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An Improved Version of the Hairpin Ribozyme Functions as a Ribonucleoprotein Complex[†]

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ABSTRACT: Most RNA molecules that are endowed with catalytic activity function in the form of ribonucleoproteins within cells. These complexes are frequently large, poorly defined, and difficult to study. As a model system to study biological catalysis by ribonucleoproteins, we have modified the hairpin ribozyme by inserting an RNA structure that serves as a binding site for bacteriophage R17 coat protein in the form of an extension to ribozyme helix 4, which lies at the periphery of the catalytic domain. In the absence of protein, we find that incorporation of the protein-binding domain increases the catalytic efficiency of the hairpin ribozyme by 2-fold for the cleavage reaction and 16-fold for the ligation reaction. This increase in activity correlates with an increase in the proportion of molecules which fold into the active tertiary structure, as measured by a UV cross-linking assay. Mobility-shift and filter-binding assays of complex formation show that R17 coat protein binds to the chimeric ribozyme with a dissociation constant essentially identical to that of the isolated protein-binding domain; no binding of the protein to the unmodified ribozyme could be detected. The kinetics of cleavage and ligation reactions are not altered by the presence of saturating concentrations of coat protein, and competition studies demonstrate that the protein remains bound to the ribozyme throughout the catalytic cycle. These studies establish that the hairpin ribozyme can be engineered to function efficiently in the form of a ribonucleoprotein *in vitro* and will serve as the basis for future experimentation to understand mechanisms of protein modulation of catalytic RNA activity, and to introduce other protein-binding domains, for example, HIV-1 *rev*-binding and *tar* elements, which may be useful for influencing subcellular localization, regulating intracellular activity, or generating ribozymes that also function as "decoys" in antiviral applications.

Ribozymes are RNA molecules that have been shown, through *in vitro* studies, to catalyze reactions in the absence of proteins. Although a number of different catalytic RNA motifs have been characterized, many biological reactions are known to be mediated not by RNA alone but by ribonucleoprotein (RNP)¹ complexes. RNP-mediated reactions lie at the heart of central cellular pathways, including nuclear pre-mRNA splicing catalyzed by the spliceosome, ribosomal protein synthesis, and protein translocation across membranes mediated by the signal recognition particle (SRP) (for review, see Wittop Koning and Schümperli (1994)).

Even in the case of RNA molecules that function without proteins *in vitro*, there is strong genetic and biochemical evidence that protein factors are essential for catalytic function *in vivo*. Two noteworthy examples are ribonuclease P, where a protein cofactor (C5 protein in *Escherichia coli*) is required *in vivo* but not at high ionic strength *in vitro* (Guerrier-Takada *et al.*, 1988; Reich *et al.*, 1988), and a

group I intron in yeast mitochondria, *cobI2*, where a protein splicing factor (the product of the nuclear gene *CBP2*) is required for splicing *in vivo* but not under high salt conditions *in vitro* (Gampel *et al.*, 1989).

How are the structural requirements for catalytic function distributed between the RNA and protein components of catalytic RNP complexes? While this is clearly a critical question in understanding many essential biological catalysts, it has proven to be very difficult to address experimentally. The structural complexity of many RNP complexes is daunting. For example, 30–100 proteins and several RNA species are involved in spliceosome assembly and function (Moore *et al.*, 1993), while more than 50 proteins and several RNAs are present in the ribosome (Noller (1993) and references therein). Through the concerted efforts of a large number of groups, steady progress is being made in the functional dissection of large RNP complexes. However, simpler systems in which a single protein subunit and a single RNA subunit associate to form a catalytic heterodimeric RNP enzyme are more likely to provide fundamental insights in a shorter time.

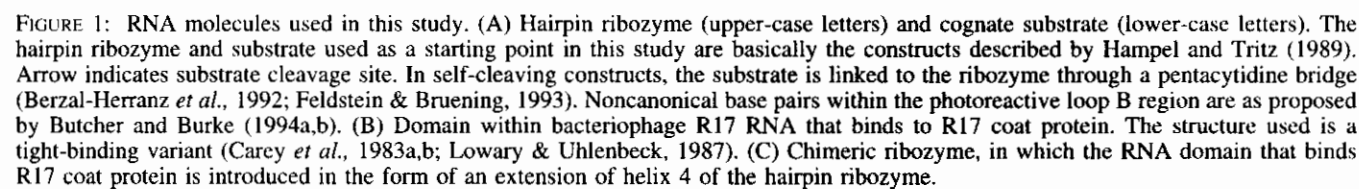
Only a small number of relatively well-defined heterodimeric RNP enzymes are currently available for investigation. Ribonuclease P is composed of a 130 kDa RNA molecule in which resides the catalytic activity, and a 14 kDa protein (Altman *et al.*, 1993). The protein component is required for cellular activity, although its function remains somewhat unclear. It has been proposed to function as an electrostatic shield allowing two negatively charged RNA

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¹ Abbreviations: R17cp, coat protein from bacteriophage R17; RNP, ribonucleoprotein; Hp, hairpin ribozyme; HpR17, hairpin ribozyme containing an RNA binding site for R17 coat protein; R17bs, RNA hairpin constituting a binding site for R17 coat protein.



Some progress in understanding the role of relatively nonspecific proteins in ribonucleoprotein-catalyzed reactions has recently emerged, through studies of the effect of RNA chaperone-like proteins on catalytic activity of the hammerhead ribozyme. These studies suggest that RNA-binding proteins can stimulate ribozyme-catalyzed reactions by facilitating annealing and/or unwinding of the substrate with the ribozyme, and also in resolving misfolded RNA structures. This has been shown for the hammerhead ribozyme and a group I intron with different proteins (Bertrand & Rossi, 1994; Coetzee *et al.*, 1994; Herschlag *et al.*, 1994; Tsuchihashi *et al.*, 1993).

the generation of a catalytic RNP heterodimer through the introduction of a protein-binding domain into a small (50 nt, 17 kDa) catalytic RNA, the hairpin ribozyme. The hairpin ribozyme is derived from a plant RNA replicon, the minus strand of the satellite RNA associated with tobacco ringspot virus (Feldstein *et al.*, 1989; Hampel & Tritz, 1989; Haseloff & Gerlach, 1989; for review see Burke (1994)). The secondary structure of the ribozyme (Hampel *et al.*, 1990; Berzal-Herranz *et al.*, 1993; Butcher & Burke, 1994b) consists of two short intramolecular helices (H3 and H4, Figure 1) separated by a large asymmetrical internal loop (loop B). Substrate binding occurs through the formation of two short intermolecular helices (H1 and H2) which flank a symmetrical internal loop containing the cleavage site. In the presence of divalent cations, the ribozyme catalyzes a site-specific and reversible transesterification reaction, resulting in the release of RNA products containing 5'-hydroxyl and 2',3'-cyclic phosphate termini, or in the ligation of two RNA molecules containing such end structures.

