Dynamics of Thermal Motions within a Large Catalytic RNA Investigated by Cross-linking with Thiol−Disulfide Interchange

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Received March 7, 1997[⊗]

Abstract: Conformational dynamics have been studied in proteins, but the extent of thermal motion within large compact RNA molecules, including ribozymes, has not been systematically explored. Methods are developed for the incorporation of the alkyl-phenyl disulfide **1** and the alkyl thiol **2** at specific ribose 2′-positions within RNA. These groups react under mild conditions to form disulfide cross-links by thiol−disulfide interchange. When incorporated on opposite faces of a short, continuous RNA helix, these reactants do not form a disulfide cross-link. In contrast, in an active 310-nucleotide group I ribozyme prepared by semisynthetic methods, sites separated by a distance of up to 50 Å in the structure are cross-linked at rates only 3- to 15-times slower than cross-linking between proximal sites. Furthermore, ribozymes with normally distant sites held together by a disulfide cross-link retain substantial catalytic activity. These results reveal unexpectedly large thermal motions between domains of a compact folded ribozyme.

The ability of RNA to switch from one conformation to another is well documented.[1] Such conformational changes can be part of a ribozyme catalytic cycle or induced by binding or release of a second molecule or by changing the experimental conditions. In contrast, the extent of thermal motion within RNA structures at constant conditions has not been investigated experimentally. Measurements of thermal motions within large biomolecules have been limited to protein systems, such as the galactose/glucose binding protein of *E. coli*, where relative thermal motions between adjacent α-helices as large as 15 Å were observed.[2] In a study of rhodopsin, rotational motions of independent helices were observed and found to be required for function.[3] To investigate dynamics of thermal motions, thiol groups (cysteines) were incorporated site-specifically at positions throughout these protein molecules. Thermal motions were then "trapped" by disulfide bond formation.

The goal of the present work was to apply such a trapping approach to a large catalytic RNA molecule (ribozyme). What is the range of thermal motions between helical domains within a ribozyme, and are such motions compatible with catalytic activity? Presented herein are procedures for incorporation of thiol modifications within a large RNA. Also presented is the application of a method of disulfide trapping via thiol−disulfide interchange that is shown to give high reactivity and selectivity and to be chemically compatible with RNA. These methods were used to investigate dynamics in a 310-nucleotide ribozyme derived from the self-splicing group I intron of *Tetrahymena thermophila*. The rate and distance dependence of formation of different disulfide cross-links within the ribozyme reveal a surprising amount of flexibility, supporting the conclusions that thermal motions between adjacent helical domains of at least 50 Å occur and that these long-range motions are compatible with ribozyme catalysis.

Disulfide Cross-linking in RNA by Thiol−Disulfide Interchange

Disulfide cross-linking has recently been used to evaluate existing structural models in the hammerhead ribozyme[4] and $tRNA^{Phe}$.[5] We have found it necessary, however, to develop new cross-linking procedures that are amenable for large catalytic RNAs and that allow kinetic characterization of cross-linking. In the aforementioned protein and RNA systems, disulfide cross-linking was effected by oxidation of two thiols, a reaction that is prohibitively slow in the absence of a catalyst. Redox-active metal complexes, such as copper(II) phenanthroline, oxidize cysteine-thiols in proteins[6] but are incompatible with RNA because they promote cleavage of the ribose backbone.[7] In addition, large structured RNAs often contain binding sites for multivalent metal ions, further complicating analysis. Sigurdsson et al. employed sulfoxide catalysis to form disulfides in the hammerhead ribozyme,[4] but the required experimental conditions (dimethyl sulfoxide ≥ 50%) and time scale (∼30 h) are not appropriate for the present study.

This work demonstrates the utility of thiol−disulfide interchange (Scheme 1) as a means of mild disulfide bond formation in RNA systems. The ribose 2′-position within an RNA duplex is located on the outer surface of the helix in an orientation favorable for interhelix contact and was therefore chosen as the position of modification.[8] Synthesis of generalizable cross-link

