labelling method.

The pseudo first order rate constant of the reaction was determined by fitting the data for the formation of product to the equation for kinetics of a first order reaction (Equation 1).

$$\%P = P_\infty \left[1 - \exp\left(-\frac{[\mathrm{Rz}]k_{\mathrm{cat}}}{K_M}t\right)\right] \quad \mathbf{1}$$

where $\%P$ is the percentage product at time t, P_∞ is the % product at the end point and [Rz] is the concentration of ribozyme.

Evaluation of the effects of phosphorothioate substitution

The rate of reaction and substrate consumption of phosphorothioate-modified substrates was determined under pseudo first order conditions as described above. In order to measure the rate of reaction of G620psA621, it was necessary to increase the ribozyme concentration from 75 to 375 nM.

Manganese rescue kinetic experiments

Manganese rescue experiments were carried out in the same manner as pseudo first order experiments with a final buffer concentration of 20 mM $MgCl_2$, 5 mM $MnCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0.

RESULTS

Synthesis, purification and characterization of oligoribonucleotides

The ribozyme and substrate used in our experiments is shown in Figures 1c and 2a, respectively. The ribozyme was prepared by *in vitro* transcription from a DNA template as described

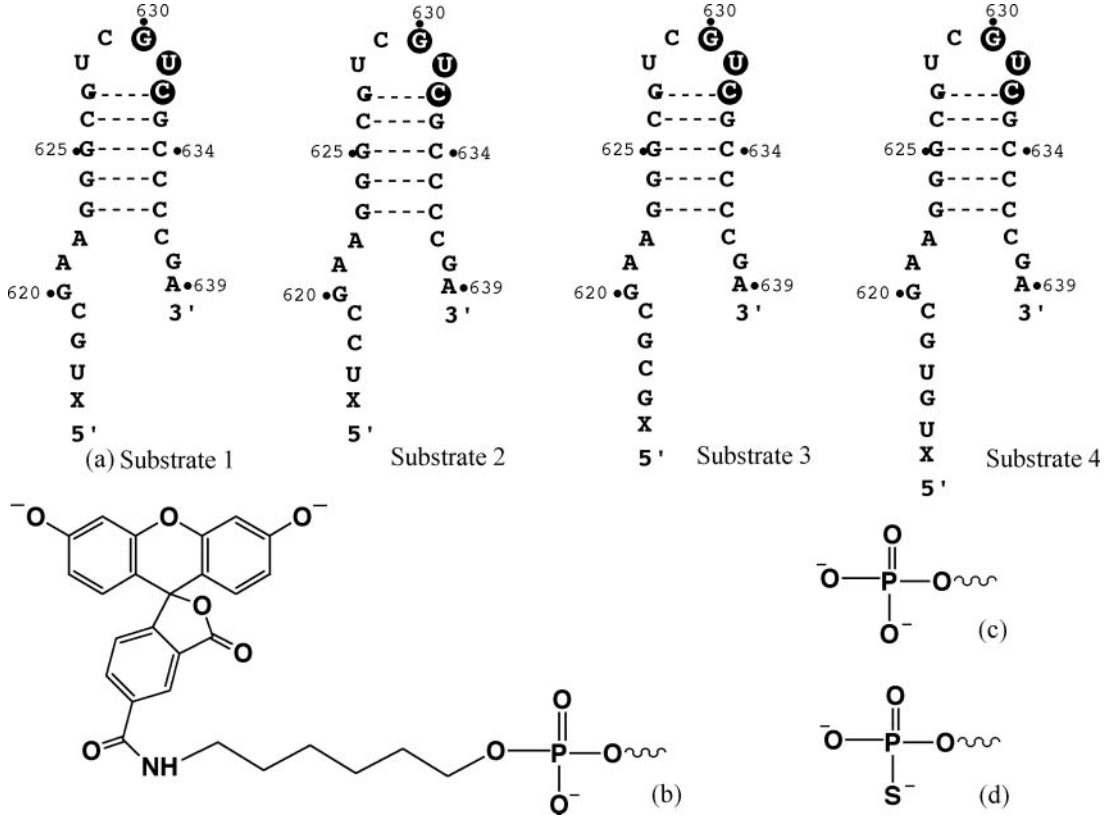

Figure 2. (**a**) The substrates used in this study with either fluorescent (X = **b**) or radiolabelled (X = **c**) 5′-termini are: substrate 1 containing the natural VS ribozymes sequence (23 nt); substrate 2 G618C (23 nt); substrate 3 U617C, G616 (24 nt); substrate 4 U617C, G616, U615 (25 nt). Substrates were either radioactively (X = c) or fluorescently labelled (X = b). The modified substrates used in this study are fluorescently labelled variants of substrate 2, where a phosphorothioate internucleoside linkage (**d**) replaces one of the internucleoside phosphate diester bonds between G620 and G638.

Table 1. The results of the steady state analysis of the catalytic parameters of various VS ribozyme substrates shown in Figure 2a

Substrate	Radioactive substrate			Fluorescent substrate		
	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
Sub 1	2.2 ± 0.10	2.3 ± 0.32	0.95	2.5 ± 0.58	3.3 ± 1.71	0.75
Sub 2	1.0 ± 0.04	0.5 ± 0.09	1.82	2.5 ± 0.2	1.1 ± 0.2	2.32
Sub 3	1.3 ± 0.16	2.3 ± 0.84	0.55	1.2 ± 0.05	1.9 ± 0.34	0.60
Sub 4	1.5 ± 0.02	1.9 ± 0.01	0.78	0.9 ± 0.02	1.3 ± 0.08	0.70
Sub 2*	ND	ND	ND	4.4 ± 0.2	0.7 ± 0.1	6.18

Experimental details are given in the Materials and Methods section. Radioactive data for substrate 2 is taken from reference (18). All data were obtained using 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0, except for sub 2*, where data is obtained with 20 mM $MgCl_2$, 5 mM $MnCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0. All data are the average of at least three independent experiments.

