Efficient, pH-Dependent RNA Ligation by the VS Ribozyme in Trans[†]

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ABSTRACT: The VS ribozyme acts as a very efficient ligase in trans when the 5′ cleavage product is prevented from dissociation by an extended helix Ia in the substrate. Provided that the length of this helix is ≥10 bp, the substrate becomes ∼80% ligated by the ribozyme acting in trans. Most of the nucleotides that have been shown to be important for cleavage are similarly important for ligation, including the critical A756 of the active site. The exception to this is C755. The variant ribozyme C755A has almost normal cleavage activity, whereas the rate of ligation is reduced 70-fold. It is therefore likely that this nucleotide plays a specific role in the organization of the termini of the ligation substrates. We have found that the rate of the trans ligation reaction depends on pH, corresponding to the protonation/deprotonation of a group with a pK_A of 5.6. A model is suggested whereby the approach to equilibrium is catalyzed by the ribozyme catalyzing the ligation reaction in its deprotonated state (rate 1.05 min^{-1}) and the cleavage reaction in its protonated state (rate 0.18 min^{-1}). A756 is a candidate for the nucleobase undergoing protonation/deprotonation.

The nucleolytic ribozymes are a group of self-cleaving RNA species, mainly involved in the processing of replication intermediates of small circular RNA species. There are four distinct members of this class of ribozymes: the hammerhead (*1*, *2*), hairpin (*3*), hepatitis delta virus (HDV)[1] (*4*), and Varkud satellite (VS) (*5*) ribozymes. These are relatively small catalytic RNA species that bring about a self-catalyzed site-specific cleavage of the phosphodiester backbone by means of a transesterification reaction in which the adjacent 2′-oxygen atom attacks the 3′-phosphorus with departure of the 5′-oxygen, to generate a cyclic 2′,3′-phosphate product. The cleavage reaction is accelerated by a factor of 10^5-10^6 by the action of the ribozyme (*6*).

In principle, the transesterification reaction of the nucleolytic ribozyme is reversible, with an attack of a 5′-oxygen atom on the phosphorus of a cyclic 2′,3′-phosphate with departure of the 2′-oxygen, leading to a ligation reaction between RNA species of appropriate sequence. Thus an equilibrium between cleavage and ligation should exist, the position of which will depend on the individual system. The ligation reaction will be favored enthalpically due to strain in the cyclic phosphate, but there are clearly important entropic factors that will influence the equilibrium. Thus the reaction will be driven toward cleavage by dissociation of the products, and this is frequently the situation which prevents the observation of a ligation reaction. However, the ligation reaction can be favored if the ribozyme complex is stabilized. In the natural form of the hairpin ribozyme based on a four-way junction (*7*), the position of the equilibrium has been shown to be strongly biased toward ligation (*8*), and cross-linking of helices I and II of the hammerhead ribozyme resulted in an acceleration of the ligation reaction by a factor of 25 (*9*). In the present work we have found that another nucleolytic ribozyme, the VS ribozyme, can carry out efficient ligation reactions in trans.

The Varkud satellite RNA is an abundant transcript found in the mitochondria of some natural isolates of *Neurospora*. It was found that the RNA contains an element that induces self-cleavage of the phosphodiester backbone (*5*), involved in the processing of replication intermediates (*10*). The RNA can be reduced to a minimal ribozyme of about 150 nt. The secondary structure (*11*) includes a stem−loop (helices Ia and Ib) in which the cleavage occurs, which is located 5′ to an H-shaped ribozyme (helices II−VI) comprising six helical segments organized by two three-way junctions (Figure 1). The VS ribozyme is the largest of the nucleolytic ribozymes and the only one for which there is no crystal structure. However, the global shape of the ribozyme has been deduced by an analysis of the conformations of the two three-way junctions and the angular relationship between them (*12*, *13*). In this structure there is a coaxial alignment of helices IV, III, and VI, from which helices V and II project laterally with a dihedral angle of approximately 75°. The substrate stem−loop binds into the cleft formed between helices II and VI (*13*) and makes a loop−loop interaction with stem−loop V (*14*). The probable active site of the ribozyme lies within helix VI, in the internal loop containing A730 (hence termed the A730 loop) (*15*−*17*). It is likely that, on docking into the helix II−VI cleft, the substrate makes an intimate association with the A730 loop, thereby creating the local environment in which catalysis can proceed.

While there has been extensive study of the cleavage reaction of the VS ribozyme, there have been very few

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[1] Abbreviations: VS, Varkud satellite; HDV, hepatitis delta virus; NMR, nuclear magnetic resonance; NAIM, nucleotide analogue interference mapping; UV, ultraviolet.

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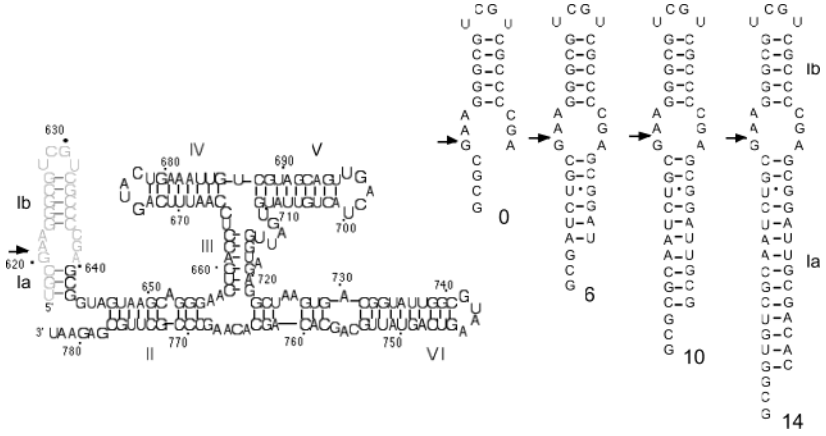