The structure of helix 4 is important for the work presented here. According to mutational and chemical modification data, helix 4 in the naturally occurring version of the ribozyme contains only three base pairs (Berzal-Herranz *et al.*, 1993; Butcher & Burke, 1994b). Published work has shown that (i) the sequence of helix 4 can be manipulated as long as base-pairing is maintained (Berzal-Herranz *et al.*, 1993), (ii) helix 4 can be extended beyond 3 bp (Chowrira & Burke, 1992; Anderson *et al.*, 1994), and (iii) the loop capping helix 4 can be replaced by three base pairs (Chowrira

& Burke, 1992), all without a measurable decrease in catalytic proficiency.

The coat protein of bacteriophage R17 (R17cp) participates in translational control of gene expression by binding to a translational operator within the viral RNA. The same interaction is also important for the initiation of phage assembly. The operator takes the form of a 23 nt RNA hairpin structure, which has been shown to associate tightly with the coat protein (for review, see Witherell *et al.* (1991) and references therein). The sequences within the loop and loop-proximal segments of the hairpin are important for protein binding. In contrast, sequences distal to the loop do not affect binding as long as base-pairing is maintained (Witherell *et al.*, 1991).

These observations led us to reason that the coat protein binding site from R17 RNA could be incorporated into helix 4 of the hairpin ribozyme without compromising its catalytic activity. However, it was not possible to confidently predict whether R17 coat protein would bind to the chimeric structure, or how catalytic activity of the ribozyme would be affected by binding of the protein. In the work described here, we draw four major conclusions. First, incorporation of the protein-binding domain within helix 4 results in a modest increase in the catalytic activity of the ribozyme in the absence of protein. Second, the protein binds to the chimeric ribozyme with an affinity equivalent to that of the isolated protein-binding domain. Third, the ribonucleoprotein complex formed between the modified ribozyme and R17 coat protein has a high level of catalytic activity. Fourth, the protein remains bound to the ribozyme throughout the catalytic cycle. These results have significant implications for further analysis of the collaboration between RNA and proteins in catalytic RNPs, for structural analysis of the hairpin ribozyme, and for cellular applications of ribozymes engineered to cleave targeted RNA sequences.

EXPERIMENTAL PROCEDURES

Purification of R17 Coat Protein. R17 coat protein was purified from an overexpressing *E. coli* clone (plasmid pTCT5 in strain BL21(λ DE3)) according to the procedure described by Gott *et al.* (1991). Briefly, cells overproducing R17 coat protein were harvested and lysed. The lysate was cleared by a first centrifugation for 50 min at 27000g, and the supernatant was subjected to further centrifugation at 186000g for 90 min. The pellet was resuspended, passed through a DEAE-Sephadex A-25 column, and then extracted with 2 volumes of cold glacial acetic acid. Samples were then dialyzed against 1 mM acetic acid. Protein concentrations were determined by optical density at 280 nm and by a Bradford assay (Bio-Rad) relative to bovine serum albumin standards. Purified coat protein was then stored in 1 mM acetic acid and 1 mM DTT at 4 °C (Gott *et al.*, 1991).

Construction of Plasmid Encoding a Hairpin Ribozyme Containing a Binding Site for R17 Coat Protein (Plasmid pHpR17). Two oligonucleotides with complementary 3' ends were synthesized. The sequences of the two oligonucleotides are 5'-CCAACGTCGACAGATCTAAACA GGACTGTCAGGGGGTACCAGGTAA TATACCACAAACATGGGTGATCCTCATGTTTG-3' and 5'-ACCAAAAGCTTTAATACGACTCACTATAGGAAACAGAG AAGTCAACCAGAGAAACACACAAA CATGAGGATCACCCATG-3'. These sequences

encode the self-cleaving hairpin ribozyme construct containing an RNA domain for binding R17 coat protein (Figure 1), located downstream of a T7 RNA polymerase promoter. The oligonucleotides were annealed and elongated with a modified T7 DNA polymerase (Sequenase, United States Biochemicals). The resulting DNA duplexes were digested with *Hind*III and *Sal*I endonucleases and then cloned in plasmid vector pUC19 (Yanisch-Perron *et al.*, 1985). The resulting construct is called pHpR17. Recombinant DNA manipulations were performed basically as described (Sambrook *et al.*, 1989). Following plasmid purification, the sequence of the construct was confirmed by DNA sequencing.

RNA Preparation. RNA molecules were transcribed from a synthetic DNA template or from the *Eco*RI-linearized plasmid pHpR17 using T7 RNA polymerase, essentially as described (Milligan & Uhlenbeck, 1989). RNA was purified by electrophoresis through 10% or 20% denaturing polyacrylamide-urea gels, visualized by UV shadowing, excised, and eluted overnight at 4 °C in 500 mM ammonium acetate, 0.1% SDS, and 1 mM EDTA. The eluates were recovered by filtration, extracted sequentially with phenol and chloroform-isoamyl alcohol, and then ethanol precipitated. RNA pellets were washed with 70% ethanol, dried, resuspended in water, and quantified by measuring absorbance at 260 nm.

For 5' end-labeling, gel-purified RNA was first dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim; 1 unit of phosphatase/50 pmol of RNA) for 1 h at 50 °C and subsequently phosphorylated using [γ -³²P]ATP and T4 polynucleotide kinase (USB). Labeled RNAs were repurified as described above.

Formation of Ribonucleoprotein Complexes. Formation of RNP complexes was analyzed using two assays—filter binding and electrophoretic mobility shifts. Filter-binding assays were conducted as described (Carey *et al.*, 1983a). A constant low quantity of ³²P-labeled RNA (15–32 fmol) was heat denatured and then renatured in 500 μ L of TMS buffer (40 mM Tris-HCl, pH 8, 12 mM magnesium chloride, 2 mM spermidine) or TMK buffer (40 mM Tris-acetate, pH 8, 10 mM magnesium acetate, 80 mM KCl). TMS buffer has been the standard for hairpin ribozyme activity assays, while TMK buffer has been the standard for R17 coat protein-binding assays. Assays were initiated by adding R17 coat protein at various concentrations (0.01 nM–1 μ M). After at least 20 min of incubation at the desired temperature, 450 μ L of the reaction mixtures was filtered through presoaked nitrocellulose filters (Millipore HAWP 0.24–0.45 μ M). The amount of complex retained on filters was determined by liquid scintillation counting. The amount of input RNA was determined by spotting a 20 μ L aliquot of the RNA-protein mixture on a filter. The fraction bound was plotted against the protein concentration. The maximum amount of complex formation varied between 40% and 100% of input RNA, depending on the RNA preparation. This is consistent with previous observations (Carey *et al.*, 1983a,b; Carey & Uhlenbeck, 1983; Lowary & Uhlenbeck, 1987; Schneider *et al.*, 1992). When saturation was clearly observed, results were normalized to 100% of retention for further calculation and for comparison of different experiments. Although a theoretical bimolecular binding curve does not always describe the experimental data accurately (Lowary & Uhlenbeck, 1987), the data points were fitted to a curve describing

theoretical bimolecular association (Pyle & Green, 1994). K_D values were then obtained from a nonlinear least-squares fit using Sigmaplot software (Jandel Scientific). The standard error reported is calculated from the fit of the observed data to the theoretical curve.