(18), whereas the substrates (Figure 2a) were synthesized and purified using standard phosphoramidite chemistry with *tert*-butyldimethylsilyl protection for the 2′-hydroxyl groups, employing FAM phosphoramidite to generate fluorescent oligoribonucleotide substrates (Figure 2b) as required. All oligonucleotides had molecular weights in good agreement with the calculated values. Radioactively labelled substrates were generated and purified post synthetically using conventional methodology.

In order to study the role of the phosphate groups in the VS ribozyme substrate (Figure 2a, substrate 2), 18 fluorescently labelled modified VS ribozyme substrates were prepared, in which a phosphorothioate replaced one of the phosphate groups in the unmodified substrate 2 between residues G620 and G638. Fluorescently labelled phosphorothioate modified substrates were synthesized using standard RNA synthesis protocols for the introduction of a phosphorothioate internucleoside linkage, (20) and were purified and separated from small contaminants of all phosphate containing material by RPHPLC. All attempts to resolve the two phosphorothioate diastereoisomers using a variety of HPLC columns and buffers were unsuccessful. A smaller subset of the phosphorothioate-containing oligoribonucleotides was also synthesized without a fluorescent reporter. These also proved refractory to separation of the constituent epimers. Phosphorothioate containing oligomers were characterized by MALDI-TOF mass spectrometry and all had molecular weights in good agreement with the calculated values and demonstrated the absence of unmodified material.

A fluorescence based assay for the VS ribozyme

Experiments to determine the kinetic parameters of enzyme-catalysed reactions of oligonucleotide substrates typically use radioactive assays. Alternatively, fluorescent substrates can be employed using appropriate detection methodology. However, a careful validation of fluorescence-based assays is required, as the addition of a fluorophore to an oligonucleotide substrate could potentially interfere with substrate binding or reaction.

Four fluorescent oligoribonucleotides were selected for investigation in *trans*-cleaving VS ribozyme reactions (Figure 2). Substrate 1 is the wild-type VS RNA sequence, and residues C619, G618 and U617 can potentially base pair with G640, C641 and G642 at the 5′-terminus of the ribozyme, forming helix Ia. Substrate 2 has been used previously by us to investigate the role of hydroxyl groups in the VS ribozyme substrate–ribozyme interaction (18), and contains a G618C mutation that would prevent such base pairing with the ribozyme. Substrate 3 is one nucleotide longer, contains a U617C mutation and can potentially still form a 3 base-pair helix Ia. This substrate has been used previously by Lilley and co-workers to study the intermolecular VS ribozyme reactions under single turnover conditions (11). Substrate 4 is a one-nucleotide extension of substrate 3, created to investigate whether moving the fluorophore further from the reaction site had any effect on catalytic parameters. For comparison, the corresponding ^{32}P-labelled substrates were also prepared.

The steady state catalytic parameters of the various substrates were analysed using both radioactive and fluorescence detection under multiple turnover conditions. Separation of fluorescent products and starting material by dHPLC used tetrabutylammonium bromide buffers as the ion-pairing reagent, as this allows single nucleotide resolution of fluorescent oligomers (21). These data are summarized in Table 1. For each substrate, results obtained from the fluorescent assay are comparable with the radioactive assay and are in line with previous determination of the kinetic parameters of VS ribozyme reactions under either steady state (3) or pre-steady state (11) conditions. The very small differences observed between the radioactive and fluorescent assays, in all cases <2-fold, are consistent with the results obtained comparing fluorescent and radioactive assays of the hammerhead (22) and hairpin ribozymes (23,24). The magnitude of the errors determined by radioactive and fluorescent assay are comparable. Neither the length of 5′-termini (3, 4 or 5 nucleotides) nor the ability to form base-pair interactions with the ribozyme in either assay had any major effect on the catalytic parameters, in accord with the previous observation that this portion of the substrate is not required for efficient cleavage (3).

Previous work on ribozyme reactions has demonstrated that monitoring first order reactions to near completion is informative of substrate consumption during the reaction, which in turn gives insights into the availability of correctly folded substrate or ribozyme–substrate complex (25). The failure of ribozyme reactions to reach completion, and/or the observation of biphasic kinetics has been explained by inactive conformations of substrate or ribozyme–substrate complex (25–27). As the K_M of the substrates used in this study are relatively high, and would require large amounts of ribozyme to affect single turnover conditions that produced maximal rate, we elected to study substrate consumption under pseudo first order conditions, where [S] < [Rz] < K_M. The rate of reaction under these conditions is $k_{cat}[Rz]/K_M$. The results of this analysis are given in Table 2. Typical of many ribozyme

Table 2. The results of pseudo first-order reactions of the various unmodified substrates under conditions where [S] < [E] < K_M