FIGURE 1: Sequence and secondary structure of the VS ribozyme (*11*) and substrates used in these studies. The sequence of the ribozyme (left) in its natural cis-acting form. In this form the stem−loop substrate (shown in gray) is covalently connected through the 5′-end of helix II, forming a 3 bp helix Ia (including a terminal U·G mismatch). Cleavage occurs at the arrowed position. The numbering of the nucleotides and the conventional naming of the helices are shown. The trans-acting form of the ribozyme begins at G640 (shown in black). The sequences of four cleavage substrates are shown (right). These are named according to the number of base pairs (including the U·G mismatch) in helix Ia. The ligation substrates had the same sequence, with a break at the arrowed position.

studies of the reverse, ligation reaction. In an early study, Saville and Collins reported the cyclization of monomeric VS RNA generated from multimeric RNA transcribed in vitro (*18*). While this indicates that the VS ribozyme can carry out ligation when it is embedded in the complete VS RNA, evidence for such activity in the minimal ribozyme has been harder to come by. Jones, Ryder, and Strobel (*19*) explored the effect of adding further VS sequences to the 5′ and 3′ termini of the minimal ribozyme, thereby creating a new, extensively mismatched helix (termed VII), and consequently a new three-way junction with helices Ia and II. They found that this species exhibited weak ligase activity, capable of generating a few percent ligated product. However, they isolated sequences that were mutated in the new helix VII that gave elevated levels of ligation.

In the present study we have examined the potential ligation activity of the minimal VS ribozyme acting in trans. We turned our attention to the substrate, reasoning that the ligation activity might be revealed if helix Ia were extended to act as a guide helix for the 5′ substrate. We find that provided this substrate is bound to the 3′ substrate by 10 bp, a very efficient ligation reaction can be catalyzed by the VS ribozyme in trans, with rates of 5 min^{-1} or higher. We have analyzed the sequence requirements for the ligation reaction and also found that the reaction rate is pH dependent, corresponding to the deprotonation of a group with a pK_A of 5.6.

MATERIALS AND METHODS

Transcription of RNA. RNA for cleavage activity experiments was synthesized by transcription using T7 RNA polymerase (*29*) from double-stranded DNA templates. Templates for transcription of ribozymes were made by recursive polymerase chain reactions from synthetic DNA oligonucleotides. RNA was purified by electrophoresis in 5% or 20% polyacrylamide gels containing 7 M urea. RNA was recovered by electroelution into ammonium acetate and precipitation with ethanol at −20 °C.

RNA Substrates. For most experiments we used VS ribozyme 1 as defined by Lafontaine et al. (*12*), having the sequence (written 5′ to 3′) GCGGUAGUAAGCAGGGAACUCACCUCCAAUUUCAGUACUGAAAUUGUCGUAGCAGUUGACUACUGUUAUGUGAUUGGUAGAGGCUAAGUGACGGUAUUGGCGUAAGUCAGUAUUGCAGCACAGCACAAGCCCGCUUGCGAGAAU.

For analysis of the effect of varying the length of helix V, the following sequences were used (5′ sequences only shown): 5 bp, CGUAG; 7 bp, CGUAGCA; 9 bp (WT), CGUAGCAGU; 10 bp, CGUAGCGAGU; 11 bp, CGUAGCGUAGU; 12 bp, CGUAGCGUCAGU.

For analysis of the effect of varying the length of helix III, the following sequences were used (5′ sequences only shown): 5 bp, CCACC; 6 bp, CUCACC; 7 bp, CUCUACC; 8 bp, CUCGUACC; 9 bp, CUCGCUACC; 10 bp, CUCGACUACC.

Other sequence variations summarized in Table 1 were introduced into the ribozyme as indicated in the text.

The cis-ligating ribozyme was created from the following sequence by self-cleavage: GCGCGAAGGGCGUCGUCGCCCCGAGCGGAUUGCGACAC$(U)_n$GUAGUAAGCAGGGAACUCACCUCCAAUUUCAGUACUGAAAUUGUCGUAGCAGUUGACUACUGUUAUGUGAUUGGUAGAGGCUAAGUGACGGUAUUGGCGUAAGUCAGUAUUGCAGCACAGCACAAGCCCGCUUGCGAGAAU, where $n = 10$, 20, or 30.

*Substrates for Cleavage Reaction*s. Substrate 1 (*12*) was extended in the 5′ direction using the natural VS sequence, with a 5′ unpaired GCG for labeling purposes. It was extended to the 3′ by adding sequence complementary to the 5′ end: 0 bp (substrate 1), GCGCGAAGGGCGUCGUCGCCCCGA; 6 bp, GCGAUCUGCGAAGGGCGUCGUCGCCCCGAGCGGAU; 8 bp, GCGCAAUCUGCGAAGGGCGUCGUCGCCCCGAGCGGAUUG; 10 bp, GCGCGCAAUCUGCGAAGGGCGUCGUCGCCCCGAGCGGAUUGCG; 14 bp, GCGGUGUCGCAAUCUGCGAAGGGCGUCGUCGCCCCGAGCGGAUUGCGACAC.

*Substrates for Ligation Reaction*s. The two RNA species required to generate each ligation substrate were generated in separate cleavage reactions with VS ribozyme. These had the same base sequence as the cleavage substrates but included the break between nucleotides 620 and 621 with the required 2′,3′-cyclic phosphate and 5′-hydroxyl termini.

Efficient production of substrates was obtained by truncation of the 5′ substrate construct after A639 and by addition of the minimal GCGCG 5′ to A622 for the 3′ substrate.

Analysis of Ribozyme Kinetics. Ribozyme kinetics were analyzed in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM KCl, and 2 mM spermidine (*11*), except where indicated.