Electrophoretic mobility-shift assays for visualization of RNA-protein complex formation on polyacrylamide gels were done essentially as described by Lowary and Uhlenbeck (1987). A constant amount of 5'-labeled RNA (10 pM) was denatured at 90 °C for 1 min and then renatured for 20 min on ice in TMS buffer. The RNA was then incubated in siliconized tubes with increasing concentration of coat protein (1 pM–0.1 μ M) for 2 h on ice. Glycerol was added to a final concentration of 5% (v/v), and then the samples were loaded on a 6% nondenaturing polyacrylamide gel containing 12 mM magnesium acetate and 40 mM Tris-acetate (pH 7.5). Electrophoresis was at 150 V for 10 h at 4 °C.

Formation of Ribozyme-Substrate Complexes. Ribozyme-substrate interactions were analyzed using a mobility-shift assay, essentially as described (Fedor & Uhlenbeck, 1990). A radiolabeled, noncleavable substrate analog (mutation $G_{+1}A$) was employed; its affinity for the ribozyme reflects the binding of the wild type substrate since it competitively inhibits the cleavage of the wild type substrate with a K_i close to the K_M describing the reaction (Chowrira *et al.*, 1991). The $G_{+1}A$ analog (10 pM) was mixed in siliconized tubes with increasing concentrations of unlabeled ribozyme (0, 0.1, 1, 10, 20, 50, and 100 nM) and denatured for 1 min at 90 °C in TMS buffer. Renaturation and binding were allowed to proceed for 2 h on ice. Following addition of glycerol to 5%, samples were loaded on a nondenaturing 15% polyacrylamide gel containing 12 mM magnesium acetate and 40 mM Tris-acetate (pH 7.5). Samples were electrophoresed at 11 W for 13 h at 4 °C.

Ribozyme Activity Assays. Single-turnover cleavage reactions were performed under conditions of ribozyme excess (10, 20, and 100 nM) over radiolabeled substrate (<1 nM; Chowrira *et al.*, 1991; Hampel & Tritz, 1989; and this work). Ribozyme and substrate were denatured separately for 1 min at 90 °C in reaction buffer (TMS or TMK), then renatured on ice for 20 min, and finally brought to reaction temperature (25 or 37 °C) for 20 min. Reactions were initiated by mixing an equal volume of ribozyme and substrate. Aliquots of the reaction (10 μ L) were removed, quenched with an equal volume of urea loading buffer (10 M urea, 20% sucrose, 0.5% SDS, 0.02% xylene cyanol, 0.02% bromophenol blue, 160 mM Tris-HCl (pH 7.5), 160 mM boric acid, 4 mM EDTA), and immediately frozen on dry ice. Reaction products were separated by electrophoresis through a 20% polyacrylamide-7 M urea gel, and gels were quantitated using a Bio-Rad GS-250 Molecular Imager. Results were then plotted and fitted by linear regression analysis to the equation $\ln(y) = -kt + b$, where y is the unreacted fraction of the reactants, t the time, and k the rate constant for the reaction (Thill *et al.*, 1993). Reactions were observed to be first order until about 60% of the substrate was cleaved. Variation of the substrate concentration was observed to have no effect on reaction rate as long as the initial substrate concentration was lower than the ribozyme concentration. Because the reaction rate was proportional to the ribozyme concentration for the three concentrations tested, a value of k_{cat}/K_M was obtained by dividing k by the ribozyme concentration (Chowrira *et al.*, 1993b; Herschlag & Cech, 1990).

For kinetic analysis under multiple-turnover conditions, samples were prepared and reactions initiated as described above for single-turnover kinetics. Substrate concentration (10, 20, 40, 50, 75, 100, 200, 500, 1000 nM) was at least 5 times more than the ribozyme concentration (2 nM). Under these experimental conditions, saturation was observed between 200 and 500 nM substrate. Reactions were incubated for 5 min, where the extent of reaction was less than 5%. Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation by nonlinear regression analysis using "kcat" software (Biometallics). Reported error values reflect the fit of the experimental points to the theoretical curve.

Ligation Assays. Ligation assays utilize self-cleaving molecules, in which the 5' end of the substrate is tethered through a short linker to the 3' end of the ribozyme (Berzal-Herranz *et al.*, 1992; Feldstein & Bruening, 1993; Komatsu *et al.*, 1994). These molecules were transcribed from linearized plasmids in the presence of [α - 32 P]CTP. In these reactions, RNA self-cleavage occurs during transcription, and the larger cleavage product (Rz-5'P), corresponding to the ribozyme tethered to the 5' cleavage product of the substrate, was gel-purified as described above. The ligation substrate, corresponding to the 3' cleavage product of the substrate (3'P), was transcribed from synthetic DNA oligonucleotides as described above, gel-purified, and enzymatically dephosphorylated. Ribozyme (Rz-5'P) and ligation substrate (3'P) were then mixed on ice in TMS buffer and denatured for 2 min at 90 °C, and ligation reactions were allowed to proceed during a 4 °C incubation, which has been shown to be optimal for maximizing the extent of ligation (A. Berzal-Herranz and J. Burke, unpublished results). Reactions were stopped by addition of an equal volume of loading buffer, and reaction products were separated on a 10% polyacrylamide-7 M urea gel and quantitated as described above. The apparent reaction rate constant k_{obs} was calculated as described in the section on single-turnover kinetics above.

UV Cross-Linking Analysis. Cross-linking of the photo-reactive domain within loop B of the unmodified hairpin ribozyme was performed as described by Butcher and Burke (1994a). Briefly, gel-purified radiolabeled RNA was denatured at 70 °C for 2 min and then renatured for 20 min on ice in TMS. Twenty-microliter aliquots were irradiated at a distance of 1 cm with a 254 nm UV light source. Irradiated samples were then analyzed by electrophoresis through a 15% denaturing polyacrylamide-urea gel, and reaction products were quantitated.

RESULTS

Introduction of a Protein-Binding Domain into the Hairpin Ribozyme Structure. We utilized a variant R17 coat protein-binding domain containing the $A_{10}C$ substitution (Figure 1), which had been previously shown to stabilize the ribonucleoprotein complex (Carey *et al.*, 1983b; Lowary & Uhlenbeck, 1987). To avoid disruption of essential structural elements within the hairpin ribozyme, we deleted the terminal loop of helix 4 and elongated the helix using the sequences that constitute the protein-binding domain (Figure 1). A synthetic DNA template was constructed and cloned, and RNA transcripts were generated, as described in the Experimental Procedures. The resulting chimeric hairpin ribozyme containing the protein-binding domain is designated HpR17.