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

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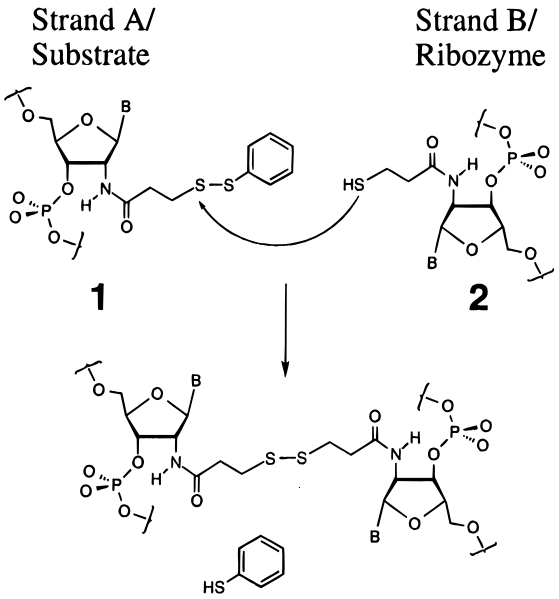
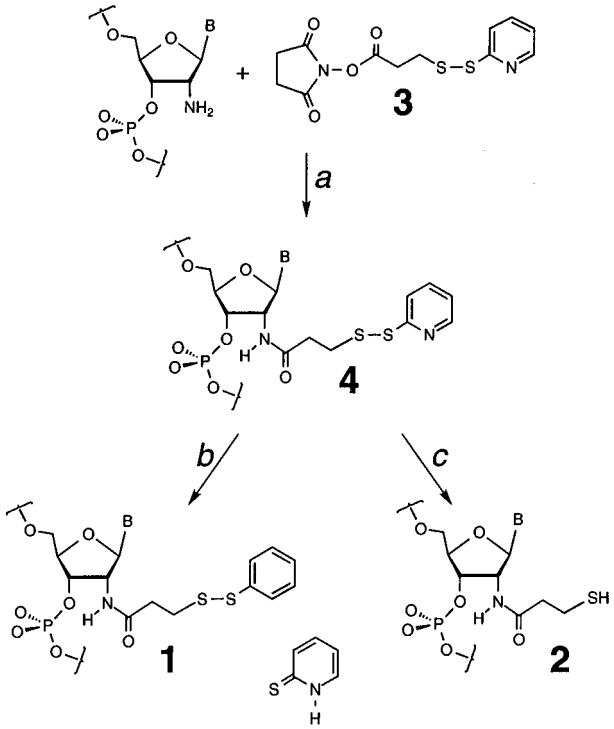
S0002-7863(97)00742-7 CCC: $14.00

Scheme 1



Scheme 2. Preparation of Cross-Link Precursors **1** and **2**[a]



[a] Reaction conditions: (***a***) RNA with site-specific 2′-amino-2′-deoxyribose substitution (50−100 μM), aqueous $NaB(OH)_3$ (100 mM, pH 8), NaCl (100 mM), **3** (50 mM), DMF (10% v/v), 37 °C, 15 min. (***b***) C_6H_5-SH (70 mM), 1/1 v/v aqueous $NaB(OH)_3$ (100 mM, pH 8)/ethanol, 23 °C, 2 min. (***c***) Dithiothreitol (100 mM), aqueous $NaB(OH)_3$ (100 mM, pH 8), 37 °C, 30 min.

precursors **1** and **2** was achieved by incorporation of a unique 2′-amine followed by reaction with activated ester **3** (Scheme 2). A 2′-amine is much more nucleophilic than the exocyclic amines of bases and provides a selective handle for modification. Disulfide exchange or reduction of intermediate **4** then yields products **1** and **2**, respectively (Scheme 2).[9]

A preliminary evaluation of the cross-linking reaction was performed within a simple 22-base pair RNA duplex (Figure 1). The disulfide **1** and thiol **2** were placed on opposite strands, which were then allowed to anneal. In helix **a**, where the reactive moieties were directly across the minor groove, cross-linking occurred too rapidly to measure at pH 7.5 and within 15 min at pH 4.5. In contrast, in helix **b**, where the modified positions were on opposite faces separated by eight base pairs, no cross-linking was observed after 4 h at pH 7.5. The difference in cross-linking rates between helices **a** and **b** demonstrate at least five orders of magnitude dynamic range for this reaction.[10] These results are consistent with studies of helix flexibility, from which one would predict that an RNA helix like **b** has insufficient flexibility to accommodate cross-linking.[11]

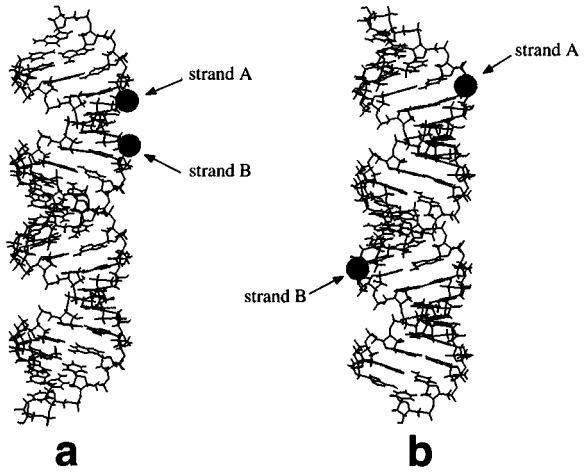


Figure 1. Thiol−disulfide interchange in a simple RNA helix. Spheres represent 2′-modifications.