Cleavage reactions were carried out under single turnover conditions, using trace concentrations (~1 nM) of radioactively 5′-^{32}P-labeled substrate in the presence of a large excess of ribozyme (1 μM). Substrate and ribozyme were incubated individually in kinetic buffer at 37 °C for 15 min and then mixed to initiate the reaction. Aliquots of 2 μL were removed at different times, and the reaction was terminated by addition of 8 μL of 95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromophenol blue. Substrate and product were separated by electrophoresis in a 20% polyacrylamide gel containing 7 M urea using 8 × 10 cm plates. They were quantified by exposure to a storage phosphor screen and imaging (Fuji). Data were fitted to single exponential functions by nonlinear regression analysis (Kalaidagraph, Abelbeck Software).

Ligation reactions were performed using trace concentrations of radioactively α-^{32}P-labeled 3′ substrate (2 nM), a higher concentration (100 nM) of the 5′ substrate, and an excess of ribozyme (1 μM, except in the ribozyme titration experiment of Figure 3). The two substrate species were incubated together, and the ribozyme was separately incubated in parallel in kinetic buffer at 25 °C for 15 min. These were mixed to initiate the reaction. Aliquots of 2 μL were removed at different times, and the reaction was terminated by addition of 8 μL of 95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromophenol blue. Substrate and product were separated by electrophoresis in a 15% polyacrylamide gel containing 7 M urea using 33 × 39 cm plates. They were quantified by exposure to a storage phosphor screen and imaging (Fuji). Data were fitted to double exponential functions by nonlinear regression analysis (Kalaidagraph, Abelbeck Software). Ligation was studied as a function of pH by measurement of rates using the following buffers: acetate, pH 5.0, 5.5, and 6.0; KMES, pH 5.5, 6.0, and 6.5; KPIPES, pH 6.0, 6.5, and 7.0; Tris-HCl, pH 7.0, 7.5, 8.0, 8.5, and 9.0.

Ligation reaction kinetics for the cis-acting construct (Figure 7) were analyzed in kinetic buffer. Substrate and ribozyme were separately preincubated, and the reaction was initiated by mixing. Substrate and product were quantified by denaturing gel electrophoresis and phosphorimaging as above.

RESULTS

Effect of the Length of Helix Ia on Ribozyme Activity. In our model of the global structure of the VS ribozyme and its interaction with the substrate stem−loop (*13*), the open helix (Ia) of the substrate projects away from the ribozyme and should encounter no steric clash with the body of the ribozyme. In the natural ribozyme this helix is only 3 bp in length (including one U·G mismatch), and in our kinetically best behaved substrate even this short helix is replaced by a single-stranded section comprising the 5′ terminus (*12*). We therefore investigated the effect of artificially increasing the length of helix Ia as a perfectly base-paired helix of 6, 8, 10, or 14 bp (Figure 1). These radioactively 5′-^{32}P-labeled substrates were each incubated with the ribozyme in trans under single turnover conditions, and the products were examined by separation on a polyacrylamide gel and phosphorimaging (Figure 2). The original substrate lacking a base-paired helix Ia is cleaved to ≥80%, with an observed rate of 1 min^{-1} in agreement with previously observed behavior. However, the presence of a helix Ia of 6 or 8 bp results in a significant reduction in the rate of ribozyme cleavage, with rates of k_{obs} = 0.1 and 0.05 min^{-1}, respectively, similar to values observed previously for the substrate with the natural 3 bp helix (*12*). Moreover, the plateau extent of cleavage is reduced to 60% with the 8 bp helix. Further increase in length of helix Ia accentuates these effects, so that the substrates with 10 and 14 bp helices are cleaved to only ~10%, with rates of k_{obs} = 0.03 and 0.06 min^{-1}, respectively.