Substrate	Radioactive substrate		Fluorescent substrate	
	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	P_∞ (%)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	P_∞ (%)
Sub 1	1.2 ± 0.07	69 ± 1	0.6 ± 0.07	79 ± 2
Sub 2	3.3 ± 0.02	71 ± 1	3.4 ± 0.26	71 ± 2
Sub 3	1.5 ± 0.09	66 ± 1	1.5 ± 0.04	73 ± 3
Sub 4	2.2 ± 0.24	63 ± 2	1.0 ± 0.17	74 ± 4

The first-order rate constant was normalized for enzyme concentration and is therefore k_{cat}/K_M. The % product at end point (P_∞) is generated from Equation 1. Reactions contained 10 nM substrate, 75 nM ribozyme, 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0. All data are the average of at least three independent experiments.

reactions, the end point of the reaction derived from fitting a first order rate equation (% product at end point P_∞, Equation 1) is <100%, but in most cases is relatively high. McLeod and Lilley have recently studied the yield of products of radioactive substrate 3, in a reaction containing 1 nM radioactive substrate 3, 1μM ribozyme, 10 mM $MgCl_2$, 25 mM KCl, 2 mM spermidine and 50 mM Tris–HCl, pH 8.0 and report this value to be ∼80% (28). In contrast, our reactions were carried out under pseudo first order conditions with 10 nM substrate 3 (with either radioactive or fluorescent labelling), 75 nM ribozyme, 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0 and produce end points of 66% (radioactive assay) and 73% (fluorescent assay). Spermidine, a non-essential component of VS ribozyme reaction mixtures, has been omitted from our reactions, as it is possible that the polyamine fulfils some of the roles of the divalent metal ion and may therefore complicate later studies. The concentration of magnesium ions is also elevated with respect to that used by McCleod and Lilley to a value greater than the magnesium ion dissociation constant of the VS ribozyme in a *trans*-cleavage reaction (16 mM) (18). The differing buffer components may explain the small discrepancies in the product yield of the two radioactive assays. We selected substrate 2 for use in further experiments as this displayed the greatest amount of product at end point using either assay.

Properties of phosphorothioate containing substrates

The rates of reaction of the modified substrates, containing a single replacement of one of the phosphate groups between nucleosides G620 and G638 with a phosporothioate internucleoside linkage, were determined under pseudo first order conditions. The results of this analysis are shown in Figure 3. The majority of phosphate groups in the VS ribozyme substrate are tolerant of phosphorothioate modification, indeed in several cases, introduction of a phosphorothioate internucleoside linkage is mildly stimulatory to reaction. Four phosphorothioate-modified substrates produced a significant decrease in the rate of reaction. The data concerning these modified substrates are listed in Table 3. Introduction of a phosphorothioate linkage between nucleosides A621 and A622, one phosphate linkage downstream of the scissile bond, produced a 6-fold decrease in the rate constant. G630psU631 and U631psC632, with phosphorothioate modifications both located within the loop region of stem–loop I that interacts with the loop of stem V of the ribozyme, impaired reaction 3-fold. Replacement of the scissile phosphate diester bond with a phosphorothioate (G620psA621) slowed the rate of reaction 500-fold.

As HPLC separation of the *R*p and *S*p phosphorothioate epimers of the phosphorothioate-modified substrates had proven impossible, we chose pseudo first-order conditions as an initial screen for the modified substrates, as this produced information about the consumption of substrate within the reaction. As each modified substrate was a mixture of two diastereoisomers, the possibility exists that one isomer could be a substrate for the ribozyme, whereas the other could be refractory to cleavage. Thus, monitoring the consumption of substrate during the reaction, allows us to identify modified substrates where this has possibly occurred. For the majority of positions of phosphorothioate modification within the VS ribozyme substrate, the % product at end point of the reaction (40–55%) was lower than that for the unmodified substrate (60–75%). However, four modified substrates produced very much lowered % product at end point, namely A621psA622, A622psG623, U631psC632 and G620psA621 (Table 3). It is therefore possible that one of the phosphorothioate isomers, which results from modification at each of these positions is either a very poor or a non-cleavable substrate.

Are decreased yields of product the result of non-cleavable conformers or a non-cleavable or a very poor substrate phosphorothioate diastereoisomer?

In our hands, providing protocols for pre-incubation and denaturation of buffered substrate are rigidly adhered to (see Materials and Methods section), the unmodified VS ribozyme substrate (substrate 2, Figure 2a), gives reproducible yields of 60–75% product in a pseudo first-order kinetics experiment (Table 2). The most likely explanation of this phenomenon is that the stem–loop VS ribozyme substrate exists in more than one conformation, but that only one of these can undergo reaction. To test if the unmodified VS ribozyme contained inactive conformers, reaction was allowed to reach completion under pseudo first-order conditions (t = 60 min) and then the reaction mixture was denatured at 90°C for 1 min, and the reaction was then monitored for a further 30 min. Further reaction was not stimulated by this procedure (data not shown), although some non-specific cleavage of the substrate was observed, presumably due to heating in the presence of divalent metal ions. Denaturation and degradation of the ribozyme is also likely to take place under these conditions. We therefore repeated the experiment and after denaturation added a pre-activated ribozyme solution to equal the concentration of ribozyme present in the initial reaction (Figure 4). As a control the same pre-activated ribozyme was added to a non-denatured reaction mixture (data not shown), increasing the concentration of ribozyme in these experiments. The combination of denaturation and addition of pre-activated ribozyme produced further reaction of the substrate, whereas the control wherein denaturation was not carried out produced no further reaction. Assuming a simple two-component equilibrium of 63% reactive conformer (end point of the reaction shown in Figure 4 prior to denaturation and addition of ribozyme) and 37% unreactive conformer, a further 23% reaction would be expected after