Table 1: Single-Turnover Kinetic Efficiencies for Ribozymes^a

ribozyme	buffer	T (°C)	k_{cat}/K_M (min ⁻¹ μM ⁻¹)	k_{cat}/K_M (relative)
Hp	TMS	37	3.1	1.0
HpR17	TMS	37	5.6	1.8
Hp	TMS	25	2.1	0.7
HpR17	TMS	25	4.6	1.5
Hp	TMK	25	1.2	0.4
HpR17	TMK	25	2.7	0.9

^a Reactions were carried out as described in Experimental Procedures. Hp, unmodified hairpin ribozyme, as shown in Figure 1A. HpR17, chimeric hairpin ribozyme containing an RNA domain for binding of R17 coat protein, as shown in Figure 1C. TMS buffer contains 40 mM Tris-HCl, pH 8, 12 mM magnesium chloride, and 2 mM spermidine. TMK buffer contains 40 mM Tris-OAc, pH 8, 10 mM magnesium acetate, and 80 mM KCl. Similar results were observed in the presence of 0.5 μM R17 coat protein.

Table 2: Steady-State Kinetic Parameters Derived from Reactions under Multiple-Turnover Conditions^a

ribozyme	coat protein	K_M (nM)	k_{cat} (min ⁻¹)	k_{cat}/K_M (min ⁻¹ μM ⁻¹)	k_{cat}/K_M (relative)
Hp		62 ± 15	0.25 ± 0.02	3.8	1.0
Hp	0.5 μM	67 ± 20	0.24 ± 0.03	3.6	0.95
HpR17		51 ± 4	0.31 ± 0.005	6.1	1.6
HpR17	0.5 μM	58 ± 10	0.32 ± 0.02	5.5	1.4

^a Reactions were carried out as described in Experimental Procedures. Ribozyme concentration was 2 nM, and substrate concentrations were 10, 20, 40, 50, 75, 100, 200, 500, and 1000 nM. Reactions were carried out in TMS buffer at 37 °C; less than 5% of the input substrate was cleaved under these conditions. R17 coat protein was added to the indicated reactions to a final concentration of 500 nM, a concentration shown to be sufficient for saturation of the chimeric ribozyme. Hp, unmodified hairpin ribozyme. HpR17, chimeric hairpin ribozyme containing an RNA domain for binding of R17 coat protein.

Introduction of the Protein-Binding Domain Increases the Catalytic Activity of the Ribozyme. We investigated the effect of introducing the protein-binding domain on the catalytic activity of the hairpin ribozyme in the absence of protein. Catalytic assays of cleavage and ligation were conducted using both the parental ribozyme (Hp, Figure 1A) and the HpR17 ribozyme containing the protein-binding domain (Figure 1C). First, we compared the activities of chimeric and unmodified ribozymes in standard TMS reaction buffer (40 mM Tris-HCl, pH 7.5, 12 mM MgCl₂, 2 mM spermidine; Hampel & Tritz, 1989; Chowrira *et al.*, 1993b) at 25 and 37 °C. Results of both single-turnover and multiple-turnover kinetic analysis (Tables 1 and 2; Figure 2) show that incorporation of the protein-binding domain does not inhibit catalytic activity of the ribozyme. Rather, we observe a modest but highly reproducible increase in cleavage activity, such that catalytic efficiency (k_{cat}/K_M) is 1.5–2.1-fold higher when the protein-binding domain is present. The increase in catalytic efficiency has been consistently observed with different preparations of RNA, in the presence or absence of monovalent salts, at 25 or 37 °C (Table 1). Determination of the Michaelis–Menten parameters of the reaction during multiple-turnover reactions showed that the improvement is due to both a modest increase in k_{cat} and a slight decrease in K_M (Table 2).

Buffers containing monovalent inorganic salts may reflect more accurately the environment that would be encountered by ribozymes *in vivo* (Chowrira *et al.*, 1993a). Therefore, we determined the effect of potassium ions on the reaction kinetics of our new construct (Table 1 and data not shown).

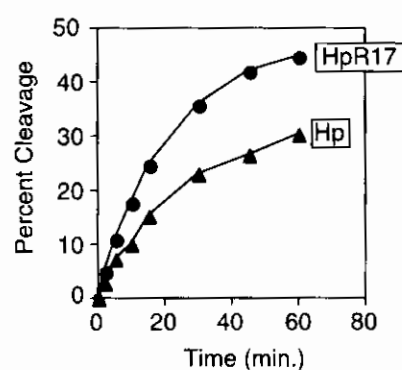


FIGURE 2: Time course of substrate cleavage catalyzed by chimeric and unmodified hairpin ribozymes. Reactions were carried out as described in Experimental Procedures. Unlabeled ribozymes (2 nM) and 5'-³²P-labeled substrate (10 nM) were incubated in TMS buffer at 37 °C for 0, 3, 6, 9, 12, 15, 20, and 30 min. Samples were separated on denaturing polyacrylamide gels and quantitated using a Bio-Rad GS250 molecular imaging instrument, as described in Experimental Procedures. No protein was present during these reactions.

HpR17 shows exactly the same sensitivity to KCl as the unmodified Hp ribozyme ($K_i = 50$ mM at 2 mM MgCl₂; Chowrira *et al.*, 1993a). We tested the effect of the ribonuclease inhibitor aurin tricarboxylic acid (ATA) on the reaction. The rate of cleavage is not affected by the presence of 0.1 mM ATA (B. Sargueil, Q. Yu, and J. Burke, data not shown). Therefore, we routinely included 0.1 mM ATA in all assays where protein was present, in order to prevent RNA degradation from ribonuclease activity present in the R17 coat protein preparation.

The self-cleaving version of the hairpin ribozyme undergoes cleavage during transcription (Berzal-Herranz *et al.*, 1992), yielding the ribozyme attached to the 5' cleavage product ending with a 2',3'-cyclic phosphate (Rz-5'P), and releasing a nine nucleotide substrate fragment (3'P) having a 5'OH terminus. The large cleavage product, Rz-5'P, can covalently link itself to a RNA substrate corresponding to the 3'P in a ligation reaction that is the chemical reverse of the cleavage reaction. We investigated the effect of the protein-binding domain on the ligation reaction. Interestingly, the chimeric ribozyme construct accumulated the ligation product more rapidly than the unmodified ribozyme (Figure 3A) and resulted in a higher extent of ligation at all substrate concentrations tested (Figure 3B).

Under the conditions used (TMS buffer at 4 °C), the unmodified hairpin ribozyme accumulates only 30–40% of the substrate in the ligated form, while the HpR17 construct increases accumulation of the ligation product up to 60% of input substrate under the same conditions. We compared the substrate concentration dependence of the reaction for the two ribozyme constructs. Typically, the ligation reaction is driven by adding a large excess of substrate. As shown in Figure 3B, the reaction with the unmodified ribozyme construct reaches an optimum extent of ligation in the presence of a 100-fold molar excess of substrate. In contrast, the chimeric HpR17 construct requires only a 6-fold excess of substrate to achieve the maximum extent of ligation.