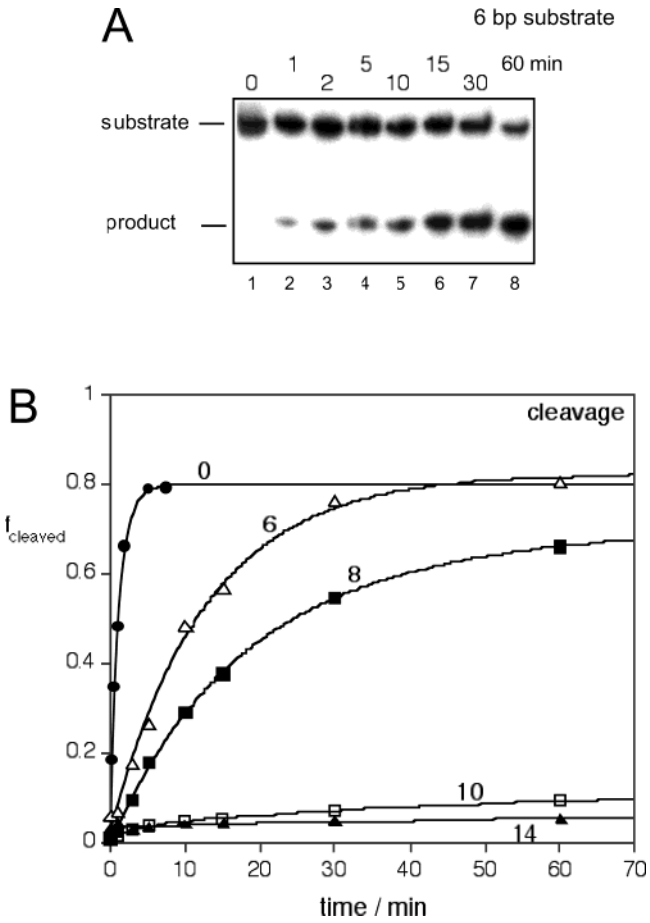


FIGURE 2: Cleavage reaction of the VS ribozyme in trans as a function of the length of helix Ia of the substrate. Radioactively labeled substrate was incubated with ribozyme under single turnover conditions as a function of time, and conversion into product was analyzed by denaturing gel electrophoresis and phosphorimaging. (A) The time course is shown for the 6 bp substrate, where the samples were incubated with ribozyme for 0, 1, 2, 5, 10, 15, 30, and 60 min (tracks 1−8, respectively). (B) The fraction cleaved is plotted against time for each substrate, and the data are fitted by a single exponential function in each case. Note that as the length of helix Ia increases, the cleavage reaction becomes slower and the extent of cleavage is significantly reduced. Symbols: 0 bp substrate, closed circle; 6 bp substrate, open triangle; 8 bp substrate, closed square; 10 bp substrate, open square; 14 bp substrate, closed triangle.

An Efficient Ligation Reaction in Trans. In view of our structural model of the VS ribozyme plus substrate, we felt that the effect of helix Ia length on cleavage activity was unlikely to result from an effect on the cleavage reaction per se. Instead, we investigated the alternative possibility that the reverse ligation reaction was becoming more efficient with these substrates. In principle, the cleavage reactions of the nucleolytic ribozymes are reversible; for example, the hairpin ribozyme is an efficient ligase provided the natural form of the ribozyme stabilized by its four-way junction is used (*8*). A ligation reaction has been reported for the complete VS RNA (*18*), but this is probably not observable in the forms of the substrate with the minimal helix Ia due to rapid loss of the short 5′ product of cleavage. Extension of helix Ia would have the effect of retaining the 5′ product due to base pairing with the 3′ extension of the substrate, increasing the probability of religation after cleavage. Strobel and colleagues (*19*) have demonstrated a ligation reaction in an extended cis-acting form of the VS ribozyme, probably for similar reasons.

We therefore decided to investigate the ligation activity of the VS ribozyme in trans on substrates in which the two RNA molecules to be ligated are held together by the base pairing of helix Ia (Figure 3). The length of helix Ia was either 6, 10, or 14 bp. The 5′ and 3′ substrates for the ligation reaction were separately generated using VS cleavage reactions, ensuring that these had the correct cyclic 2′,3′-phosphate and 5′-OH termini, respectively. The results are shown in Figure 3A,B, where it can be seen that the substrates are now efficiently ligated by the ribozyme. With the longer helix Ia, almost 80% of the substrate ends up in the ligated form, with a length ~10 bp required for full stability and hence retention of the cleavage product. Unlike the cleavage reaction, we required two exponential functions to fit the ligation data, with a faster rate of 1.1 min^{-1} under the conditions of the experiment. Thus the base pairing of the two substrates through helix I clearly results in a very efficient ligation reaction by the VS ribozyme acting in trans.

We studied the reaction as a function of ribozyme concentration (Figure 3C). In contrast to the cleavage reaction (*15*) we observed no saturation of the rate up to a ribozyme concentration of 20 μM, and rates of >7 min^{-1} were obtained. We could not measure the affinity for the substrate from these data, although it is clear that the ligation substrate is bound more weakly than the type I cleavage substrate.

Effect of Ribozyme Sequence Changes on Ligation Reaction. We and others (*12*, *13*, *16*, *20*) have previously examined the effect of many sequence variations in the different regions of the VS ribozyme on the cleavage activity. Most changes that lower cleavage rates can be characterized as structural effects, including the two three-way helical junctions, the base bulges, and the lengths of helices III and V (*12*, *13*). The main exception to this is the internal loop containing A730 (termed the A730 loop) of helix VI. Almost all sequence changes in the A730 loop lead to pronounced reduction in cleavage activity without changing the folding properties of the ribozyme (*15*, *16*), leading to the suggestion that the loop is effectively the active site of the ribozyme. If the ligation activity is essentially the reverse of the cleavage reaction, the same features of the ribozyme should be required to promote the reaction. We therefore studied the trans ligation activity under our standard conditions using the 14 bp substrate with a group of variant ribozymes chosen to illustrate the functional features determined for the cleavage reaction. The results are summarized in Table 1, including comparisons with the corresponding trans cleavage reaction. With a few exceptions, the effects on cleavage and ligation reactions are within a factor of 2 of each other.