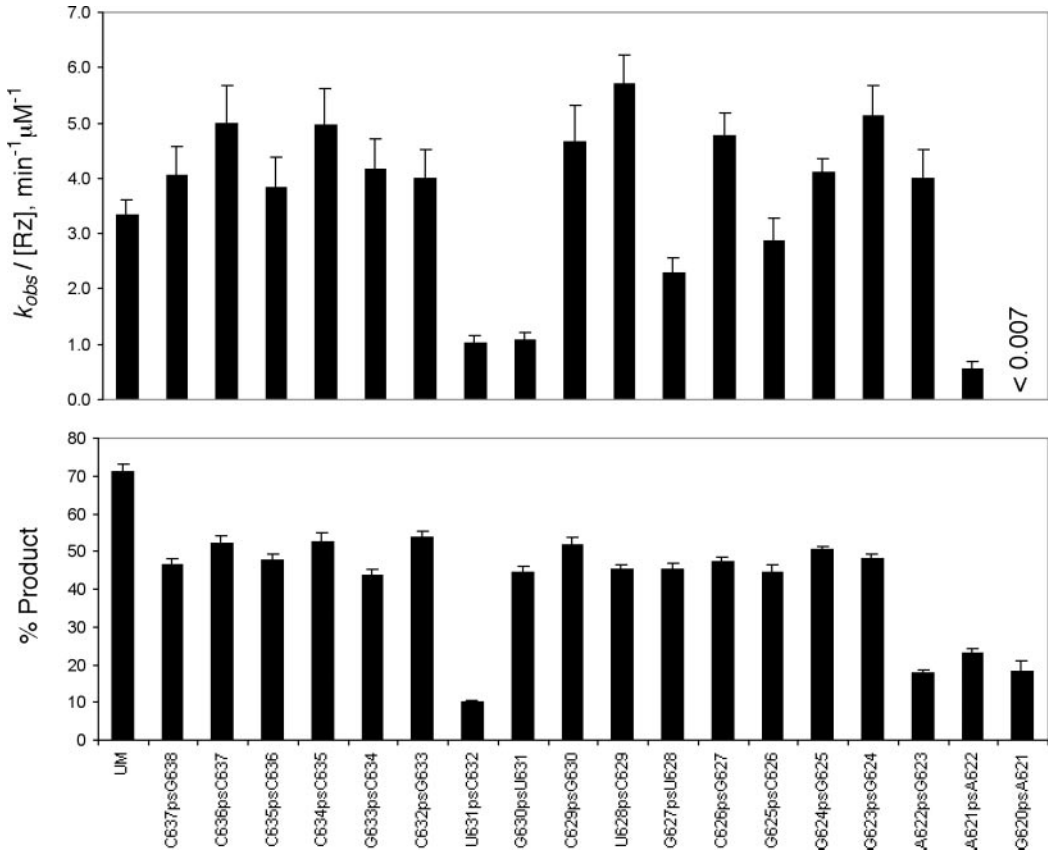

Figure 3. Evaluation of the consequences of replacement of phosphate diester with a phosphorothioate internucleoside linkage in a fluorescent VS ribozyme substrate at the position designated. UM is the unmodified substrate 2. Rates of reaction (above) and % products at end point (below) were determined under pseudo first-order conditions (10 nM substrate, 75 nM ribozyme (except for G620psA621, where 375 nM ribozyme was employed), 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0) as described in the Materials and Methods section. All data are the average of at least three independent experiments. Note that for G620psA621, the concentration of ribozyme employed to produce a measurable rate of reaction was increased from 75 nM to 375 nM, but all rate constants have been normalized with respect to ribozyme concentration to allow for this.

Table 3. The results of pseudo first-order reactions of phosphorothioate modified substrates under conditions where [S] < [E] < K_M

Cofactor	25 mM Mg^{2+}			20 mM Mg^{2+}/5 mM Mn^{2+}		
Substrate	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	P_∞ (%)	$(k_{cat}/K_M)_{rel}$	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	P_∞ (%)	$(k_{cat}/K_M)_{rel}$
Sub 2	3.4 ± 0.19	71 ± 2	1.00	7.1 ± 0.57	80 ± 2	1.00
U631psC632	1.1 ± 0.18	10 ± 1	0.32	6.4 ± 0.93	20 ± 1	0.90
G630psU631	1.0 ± 0.14	44 ± 2	0.31	3.4 ± 0.77	61 ± 2	0.48
A621psA622	0.6 ± 0.17	23 ± 2	0.17	3.9 ± 0.59	39 ± 2	0.55
G620psA621	$6.9 \pm 0.8 \times 10^{-3}$	18 ± 3	2×10^{-3}	$8.2 \pm 1.1 \times 10^{-3}$	47 ± 2	1.1×10^{-3}

The first-order rate constant was normalized for enzyme concentration and is therefore k_{cat}/K_M. The % product at end point (P_∞) is generated from Equation 1. Reactions were carried out in buffers containing either 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0 or 20 mM $MgCl_2$, 5 mM $MnCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0. The relative rate constant $(k_{cat}/K_M)_{rel}$ was generated by dividing the observed rate constant by that obtained for unmodified substrate 2 in the same buffer. All data are the average of at least three independent experiments.

re-equilibration. The amount of reaction stimulated by the denaturation procedure (a further 18%) approaches, but does not reach, this level.