One explanation for the increased ligation rate and extent of the chimeric ribozyme could be due to a stabilization of the ribozyme–substrate complex. To test this possibility, we determined the affinities of both ribozymes for a noncleavable substrate analog using a mobility-shift assay.

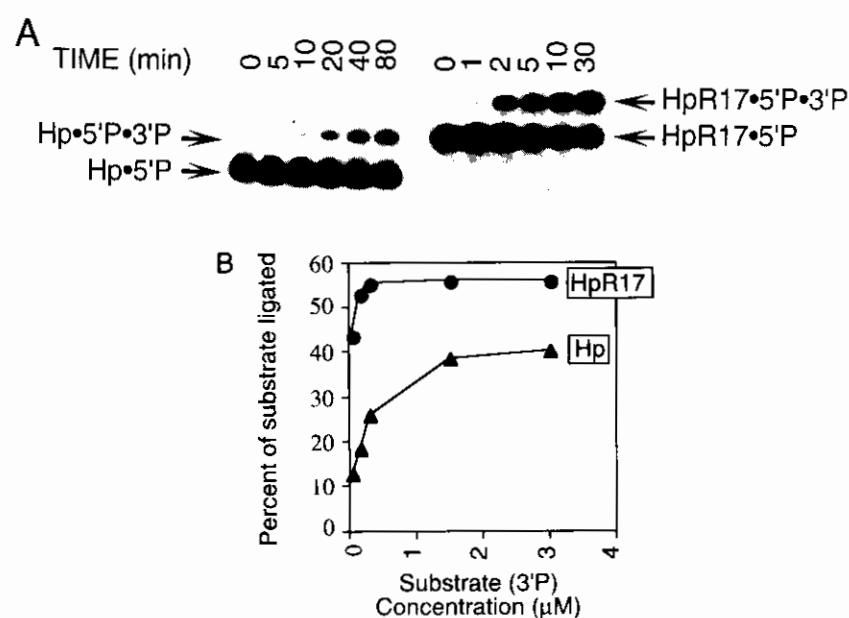


FIGURE 3: Ligation reactions catalyzed by chimeric and unmodified hairpin ribozymes. Reactions involved ligation of an oligonucleotide corresponding to the 3' cleavage product (3'P) and containing a 5'-hydroxyl group, to ribozyme molecules connected to the 5' cleavage product through a pentacytidine linker (see Figure 1A). These latter molecules terminate in a 2',3'-cyclic phosphate. No protein was present during these reactions. (A) Uniformly ^{32}P -labeled ribozyme-containing fragments derived from self-cleavage of the unmodified hairpin ribozyme (Hp-5'P, 54 nM) and chimeric ribozyme (HpR17-5'P, 34 nM) were mixed with cold 3'P (300 nM) in TMS buffer. The mixture was heat-denatured for 2 min at 90 °C, and then the ligation reactions were allowed to proceed at 4 °C for the indicated time. Samples were separated on denaturing polyacrylamide gels and quantitated as described in Experimental Procedures. (B) Experiment analyzing the effect of varying the concentration of ligation substrate on ligation efficiency. Uniformly ^{32}P -labeled Hp-5'P (40 nM) and HpR17-3'P (30 nM) RNA molecules were mixed with unlabeled ligation oligonucleotide (3'P) at concentrations of 30, 150, 300, 1500, and 3000 nM in TMS buffer. The mixtures were heated to 90 °C for 2 min, and then reactions were allowed to proceed at 4 °C for 30 min. Reactions were analyzed as described in the legend to Figure 2.

No significant difference in binding of the noncleavable substrate analog G_{+1}A (see Experimental Procedures) was detected for Hp and HpR17 (data not shown). We also attempted to use the mobility-shift analysis to examine the affinities of the two ribozymes for the 9 nt ligation substrate, 3'P. In each case, we were unable to detect the ribozyme-substrate complex, even in the presence of a vast excess of the ribozyme (2 μM) over substrate (<1 nM).

Because the ligation product is subject to cleavage, caution must be used in interpreting the results of the ligation experiments. That is, increased accumulation of the ligation product could be due to enhanced ligation and/or due to inhibited cleavage. However, the initial reaction rates were determined during the accumulation of only the first 15% of ligation product, under conditions where the ligation is favored due to a very large excess of the ligation substrate and a low temperature which inhibits dissociation of the cleavage products. Introduction of the protein-binding domain enhances the initial rate of ligation by 15–20-fold under the conditions utilized (Figure 3A).

The Protein-Binding Domain Enhances Folding of the Ribozyme into the Catalytically Active Conformation. Previously, we showed that a high-efficiency UV-induced cross-link forms between bases G_{21} and U_{42} within loop B of the unmodified hairpin ribozyme at modest irradiation levels and that the cross-linkable structure reflects the catalytically active conformation of the ribozyme (Butcher & Burke, 1994a,b; Figure 1). The extent of cross-linking monitors formation of the "UV-loop" tertiary structure element and can be used to estimate the fraction of the ribozyme that is folded into the active conformation. We used the UV cross-linking assay to assess potential structural differences between the two ribozyme constructs. In a time course study,

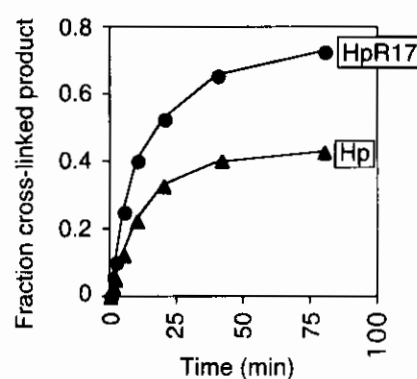


FIGURE 4: Time course of UV cross-linking. Cross-linking reactions were carried out on unmodified RNA as described by Butcher and Burke (1994) and summarized in Experimental Procedures. 5' end-labeled RNA (approximately 1 nM) was irradiated for 0, 1, 2, 5, 10, 20, 40, and 80 min. Cross-linked molecules were separated from unreacted material using a 15% denaturing polyacrylamide gel and quantitated on a molecular imaging instrument.

the HpR17 construct containing the protein-binding domain cross-links about 2-fold faster ($k_{\text{obs}} = 0.05 \text{ min}^{-1}$) and accumulates almost 2-fold more cross-linked product than the parental hairpin ribozyme construct ($k_{\text{obs}} = 0.025 \text{ min}^{-1}$; Figure 4). Thus, the 2-fold increase in catalytic efficiency correlates closely with a 2-fold enhancement in cross-linking rate. From these results, we infer that stabilization of helix 4 has a primary effect on folding, acting to increase the fraction of ribozymes that are folded into an active conformation.