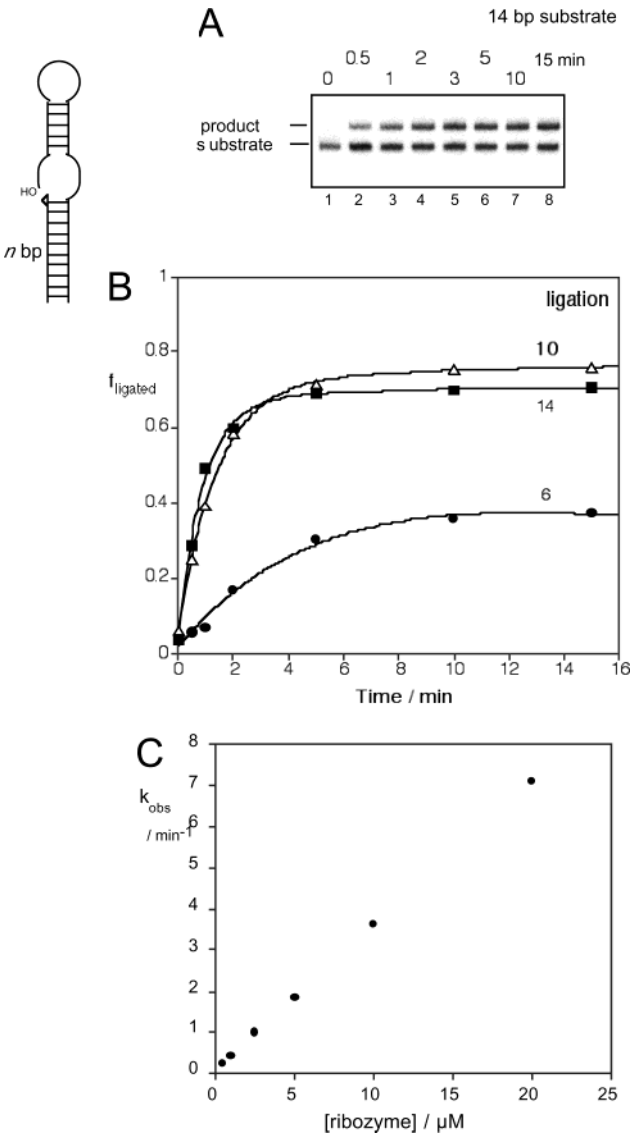


FIGURE 3: Ligation reaction of the VS ribozyme in trans as a function of the length of helix Ia of the substrate. The design of the ligation substrate is shown schematically on the left. Radioactively labeled substrate was incubated with ribozyme under single turnover conditions as a function of time, and conversion into product was analyzed by denaturing gel electrophoresis and phosphorimaging. (A) The time course is shown for the 14 bp substrate, showing good conversion into the ligated product. The samples were incubated with ribozyme for 0 s, 30 s, 1 min, 2 min, 3 min, 5 min, 10 min, and 15 min (tracks 1−8, respectively). (B) The fraction ligated is plotted against time for each substrate, and the data are fitted by two exponential functions in each case. Note that as the length of helix Ia increases, the ligation reaction becomes faster and the extent of ligation is increased. Symbols: 6 bp substrate, closed circle; 10 bp substrate, open triangle; 14 bp substrate, closed square. (C) The rate of ligation was measured for the 14 bp substrate as a function of ribozyme concentration. The reaction rate increases linearly, with no indication of saturation at these ribozyme concentrations.

Three-Way Junctions. The three-way helical junctions play a key role in organizing the three-dimensional structure of

Table 1: Rate Constants for Ligation (14 bp Substrate) and Cleavage (0 bp Substrate) Measured for Sequence Variants of the VS Ribozyme[a]

		k_{obs}/min^{-1}	
		ligation	cleavage
natural sequence		0.9	1.0
helix II	ΔA652	0.054	0.013
helix III	5 bp	0.002	<0.001
	6 bp	0.13	0.14
	7 bp	0.68	0.85
	8 bp	0.14	0.09
	9 bp	0.002	0.004
	10 bp	0.002	<0.001
helix V	5 bp	<0.001	<0.001
	7 bp	<0.001	0.047
	9 bp (natural)	0.90	1.00
	10 bp	0.10	0.031
	11 bp	0.002	0.003
	12 bp	0.002	<0.001
	A698U	0.005	<0.001
helix VI	ΔA725, A726	0.002	0.007
	Δ8 bp distal	0.34	0.46
2-3-6 junction	A656C	0.046	0.003
	G768U	0.076	0.016
	C765U	0.39	0.56
3-4-5 junction	U664A	0.27	0.12
	C665A	0.067	0.15
	ΔU686	0.007	<0.001
	U686A	0.034	0.006
	U710C	0.002	0.03
	U710A	0.007	0.002
	G711U	0.13	0.9
	A712U	0.008	0.005
	A712G	0.024	0.006
	U713A	0.12	0.02
	U714A	0.28	0.14
A730 loop	C755A	0.013	0.84
	A756G	0.003	0.003
	G757C	0.049	0.015

[a] Observed ligation rate constants were measured using the trans-acting systems described in the text, under single turnover conditions, using 1 μM ribozyme in the presence of 10 mM magnesium ions. All cleavage rate constants were taken from our previously published studies except that for A698U, which was measured in this study.

the ribozyme. The 2-3-6 junction creates a cleft in which the substrate stem−loop docks, and the bases A656 and G768 are particularly important (*12*). Changing either results in major reduction of both ligation and cleavage activity. By comparison, C765 is not important for either reaction. The role of the 3-4-5 junction is to position helix V so that it can interact with the substrate loop (*13*, *20*). There is less structural insight into this junction at the present time, but there is reasonable correspondence between effects of sequence variation on both reactions. Both cleavage and ligation are particularly sensitive to substitution of nucleotides A710 and A712 or deletion of U686.

Loop−Loop Interaction. Collins and co-workers have demonstrated a base-pairing interaction between the substrate loop and that of helix V (*14*). This would be expected to be important for holding the substrate in place for both cleavage and ligation reactions. The length of this helix is therefore critical, and either increase or decrease has a major effect on the cleavage reaction (*13*). The dependence of the rate of ligation on helix V length exhibits a closely similar dependence. Furthermore, a sequence change that should to disrupt the loop−loop base pairing (A698U) has a very large deleterious effect on both reactions.

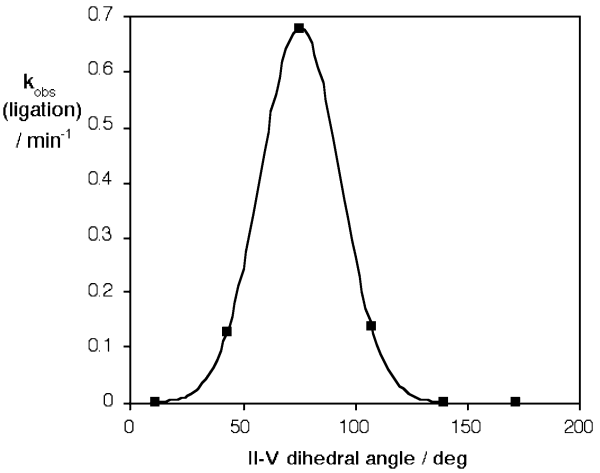


FIGURE 4: Rate of ligation in trans as a function of the length of helix III. The ligation rate was measured for the 14 bp substrate using variant ribozymes in which the length of helix III was varied about the optimal of 7 bp. The rates are plotted as a function of the calculated dihedral angle between helices II and V (*13*). The data are well fitted (line) by a model based on the assumption that the difference in activation energy between the ribozymes of different length helix III results from the energy needed to restore the dihedral angle back to the optimal in order to bind the substrate correctly (*13*).