It is possible that phosphorothioate modification of the VS ribozyme substrate will bring about changes in conformational equilibria, which results in higher amounts of non-cleavable conformers and therefore, a decrease in % product at end point. To test for this possibility, the phosphorothioate modified substrates A621psA622, A622psG623 and U631psC632 were subjected to a similar analysis, wherein after reactions reached plateau under pseudo first-order conditions, denaturation was carried out and a further aliquot of pre-activated ribozyme was added. In contrast to the unmodified substrate 2, very little further reaction was observed with any of these modified substrates. Moreover, for the modified substrates, the amount of further reaction observed after the denaturation procedure is very much less than would be predicted by a simple analysis based upon a two component equilibrium. This suggests that, although these modified substrates may contain amounts of non-reactive conformers,

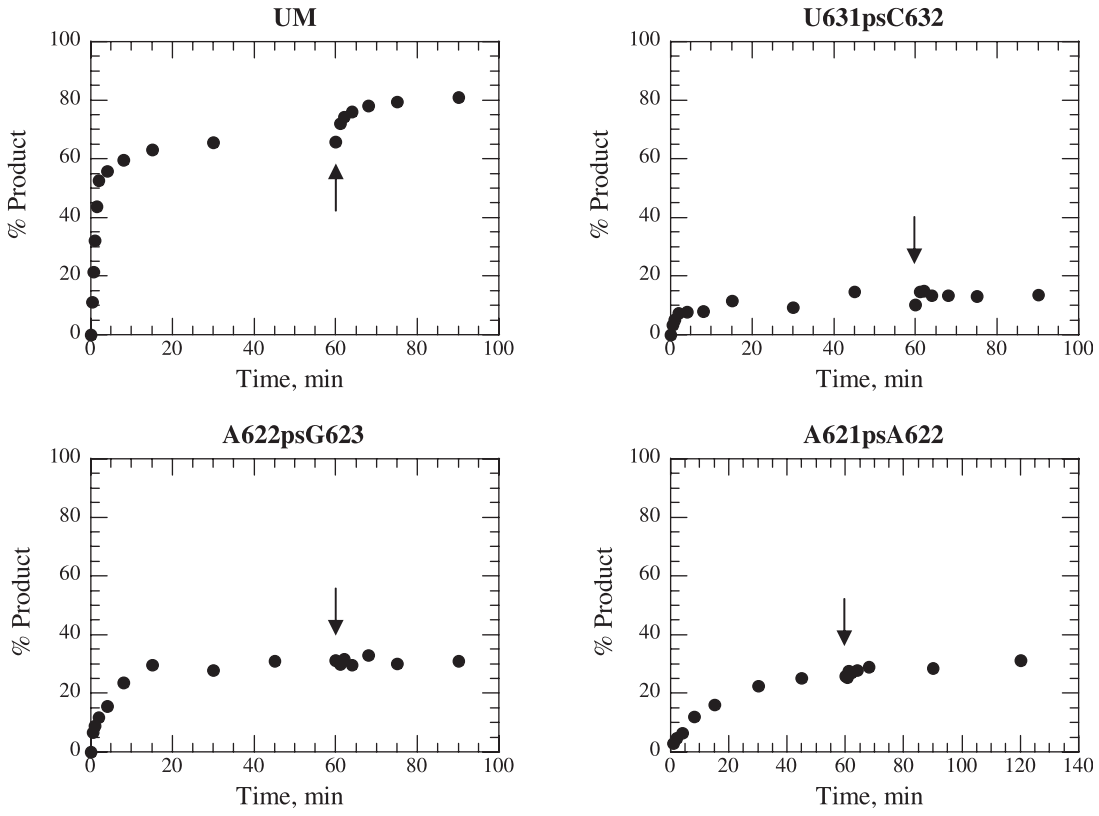

Figure 4. Reaction of unmodified substrate 2 (UM) and phosphorothioate containing substrates U631psC632, A622psG623 and A621psA622 under pseudo first-order conditions (10 nM substrate, 75 nM ribozyme, 25 mM $MgCl_2$, 25 mM KCl and 40 mM Tris–HCl, pH 8.0). The reactions have been allowed to proceed for 60 min and then heated to 90°C for 1 min and after cooling to 37°C, a further aliquot of pre-activated ribozyme was added and the reaction was then monitored again. An arrow indicates the denaturation point. In the case of UM, further reaction was stimulated by this procedure and the amount of further reaction approached that predicted if a simple two component equilibrium of active and inactive conformers is present. In the case of the phosphorothioate modified substrates, the amount of further reaction stimulated is much lower than predicted based upon simple equilibrium of conformers and suggests the presence of a non-cleavable or very poor substrate phosphorothioate diastereoisomer.

as does the unmodified substrate, the poor yield of product cannot be explained by the presence of these alone. It is therefore likely that one of the phosphorothioate diastereoisomers, which is the result of modification at A621psA622, A622psG623 and U631psC632, is either a very poor or a non-cleavable substrate. Due to a very slow rate of reaction, it was not possible to carry out this analysis with G620psA621.