R17 Coat Protein Binds Efficiently to the Modified Hairpin Ribozyme. We analyzed the binding affinity of four different RNA constructs for R17 coat protein. These molecules are shown in Figure 1 and include (i) the chimeric hairpin

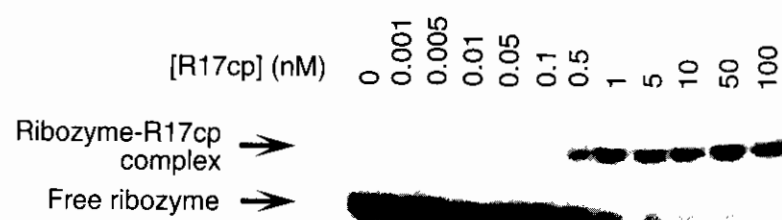


FIGURE 5: Gel mobility-shift assay of formation of the RNP complex between the radiolabeled chimeric hairpin ribozyme and R17 coat protein in TMS buffer at 4 °C. The chimeric ribozyme (HpR17, 10 pM) was incubated with the indicated concentration of purified R17 coat protein. Complexes were allowed to form for 2 h on ice. The free RNA and the complex were separated by electrophoresis through a 6% nondenaturing polyacrylamide gel containing 12 mM magnesium acetate and 40 mM Tris-acetate (pH 7.5) for 10 h at 4 °C at 150 V, as described in Experimental Procedures.

ribozyme containing the protein-binding domain (HpR17), (ii) the unmodified hairpin ribozyme (Hp), (iii) the isolated protein-binding domain (R17bs), and (iv) a mutant protein-binding domain, in which deletion of the bulged A (A₈, construct termed R17bsΔA8) decreases binding affinity by at least 3 orders of magnitude (Carey *et al.*, 1983b).

We used an electrophoretic mobility-shift assay (Figure 5) to directly visualize and quantitate formation of the ribonucleoprotein complexes. Using this method, we were readily able to detect formation of the RNP complex with the isolated protein-binding domain and with the chimeric HpR17 ribozyme. We were unable to detect complex formation with the unmodified hairpin ribozyme or with the mutated protein-binding domain. In the case of R17bs, our observed binding constant showed good correlation with previously reported results (Lowary & Uhlenbeck, 1987).

While the mobility-shift assay permits the direct visualization of the ribonucleoprotein complex, it has two potential disadvantages. First, because complex dissociation may occur during electrophoresis, the mobility-shift assay may not report an accurate equilibrium constant (Lowary & Uhlenbeck, 1987; Witherell *et al.*, 1991). Second, we observe that the method fails to detect complex formation at the higher temperatures which we used for catalytic assays (data not shown). Therefore, we also conducted filter-binding assays (Carey *et al.*, 1983a) so that an accurate characterization of RNP complex formation could be conducted under the same conditions that were used for cleavage reactions. Results are shown in Table 3 and Figure 6 and show that the R17cp binds to the ribozyme containing the protein-binding domain with the same affinity as it does to the isolated domain ($K_D = 0.3$ nM in TMS buffer at 25 °C). In contrast, the binding curve for the unmodified ribozyme is identical to the one observed with the null-mutant R17bsΔA8 and reveals a low-affinity nonspecific binding, where K_D is 3 orders of magnitude higher, consistent with previously reported work (Carey *et al.*, 1983b). In addition, no complex formation was detected when an extract from an *E. coli* strain that does not overexpress R17cp was used. Additionally, the coat protein showed no binding to our standard RNA substrate, S17 (data not shown). We conclude that introduction of the protein-binding domain into the hairpin ribozyme confers high-affinity binding for R17 coat protein onto the hairpin ribozyme and that the unmodified ribozyme shows no specific affinity for coat protein.

We monitored protein binding in the commonly used ribozyme reaction buffer (TMS) and a buffer believed to be somewhat more physiological (TMK) at 37 and 25 °C. The K_D values obtained (Table 3) are again in good agreement

Table 3: Dissociation Constants for Formation of RNA-Protein Complexes^a

RNA	T (°C)	buffer	K_D (nM)
R17bs	25	TMK	4.0
R17bs	25	TMS	0.31
R17bsΔA8	4	TMK	> 1000
R17bsΔA8	25	TMK	> 1000
R17bsΔA8	25	TMS	> 1000
Hp	4	TMK	> 1000
Hp	25	TMK	> 1000
Hp	25	TMS	> 1000
Hp	37	TMS	> 1000
HpR17	4	TMK	0.25
HpR17	25	TMK	4.0
HpR17	25	TMS	0.4
HpR17	37	TMS	92

^a K_D values were obtained through filter-binding assays using the indicated buffers and temperatures for the combinations of R17 coat protein with the indicated RNA molecules. R17bs, protein-binding site (RNA hairpin) from phage R17 RNA. R17bsΔA8, R17 protein-binding site with mutation that reduces protein-binding affinity. Hp, unmodified hairpin ribozyme. HpR17, chimeric hairpin ribozyme containing an RNA domain for binding of R17 coat protein.

with previous studies (Carey *et al.*, 1983a,b; Lowary & Uhlenbeck, 1987; Schneider *et al.*, 1992). We observed that binding affinity decreases with temperature and ionic strength, as has been seen for the isolated domain (Carey & Uhlenbeck, 1983). In all of the conditions reported in Table 3, the chimeric ribozyme HpR17 RNA was saturated with coat protein at a protein concentration of 0.5 μM. To prevent degradation by cellular ribonucleases potentially copurified with coat protein, we added ATA during the RNA-protein incubations and found that the binding properties were not affected by the presence of 0.1 mM ATA (data not shown).

The Ribonucleoprotein Complex Possesses Catalytic Activity Equivalent to the RNA Alone. To assay the effect of R17 coat protein binding on the ribozyme-catalyzed cleavage and ligation reactions, the chimeric ribozyme was heat-denatured, refolded, and then preincubated with the protein for 20 min after being renatured. These conditions are sufficient for maximal extent of binding (Carey & Uhlenbeck, 1983; Lowary & Uhlenbeck, 1987). The RNP complex was then mixed with the substrate RNA to initiate the reactions. We could not detect any difference in reaction efficiencies during cleavage reactions with the HpR17 ribozyme in the presence of increasing quantities of R17 coat protein (concentration range 10^{-10} – 10^{-6} M; Figure 7). Further detailed studies of the ribozyme activity in the presence of the protein were performed with 0.5 μM of R17cp such that all RNA molecules are in the form of a complex, but low enough that the protein does not aggregate (Witherell *et al.*,

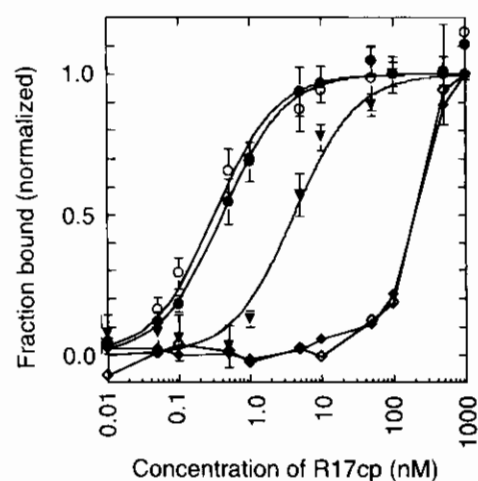


FIGURE 6: Filter-binding assay of formation of the RNP complex between the radiolabeled chimeric hairpin ribozyme and R17 coat protein at 25 °C. The assay was carried out as described in Experimental Procedures. R17bs (○), HpR17 (●) in TMS, HpR17 in TMK (▼), Hp (◆), R17bsΔA8 (◇). To facilitate comparison, results have been normalized to the value of the plateau (Carey *et al.*, 1983; Lowary & Uhlenbeck, 1987; Schneider *et al.*, 1992), except for Hp and R17ΔA8 which does not reach a plateau. Error bars represent the standard deviation from the mean for data from three independent experiments. Solid lines represent theoretical bimolecular binding curves from which K_D is estimated. Lowary and Uhlenbeck (1987) have shown that the filter-binding method generally indicates formation of a more stable RNA–protein complex than does the gel mobility-shift assay.