Length of Helix III. According to our model the length of helix III determines the dihedral angle between the two parts of the ribozyme that interact with the substrate, i.e., the binding cleft between helices II and VI and stem−loop V. Cleavage activity is very sensitive to the length of helix III, symmetrically about a maximal activity for 7 bp. As expected, the ligation activity closely parallels this dependence. The rate of ligation can be fitted to a model that assumes that the difference in activation energy derives from the requirement to restore the angle between helices II and V back to the optimal and that this depends on the square of the displacement angle (*13*). A good fit was obtained to the experimental data (Figure 4), and the values of the optimal dihedral angle (within 2°) and the torsional stiffness force constant (within 20%) for helix III were very similar to those obtained for the cleavage reaction.

A730 Loop. Most sequence changes within the A730 loop have large effects on the rate of the cleavage reaction, and A756 appears to be particularly critical such that substitution by any other nucleotide leads to 3 orders of magnitude slower cleavage (*15*). We have not studied a comprehensive set of changes for the ligation reaction, but it is clear that G757 and especially A756 are similarly important (Figure 5). The biggest difference between the effect of a sequence substitution on cleavage and ligation is for C755A. Ligation is 70-fold slower in this variant, whereas cleavage was almost unaffected. We have confirmed the small effect on the cleavage reaction using the same preparation of ribozyme that was used for the measurement of ligation rate. This is clearly a significant difference and suggests that C755 may be involved in an aspect of the ligation reaction such as ensuring that the two nucleotides are correctly aligned. This is therefore further evidence that the A730 loop is the catalytic center of the ribozyme.

pH Dependence of the Ligation Reaction in Trans. The rate of the trans ligation reaction was measured as a function of pH using the 14 bp substrate. The observed rate constant

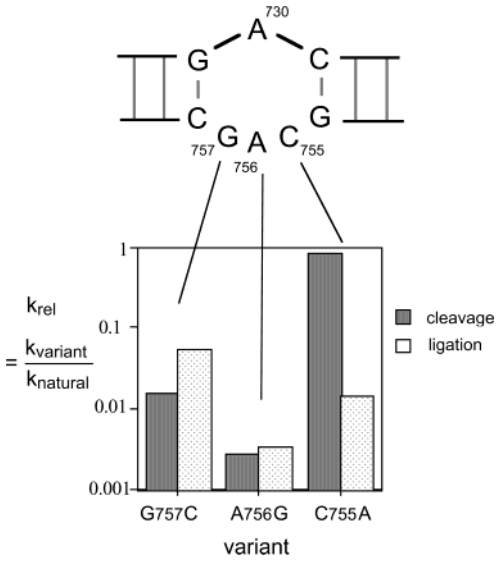


FIGURE 5: Effect of sequence variation in the probable active site on the rate of ligation and cleavage. The relative rates ($k_{rel} = k_{variant}/k_{natural}$) are plotted in the histogram for three sequence variants on the lower strand, G757C, A756G, and C755A. The largest effect for both cleavage and ligation is observed for substitution of A756, consistent with a direct role for the nucleobase (*17*, *27*). The largest difference between an effect on cleavage and ligation is clearly seen for C755, where replacement by adenine has almost no effect on cleavage but reduces the rate of ligation 70-fold.

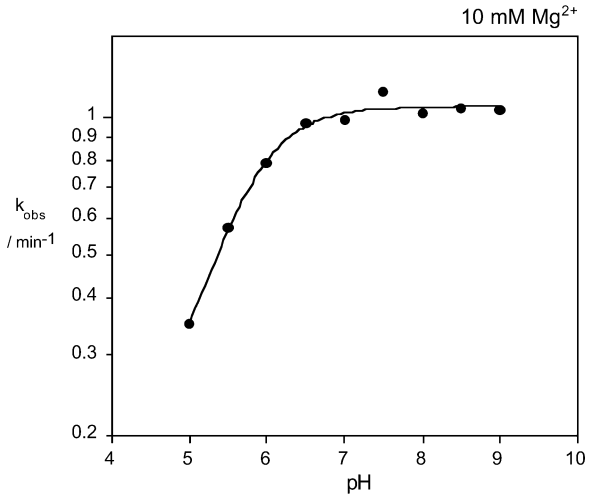
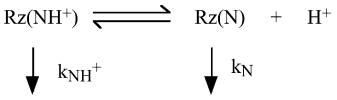


FIGURE 6: Dependence of ligation rate in trans on the pH of the reaction buffer. The observed rate constant for the ligation of the 14 bp substrate was measured under standard conditions as a function of buffer pH and plotted on a logarithmic scale. A significant dependence of ligation rate on pH is observed below pH 6 and can be fitted to eq 1 (line) as discussed in the text.