McLeod and Lilley have recently reported the ligation of a *trans*-acting VS ribozyme substrate (28). This was achieved by extending the length of helix Ia adding additional (6–14) base pairs, with substrates containing 10–14 base pairs producing the greatest extent of ligation. Permutated or extended ribozymes with longer helix Ias also display ligation probably for the same reasons (29,30). The minimal VS ribozyme substrate (substrate 1, Figure 2) forms only 3 base pairs with the ribozyme in stem–loop Ia. It has been suggested that ligation is not observable in the forms of the substrate with minimal helix Ia due to rapid loss of the short 5′-product of cleavage (28). Our studies use substrate 2 (substrate 2, Figure 2) as the unmodified VS substrate, and phosphorothioate modifications have been made to this sequence. This substrate contains a G618C mutation designed to inhibit formation of helix Ia, and is therefore even less likely to undergo ligation than substrate 1. To achieve ligation, interaction of the 5′-product with the ribozyme must be stabilized. Only one of our 18 phosphorothioate mutations, G620psA621, alters the 5′-product of the reaction producing an oligoribonucleotide terminating in a 2′, 3′-cyclic phosphorothioate rather than a 2′, 3′-cyclic phosphate. It is difficult to envisage that a single atom change of this nature could stabilize an interaction with the ribozyme to such a great extent that it could produce ligation and hence a poor yield of cleavage product, but this possibility cannot be strictly ruled out.

Manganese rescue experiments

As phosphate groups can act as direct ligands for magnesium ions in RNA structures, one possible consequence of phosphorothioate modification of RNA molecules is to perturb metal ion binding. However, the softer sulphur atom of a phosphorothioate should be capable of interaction with a softer Lewis acid such as manganese ions providing a ‘manganese rescue’ of the rate of reaction (31). Manganese rescue of phosphorothioate containing ribozymes and substrates has been widely used as a method of locating metal ion binding sites in catalytic RNAs, however, careful controls and some caution is required over the interpretation of results from these experiments (32,33).

To test for the possibility of metal ion binding sites, the rates of reaction of the phosphorothioate-modified substrate where substitution had proved inhibitory to reaction were measured in the presence of manganese ions. As manganese ions alone are reported to produce much slower rates of reaction of the VS ribozyme than magnesium ions (34), we chose a buffer containing both 20 mM $MgCl_2$ and 5 mM $MnCl_2$ that was of identical ionic strength to that used in all-magnesium containing experiments. Surprisingly, the rate of reaction of unmodified substrate 2, under both steady state (Table 1) and pseudo first-order conditions (Table 3), is enhanced in the presence of manganese/magnesium containing buffer when compared with magnesium as the sole cofactor. This suggests that although manganese ions cannot substitute effectively for magnesium ions in all the metal ion binding sites of the VS ribozyme (34), manganese ions are more effective than magnesium ions in some locations. In common with the unmodified substrate, U631psC632, G630psU631, A620psA622 and G620psA621 all have enhanced reaction rates in the presence of manganese ions. Because stimulation of all the reactions by manganese ions is a complicating factor in the identification of sites of manganese rescue, relative rate constants of the modified substrates under pseudo first-order conditions with both the magnesium only and the manganese containing buffers, have been normalized to the rate of the unmodified substrate with the same buffer, and then used to identify rescue sites [$(k_{cat}/K_M)_{rel}$, Table 3]. This controls for the stimulatory effect of the manganese/magnesium ion mixture on all reactions, one of the major sources of misinterpretation of manganese rescue experiments (32). Comparing normalized rate constants, the manganese containing buffers produce a rescue in the case of U631psC632 and A621psA622. Thus, perturbation of metal ion binding is the possible cause of the inhibitory effects of phosphorothioate substitution of the phosphate linkages between U631and C632 and A621 and A622. A very moderate rescue effect is observed with G630psU631. One possible mode of metal ion catalysis in ribozyme reactions involves the metal ion binding to one of the non-bridging oxygens of the scissile phosphodiester bond, acting as an electrophilic catalyst. Manganese rescue effects would be expected to be large if this mode of catalysis was taking place. However, the rescue observed with G620psA621 is very small.

Another surprising consequence of adding manganese ions to the VS ribozyme reactions is that the amount of substrate converted to the product at end point is greater than with magnesium as the sole cofactor for all reactions. This suggests that the presence of manganese ions adjusts the conformational equilibria of the unmodified and modified substrates to favour the presence of the more reactive conformer. Notably, in two of the cases where the presence of a non-cleavable or a very poor substrate phosphorothioate isomer was suspected, namely A621psA622, U631psC632, in the presence of manganese ions the extent of reaction does not exceed 40%, again suggesting that the behaviour of these modified substrates cannot be accounted for, by conformational equilibria alone.

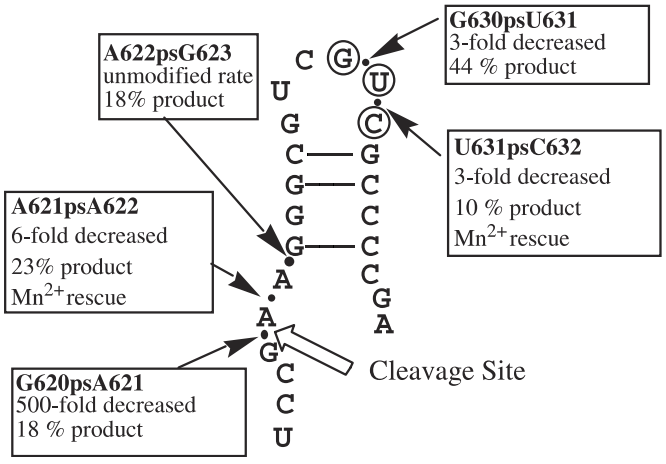

Figure 5. Summary of the sites within the VS ribozyme substrate where the substitution of epimeric phosphorothioate internucleoside diesters produces a change in the properties of the substrate. Residues involved in the proposed kissing interaction with stem–loop V are circled.