1991). Binding of coat protein to the HpR17 ribozyme has no effect on either K_M or k_{cat} for the cleavage reaction (Table 2). For the ribozyme containing the protein-binding domain, we could not detect any differences in either single- or multiple-turnover reactions carried out in either TMS or TMK buffer at 25 or 37 °C (Figure 7, Tables 1 and 2, and data not shown). Additionally, the ligation reaction at 0 °C is also insensitive to the addition of saturating concentrations of the protein. These results have been consistently obtained with different preparations of protein and RNA. The presence of coat protein has no effect on the reaction catalyzed by the unmodified ribozyme, as expected.

R17 Coat Protein Remains Bound to the Ribozyme throughout the Catalytic Cycle. We have shown that the protein binds to the chimeric ribozyme and that the modified ribozyme functions efficiently in the presence of saturating concentration of the protein. However, these results do not themselves show that the ribonucleoprotein complex is actually responsible for the observed catalytic activity. For example, binding of the substrate could induce a conformational change destabilizing the protein–ribozyme complex such that protein is released before cleavage. To determine whether the protein remains bound throughout the catalytic cycle, we first determined the protein affinity for HpR17 in the presence of a large excess of substrate under reaction conditions where cleavage proceeds continuously. As shown in Figure 8, we could not detect any difference in binding affinity in the presence or absence of substrate. Although the protein binds under cleavage conditions, the possibility remains that the protein may quickly dissociate and reassociate while the reaction occurs. To rule out this possibility, we monitored the protein–ribozyme complex stability during catalysis. In these experiments, the protein–ribozyme complex was preformed, and the cleavage reaction was initiated by adding the substrate in the presence of a large

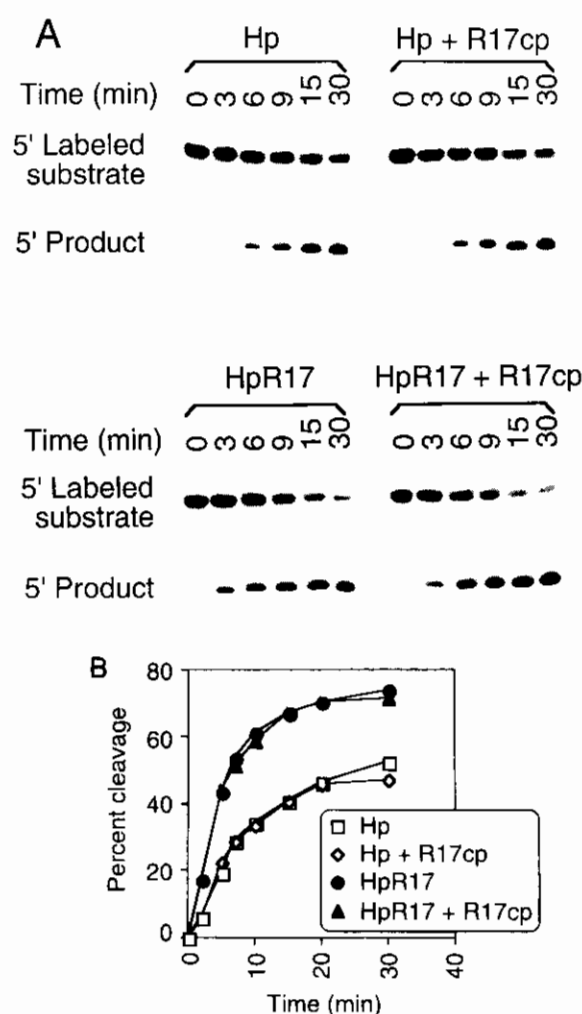


FIGURE 7: Time course of RNA cleavage reaction with Hp and HpR17 ribozyme constructs in the presence of saturating concentration of R17 coat protein. (A) Autoradiogram of time course reaction. Reactions were carried out as described in Experimental Procedures. Ribozymes (20 nM) and 5' labeled substrate (10 nM) were incubated in TMS buffer at 37 °C for the indicated time in the presence or absence of a saturating concentration (0.5 μ M) of R17 coat protein. (B) Quantitated results of the same experiment.

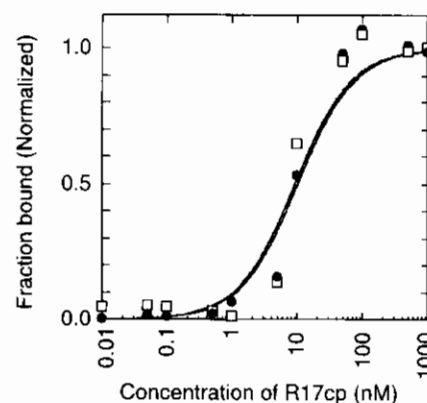


FIGURE 8: Filter-binding assays of protein binding to the chimeric ribozyme in the presence and absence of the RNA substrate at 37 °C. Protein-binding assays were carried out as described in Experimental Procedures using the chimeric HpR17 construct (0.3 pM) in the absence (●) and presence (□) of 20 nM RNA substrate for the ribozyme. Solid lines represent theoretical pseudo-first order binding curves which have been arbitrarily used to define the K_D (Lowary & Uhlenbeck, 1987).

excess of an RNA molecule constituting the R17 binding site. The excess of R17 binding site is used to trap proteins