for ligation was measured in the presence of 10 mM magnesium ions over the pH range 5.0−9.0. In contrast to the pH independence reported for the cleavage reaction (*21*), we observed that the rate of the trans ligation reaction exhibited a significant dependence on pH (Figure 6). This indicates that the ligation reaction rate is responding to the protonation of some nucleotide within the ribozyme or substrate. Below pH 7, log k_{obs} reduced linearly with pH; however, the gradient was only 0.4. These data could not therefore be fitted with a model in which only the basic form of some group was active (which would require a gradient of unity), but instead it was necessary to consider a scheme where both the protonated and unprotonated forms of an unspecified nucleotide (N) were active (see Scheme 1). In this scheme the vertical arrows denote the reactions catalyzed by the protonated and unprotonated forms of the ribozyme, with rates of ligation of k_{NH^+} and k_N, respectively. In the simpler scheme k_{NH^+} would be zero. The new scheme leads to a dependence of the rate on pH of

Scheme 1



$$k_{obs} = \frac{k_{NH^+} + k_N \cdot 10^{pH-pK_A}}{1 + 10^{pH-pK_A}} \quad (1)$$

where pK_A is the acid dissociation constant of the group undergoing protonation/deprotonation. This gives an excellent fit to the experimental data, shown by the line in Figure 6, giving a value of pK_A = 5.6. The rate constants for the two forms are $k_{NH^+} = 0.18$ and $k_N = 1.05$ min^{-1} under these experimental conditions. We have repeated our experiments at different magnesium ion concentrations and find that the pK_A of the titrating group is constant at 5.6 over the range 1−30 mM Mg^{2+}.

Conversion into a Ligation Reaction in Cis. In principle, it should be possible to connect the long-helix Ia ligation substrate to helix II using a tether. We therefore constructed a version of the ribozyme in which the 3′ end of the substrate (in which the helix Ia length was 14 bp) was connected to the 5′ end of the ribozyme helix II via an oligouridine linker of U_n, where n = 10, 20, or 30. These were incubated with the short substrate fragment with a cyclic 2′,3′ terminal phosphate as before, and the products were examined by gel electrophoresis and phosphorimaging (Figure 7). All of the tethered ribozymes carried out efficient ligation reactions, showing that the U_{10} linker was sufficient to span the distance between the lower end of the 14 bp helix Ia and the terminus of helix II.

DISCUSSION

The VS ribozyme acts as an efficient ligase in trans on a substrate that includes a double-stranded helix Ia ≥10 bp in length. Ligation reaction rates >10 min^{-1} are obtainable under some conditions. An approximately inverse correlation was observed between the rate and extent of cleavage vs ligation reactions as helix Ia was increased in size, with a critical length of approximately 6−8 bp where the reactions were equally efficient. It seems likely that when the substrate has a helix Ia that is longer than the critical length, the 5′ product with its cyclic 2′,3′-phosphate is retained so that it is subject to religation, and thus an equilibrium between the two opposing reactions is achieved. With helix Ia ≥10 bp the substrate becomes ligated to an extent of ∼80%, indicating a position of equilibrium that is biased toward ligation such that the equilibrium constant for the ligation-cleavage reaction is ∼5.

It is interesting to compare this result with that of other nucleolytic ribozymes. The hammerhead and hairpin ribozymes were originally thought to be relatively poor catalysts of the ligation reaction, yet a degree of reengineering of these species led to marked enhancement of ligase

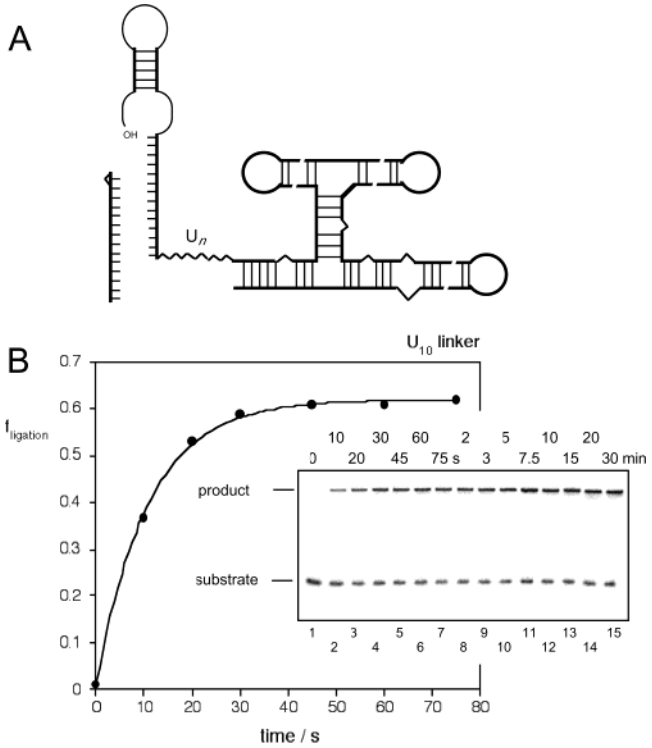


FIGURE 7: Efficient VS ligation reaction in cis. (A) The ribozyme has been converted to a cis-acting ligase by connecting the 3′-end of the 14 bp substrate via an oligouridine linker to the 5′-end of helix II of the ribozyme. (B) The progress of the ligation reaction is shown for the U_{10}-linked ribozyme, fitted to a single exponential function ($k_{obs} = 5.6$ min^{-1}). The inset shows the time course. The samples were incubated for 0 s, 10 s, 20 s, 30 s, 45 s, 60 s, 75 s, 2 min, 3 min, 5 min, 7.5 min, 10 min, 15 min, 20 min, and 30 min (tracks 1−15, respectively).

activity (*8*, *9*). The changes to these ribozymes lead to a more stable active geometry (*22*) and perhaps a subtly altered conformation of the active site (*23*, *24*).