DISCUSSION

The VS ribozyme may potentially interact with the phosphate groups of its substrate via hydrogen bonding interactions or more indirectly by making metal mediated contacts. Both of these types of interaction will be perturbed by the conversion of an internucleoside phosphate diester to a phosphorothioate linkage. The larger and more diffuse sulphur atom would be expected to form weaker hydrogen bonds than oxygen, and form weaker interactions with magnesium ions. The larger size of the sulphur atom, the longer P–S bond length and the location of greater charge on the sulphur atom compared with the equal charge distribution of the phosphate diester may also contribute to perturbing interactions (35,36). For these reasons, the conversion of phosphate diesters to phosphorothioates has been extensively used to probe RNA–RNA (37), and nucleic acid–protein interactions (38) and the mechanism of phosphoryl transfer reactions (39).

In this study, five phosphate groups within the VS ribozyme substrate have been identified which, when substituted with a phosphorothioate, are inhibitory to reaction (Figure 5). These cluster around two regions within the VS ribozyme stem–loop structure. One region is located at the cleavage site and two internucleoside linkages downstream (the three phosphate linkages between G620 and G623). Another region is situated within the hairpin loop region of the substrate, proposed to be involved in docking with the loop of stem V (the two phosphate linkages between G630 and G632). Both these regions of stem–loop I are predicted to be in intimate contact with the ribozyme (4–6,12,14).

A previous study of the effects of the introduction of phosphorothioate residues within the VS ribozyme was carried out using a substitution interference protocol on a *cis*-acting ribozyme, transcribing RNAs in the presence of small amounts of a single *S*p-nucleoside-5′-α-thiotriphosphate. Such a synthesis protocol generates only *R*p phosphorothioate linkages within the RNA, and the experimental procedure could not be used to examine the effects of *R*p phosphorothioate substitution at the scissile phosphodiester bond (19). Two phosphate groups within the stem–loop I, namely those between A621–A622 and G630–U631 were identified as important using substitution interference. The chemical synthesis of phosphorothioate

containing oligoribonucleotides used in this study generates two epimeric *R*p and *S*p modified linkages. These could not be separated, and thus in our study, each modified phosphorothioate substrate is a mixture of two diastereoisomers and inhibitory effects could arise from one or both epimers. In addition, using chemical synthesis of singly substituted phosphorothioate oligomers also allowed us to study the effects of phosphorothioate substitution at the scissile phosphodiester bond. The two phosphate residues identified as important by substitution interference are also indicated as important in our individual replacement study. In our study, phosphorothioate modification of the phosphate group between A622–G623 produced a rate of reaction similar to the unmodified ribozyme, but there is evidence that only one phophorothioate isomer participates in the reaction, from the lack of consumption of starting material. As no effect was seen at this position in the interference study, this result would only be consistent with the interference study if the non-cleavable or very poor substrate isomer were of the *S*p configuration, as this was not tested in the previous experiments. Our study also demonstrates the importance of the phosphate diester linkage between U631 and C632, manifest in a slower rate constant for a cleavable isomer and the inferred presence of non-cleavable or very poor substrate isomer. This final result is more difficult to reconcile with the previous study, as our data require some degree of inhibition of reaction with either phosphorothioate isomer.

It has previously been suggested that formation of a uridine turn within the loop region of the VS ribozyme substrate may be essential to facilitate interaction of this loop with stem–loop V (4). Such a motif results in a sharp turn in an RNA structure and requires the sequence UNR where N is any nucleotide and R is a purine. Mutation of U628 to any other nucleotide causes a severe reduction in the rate of the VS ribozyme cleavage reaction, consistent with this hypothesis (4). Formation of a uridine turn would require interaction of the N3 proton of U628 with the phosphate between residues G630 and U631 and interaction of the 2′-hydroxyl of U628 with the N7 of G630. Two previous studies have highlighted the importance of the hydroxyl group of U628 (17,18), where both 2′-deoxynucleoside and 2′-*O*-methylnucleoside substitutions are detrimental to activity, suggesting a specific requirement for the hydrogen atom of the hydroxyl function (18). The current work demonstrates the importance of the phosphate between G630 and U631, which was also noted in an interference mapping experiment (19). Thus, a body of evidence now suggests that a uridine turn within loop I is required for activity.

The lowered rate of reaction of two of the phosphorothioate containing modified VS ribozyme substrates was recovered when manganese ions were added to the reaction buffer. One site of manganese rescue occurs at the phosphate group between A621 and A622. The possibility of metal ion binding directly to this phosphate has previously been suggested based upon the observation that the sequence closely resembles a metal ion binding site observed in the hammerhead ribozyme and other RNAs (5). Furthermore, in an NMR study of a stabilized rearranged substrate, addition of magnesium ions caused chemical shift changes in two areas of the rearranged substrate, one close to the cleavage site and one within the 3′-terminal region, although the precise positioning of the metal ions could not be deduced (7). The latter site is likely to be of no functional significance, as it is within a non-essential part of the substrate. However, the close correlation between the site suggested by our study and the other region observed by NMR confirms that metal binding one phosphate upstream of the scissile bond is required for ribozyme function.