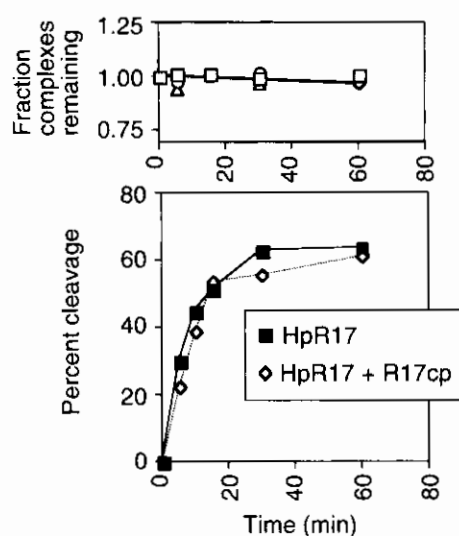


FIGURE 9: Stability of the ribozyme-protein complex during the RNA cleavage reaction. The upper part of the figure shows the stability of the HpR17-R17cp complex with time in TMK buffer at 25 °C. Complexes between 100 nM uniformly labeled chimeric HpR17 ribozyme RNA and 100 nM R17 coat protein were mixed with 500 nM unlabeled RNA substrate for the ribozyme (S17, □), or 500 nM unlabeled protein binding site as competitor (R17bs, ○), or 500 nM of both unlabeled RNA species (△), or 0.1 µg of *E. coli* tRNA (◇). In each case, binding of R17 coat protein to the radiolabeled ribozyme RNA was measured by the filter-binding assay at 5, 15, 30, and 60 min. The lower part of the figure shows the time course of RNA cleavage under similar conditions. Chimeric HpR17 ribozyme (20 nM) was preincubated with 0.5 µM R17 coat protein (◇) or mock control (■) for 20 min at 0 °C and then mixed with 2 nM S17 substrate RNA at 25 °C for 0, 3, 5, 15, 30, and 60 min.

that may dissociate from the complex during catalysis. Under these conditions, the HpR17-R17cp complex is stable for over an hour, after which most of the substrate has been cleaved (Figure 9). From these data we conclude that the cleavage reaction is catalyzed by the ribonucleoprotein complex. The extreme stability of the complex is not surprising, as Lowary and Uhlenbeck showed that this RNA-protein complex has a half-life of about 7 h in TMK and over 24 h in low-salt buffer at 0 °C (Lowary & Uhlenbeck, 1987). Because the ligation reactions were carried at 0 °C, we presume that this reaction is also catalyzed by the ribonucleoprotein.

DISCUSSION

Enhancements to the Catalytic Activity of the Hairpin Ribozyme. The 50 nt hairpin ribozyme structure defined by Hampel and Tritz (1989) has a catalytic efficiency similar to that of other *trans*-acting ribozymes, for example, the hammerhead ribozyme and hepatitis delta ribozymes. Two structural modifications have been shown to enhance hairpin ribozyme activity. Previously, we found that changing the naturally occurring U at position 39 to C enhances ribozyme activity, particularly under suboptimal divalent metal ion concentrations (Joseph & Burke, 1993). Here, we have shown that extending helix 4 through the incorporation of a protein-binding domain enhances cleavage efficiency 2-fold and ligation efficiency 16-fold. This enhancement is likely to be a general result of extending helix 4, because other helix 4 extensions have similar effects, at least on cleavage activity (Anderson *et al.*, 1994; B. Sargueil and J. Burke, unpublished data). Our cross-linking results provide insights

into the molecular basis for this enhancement, indicating that extension of helix 4 improves ribozyme folding, so that a greater proportion of the molecules fold into the tertiary structure that is required for catalytic function. However, the mechanism by which helix 4 extension enhances folding is somewhat unclear. Chemical modification results indicate that helix 4, even at 3 bp, is a very stable structural element within the ribozyme; its Watson-Crick pairing sites are inaccessible to chemical modification under both native and semidenaturing conditions (Butcher & Burke, 1994b).

In the case of the hammerhead ribozyme, it has been observed that substrate cleavage is very strongly favored over ligation of cleavage products, most likely because covalent linkage of the scissile phosphodiester is important for stabilizing the active tertiary structure of the complex by reducing entropic effects (Hertel *et al.*, 1994). In the case of the hairpin ribozyme, ligation is more readily achieved, probably due to the fact that the native ribozyme tertiary structure within the loop B domain (helix 3, loop B, and helix 4) can form in the absence of substrate (Butcher & Burke, 1994b). The results presented here are consistent with a model in which extension of helix 4 results in a more efficient folding pathway, resulting in a higher proportion of ribozymes in the active conformation. The differential effect whereby ligation is enhanced relative to cleavage may reflect increased stabilization of the complex between the ribozyme and cleavage products over that of the complex between ribozyme and uncleaved substrate.

There is great interest in the development of ribozymes to selectively cleave targeted RNA molecules in cellular and potentially therapeutic settings. While the 2-fold increase in catalytic efficiency afforded by the helix 4 extension is modest from the enzymological perspective, such a boost in activity may be very significant for *in vivo* applications.

The Hairpin Ribozyme Can Function as a Ribonucleoprotein. Recently, several groups have reported that unmodified hammerhead ribozymes can interact with nonspecific RNA-binding proteins (Bertrand & Rossi, 1994; Coetzee *et al.*, 1994; Herschlag *et al.*, 1994; Tsuchihashi *et al.*, 1993) and that these interactions can increase ribozyme activity. Our results represent, to our knowledge, the first demonstration that a specific protein-binding activity can be engineered into an RNA enzyme.

Here, we have demonstrated that the hairpin ribozyme can function in the form of a ribonucleoprotein complex with R17 coat protein. Recently, the structure of the complex between the coat protein and RNA binding domain of the very closely related bacteriophage MS2 has been determined by X-ray diffraction (Valegård *et al.*, 1994). In the crystal structure, the protein contacts the loop and 3 other nucleotides along the 23 nucleotides hairpin. Because neither extension of helix 4 nor binding of coat protein has any inhibitory effects on catalysis, we infer that the protein-binding domain is likely to project away from the catalytic core of the ribozyme in the three-dimensional folding.

Implications and Applications of Ribozymes with Ligand-Binding Sites. Our demonstration that a protein-binding domain can be introduced into the hairpin ribozyme without modifying its catalytic activity is interesting and important from two very different perspectives. First, this system provides the basis for potential development of a well-defined model system for the detailed analysis of the structure and function of catalytic ribonucleoprotein complexes. Second,

the introduction of protein-binding domains may be useful for influencing the subcellular distribution, stability, and activity of engineered ribozymes to be used for cellular RNA targeting applications. RNA molecules are distributed within cells by complex mechanisms involving proteins that have not yet been identified (Wilhelm & Vale, 1993). Sullenger and Cech have recently reported an approach for the colocalization of ribozyme and target in retroviral RNA genomes (Sullenger & Cech, 1993) that is fundamentally different from the one proposed here.

Incorporation of protein-binding domains into helix 4 of the hairpin ribozyme is clearly not limited to the binding site for R17 coat protein. A wide variety of additional proteins have been shown to have specific affinities for RNA-binding sites that take the form of hairpin structures or modified hairpins and could be incorporated into hairpin ribozymes. Furthermore, we have the ability to incorporate RNA aptamers that have been selected from random sequence pools for their ability to bind to specific ligands (Tuerk & Gold, 1990; Famulok & Szostak, 1992).

Helix II of the hammerhead ribozyme appears to be somewhat analogous in both structure and function to helix 4 of the hairpin ribozyme and may offer similar possibilities for the introduction of ligand-binding domains. However, the finding that hammerhead helix II is in close proximity to helix I in the three-dimensional structure (Pley *et al.*, 1994; Tuschl *et al.*, 1994) may mean that it will be more difficult for the chimeric ribozyme to interact with bulky ligands without interfering with catalytic activity.

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