Most of the sequence requirements in the trans-acting VS ribozyme are similar for cleavage and ligation reactions. The dependence on the sequence elements of the two three-way junctions is similar, as are the requirements for the length and loop of helix V. Both reaction rates depend in the same way on the length of helix III and, indeed, can be fitted to the model that assumes that the dihedral angle between helices II and V must be adjusted to its optimal in order to bind the substrate productively. These data suggest that the substrates are bound to the ribozyme in a similar manner for cleavage and ligation. This is consistent with our model for the three-dimensional structure of the ribozyme−substrate complex, where the lower end (i.e., helix Ia) of the substrate simply projects into the solvent unhindered. Moreover, the end of the substrate can be connected to helix II of the ribozyme by a decauridine tether without loss of activity. Strobel and co-workers (*19*) have pointed out that the natural flanking sequence of the VS ribozyme would create a new three-way junction with helices Ia and II. We could not obtain efficient ligation with this construct; our simplified trans-acting form is much better in the ligation reaction. However, Jones et al. (*19*) have selected mutations in the new helix VII that improve the efficiency of the reaction.

The probable active site of the ribozyme is the A730 loop, and as expected, sequence changes in this loop impair the rate of ligation in a major way. A756 is particularly critical for both reactions, with rates of cleavage and ligation each reduced by ≥300-fold in an A756G variant. This would be consistent with a significant mechanistic role for this nucleotide. Perhaps the most interesting aspect of the data from sequence changes in the A730 loop is the difference in the effect on cleavage and ligation rates by a sequence change at C755. We have previously found that a C755A variant behaves almost as the natural sequence in the cleavage reaction (*15*), and we have confirmed this observation in the present work. However, the same variant ribozyme preparation catalyzes a ligation reaction that is impaired 70-fold. We conclude that C755 plays an important role in the ligation reaction, such as assisting the alignment of the cyclic phosphate and/or the attacking 5′-hydroxyl in the active site. Of course, the same interactions should be made with the product in the cleavage reaction; these interactions may not be kinetically significant for the cleavage reaction, but in any case, any effect on cleavage rate would not be detected using our single turnover protocol.

While the hammerhead ribozyme exhibits a dependence of cleavage rate on pH (*25*), indicative of proton transfer in the transition state, the cleavage reaction of the VS ribozyme is almost independent of pH (*21*). By contrast, we have detected a significant pH dependence of the rate of ligation by the trans-acting VS ribozyme. The data are well fitted by a model in which some nucleotide is protonated with a pK_A of 5.6, but both the protonated and unprotonated forms are active in catalysis, with only a 6-fold difference in rate. This might be due to a pH-dependent conformational transition that has a relatively small effect on the chemistry of the ribozyme. For example, a pH-dependent structural change has been observed in the substrate by NMR (*26*). In principle, this might also affect the affinity of substrate binding. However, a second interpretation of these data is possible. Since the 5′ product is unlikely to undergo significant dissociation in the substrate with the 14 bp helix Ia, it will be expected to undergo repeated cycles of ligation and cleavage, and thus the observed rate should be the approach to equilibrium rather than that of ligation per se. In that case the observed rate constant should be the sum of the cleavage (k_{cl}) and ligation (k_{lig}) rate constants, i.e.

$$k_{obs} = k_{cl} + k_{lig} \quad (2)$$

By the principle of microscopic reversibility, if a given group is acting as a general acid in one direction, it should act as a general base in the other. Equation 1 would apply equally to this situation, but the two rates are now interpreted as those for the cleavage and ligation reactions, catalyzed by the two forms of the ribozyme in alternative states of protonation. Given that the position of equilibrium is clearly biased toward ligation for this substrate, we conclude that the ligation (i.e., the faster rate) is catalyzed by the deprotonated form of the ribozyme in this scheme. Application of this analysis gave rates of $k_{cl} = 0.18$ and $k_{lig} = 1.05$ min^{-1}. This would give an equilibrium constant for the ligation-cleavage reaction ($K_{int} = k_{lig}/k_{cl}$) of 5.8, in reasonable agreement with the ∼80% ligation for the substrates with the longer helix Ia (≥10 bp).

A756 is a strong candidate for direct nucleobase participation in the catalysis. The rates of both cleavage and ligation reactions are highly sensitive to either substitution (*15*, *16*)

or functional group modification (*17*, *27*) at this position, and this is the most sensitive nucleotide in the A730 loop. In the entire ribozyme, nucleotide 756 was the position most consistently sensitive to substitution by a wide variety of substitutions in NAIM experiments (*17*). Functional group changes indicate that the nucleobase of A756 is much more important than the ribose for cleavage activity (*27*). The effects of substitutions at nucleotide 756 are mainly due to a reduction in the rate of central conversion of the substrate into product (k_2) and have a rather minor effect on substrate binding affinity (*15*). In our three-dimensional model of the ribozyme the scissile phosphate of the substrate is naturally juxtaposed with the A730 loop. Significantly, evidence has recently been obtained indicating the physical proximity of A756 to the cleavage site; UV irradiation of a ribozyme with a covalently attached substrate containing 4-thiouridine adjacent to the cleavage site led to the formation of a strong cross-link with A756 (*28*).

While there is still no direct evidence for the participation of A756 in the chemistry of VS ribozyme activity, all of the available data are suggestive of that possibility. We should therefore ask whether A756 might be the nucleotide undergoing the protonation/deprotonation in our current experiments. The pK_A of N1 of free adenine is 3.5 and would need to be elevated by the environment of the RNA structure of the active ribozyme. A value of 5.6 would seem to be both feasible and functional. Jones and Strobel (*17*) have provided evidence that the nucleotide at position 756 undergoes a protonation or deprotonation. In particular, they observed that while substitution by either purine or 8-azaadenosine (each of which have pK_A values ~2) leads to loss of ligation, activity could be restored to that of the natural sequence by lowering the pH to 5.4. From this they concluded that the lower limit for the pK_A of A756 is 5.4, which would be consistent with our pH effects if they are ascribed to this nucleotide. Further experimentation will be required to examine these possibilities.

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