The role of the non-bridging phosphate oxygens between residues A621–A622 has also been studied using a binding and ligation assay with a permutated version of the VS ribozyme known as RZ6 P (40). Phosphorothioate substitution of either stereochemistry did not affect the apparent substrate binding affinity or the fraction of substrate capable of binding. However, both the phosphorothioate substrates reached an equilibrium between cleavage and ligation at substantially lowered ligated fraction than the unmodified substrate in the presence of $MgCl_2$. Addition of manganese increased the equilibrium extent of ligation for unmodified and phosphorothioate substrates, making it impossible to assign a specific interpretation to the effect of adding manganese ions. Zamel and Collins concluded that the non-bridging phosphate oxygens at this position are important in the VS ribozyme reaction, but that introduction of a phosphorothioate does not affect the cleavage and ligation reactions equally. These findings are consistent with the results obtained in this study, where the rate limiting step of the cleavage reaction is accelerated in the presence of manganese ions and is sensitive to phosphorothioate substitution at A621–A622. However, without the complication of a competing ligation reaction, which appears to have an alternative rate limiting step to the cleavage reaction and thus a different sensitivity to phosphorothioate substitution, we are able to detect rescue upon addition of manganese ions.

Another site of manganese rescue occurs between residues U631 and C632, located within the stem–loop, and proposed to be involved in the kissing interaction with stem–loop V. Earlier, phosphorothioate interference mapping also noted a manganese rescue effect for *R*p-G630psU631 (our nomenclature) (19), and in our hands, this is modest for the epimeric mixture, when rescue effects have been normalized to the unmodified substrate with the same cofactors. A study of the role of hydroxyl groups in the VS ribozyme–substrate interaction reported raised magnesium ion dissociation constants, when the hydroxyl groups of G630 and U628 were removed (18). It is also noteworthy that a site of manganese rescue detected in a phosphorothioate NAIM study resides between A698 and C699, two of the residues of stem–loop V proposed to be involved in the kissing interaction (19). Thus, a number of studies of altered ribozymes and substrates have implicated metal ion binding to this region of the substrate or ribozyme–substrate complex. It is possible that the interaction of residues G630, U631 and C632 with stem–loop V of the ribozyme, is more complex than simple Watson–Crick base pairing and may create a metal binding site.

Possible roles for metal ions in small ribozyme reactions include activation of the nucleophilic hydroxyl group or the leaving group by direct binding, or lowering the energy of the resultant penta-coordinate transition state by direct binding to one of the non-bridging oxygens of the scissile phosphate diester bond. Another possible role is to act as a source of hydroxyl ions at near neutral pH. If any of these modes of catalysis were taking place then it would be expected that the ribozyme reaction be accelerated when a more Lewis acidic cofactor than magnesium ion, such as manganese ions, is

supplied. Thus, a simplistic explanation of the rate enhancement observed when manganese ions are included in the reaction is that metal ions play a role in catalysis. However, the possibility of divalent metal ions acting as an electrophilic catalyst appears unlikely as manganese ions fail to stimulate the reaction substantially when the scissile bond is substituted with a phosphorothioate residue. Whilst it is possible that phosphorothioate substitution at the scissile bond could exclude metal ions from the active site resulting in no rescue effect, as is the case with one protein enzyme (41), further support for a non-catalytic role for metal ions in the VS ribozyme reaction comes from the observation of proficient VS catalysis in the presence of 4M Li_2SO_4 (42).

Replacement of the cleavage site phosphate diester linkage with a phophorothioate does produce a substantial decrease in the rate of reaction, although, this is not rescued with manganese ions. As there is no inherent difference between the chemical reactivity towards transesterification of a ribonucleoside phosphate diester and a phosphorothioate diester (43), this effect must be a consequence of the ribozyme reaction. Its origins may be non-mechanistic, for example, the differing properties of the phosphorothioate with respect to phosphate may lead to incorrect positioning of the scissile bond. Alternatively, the large effect seen upon phosphorothioate substitution may have a mechanistic explanation. It is possible that the rate of phosphoryl transfer in the VS ribozyme is accelerated by protonation of one of the non-bridging oxygens or by hydrogen bonding to the scissile bond, as both of these modes of catalysis would be inhibited upon conversion to a phosphorothioate. Several experiments have highlighted the importance of the A730 loop of the VS ribozyme and, notably, protonated A756 is a good candidate for involvement in these types of interactions within the VS ribozyme active site.

One possible explanation for the rate increase observed with the unmodified VS ribozyme in the presence of manganese containing buffers may be that the rate limiting step of the VS ribozyme cleavage reaction is a metal dependent step and this step is faster in the presence of manganese ions. In this regard, rearrangement and docking of the VS ribozyme substrate on the loop of stem V, known to be dependent on the presence of metal ions (4,5), would be a good candidate for this rate limiting step. Either or both of the metal ion binding sites identified in this work, would be possibilities for the site of metal ion interaction. Furthermore, as the cleavage activity of the VS ribozyme is pH independent (34), whereas ligation is not (28), a non-chemical rate limiting step for the cleavage reaction is a reasonable assumption. It is also possible that the enhanced yield of product observed with manganese ions has its origins in the same phenomena, although, a more complete understanding of the intermediates on the kinetic pathway of the VS ribozyme will be required before this can be fully explained.

ACKNOWLEDGEMENTS

We thank Drs I. Haq and N. H. Williams for useful discussion, Ms. E. Frary for expert technical assistance and Mr. S. Thorpe for recording the mass spectra. This work was supported by BBSRC grants 50/B11652 and 50/AF/11278. J.A.G. is a BBSRC Advanced Research Fellow.

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