Application of Thiol−Disulfide Interchange to a Ribozyme System

The *Nhe I* ribozyme (Figure 2a), a 310-nucleotide catalytic RNA derived from the self-splicing group I intron of the ciliate *Tetrahymena thermophila*, catalyzes the sequence-specific cleavage of an RNA substrate with multiple turnover (eq 1).[12]

$$5'\text{-CCCUCUAAACA-}3' + \text{G} \rightarrow 5'\text{-CCCUCU-}3' + 5'\text{-GAAACA-}3' \quad (1)$$

The reaction proceeds through a phosphotransesterification mechanism, with the 3′-hydroxyl of an exogenous guanosine molecule serving as the nucleophile, and affords a rate enhancement of $\sim 10^{11}$ over the uncatalyzed reaction.[13] Magnesium ion ($[Mg^{2+}] \geq 2$ mM) is required to attain a properly folded and active ribozyme.[14] The RNA substrate first binds to the internal guide sequence (Figure 2a, red) through base-pairing to form a six-base pair substrate helix, designated **P1**. In a kinetically separable event, the P1 helix then docks into an active-site cleft within the ribozyme core.[15] P1 docking is accompanied by

(9) The pyridyl disulfide (**4**) displayed only limited stability. Use of this disulfide also prevented inhibition of cross-linking at low pH; its reactivity increased at pH < 7 as a result of protonation of the pyridine nitrogen.

(10) For the thiol−disulfide interchange reaction, it is assumed that $\Delta \log k/\Delta \text{pH} \sim 1$ in the pH range of 4.5−7.5.

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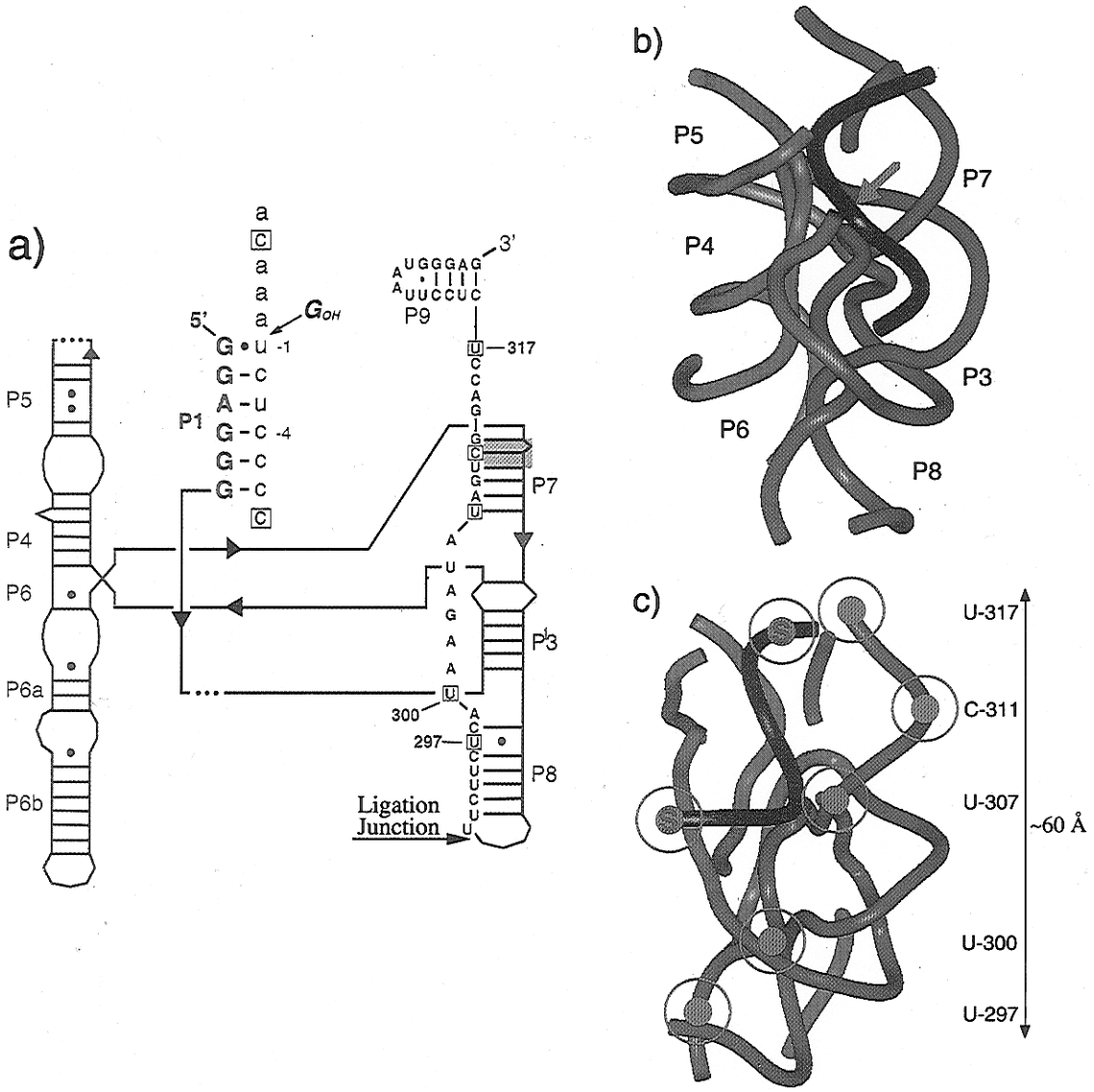

Figure 2. Secondary and tertiary structure of the *Nhe I* ribozyme core. (a) Schematic representation of the secondary structure, highlighting the P4-P6 domain (green) and the P3−P8 domain (purple) ("P" represents "paired" region). The substrate (5′-CCCCUCUAAACA-3′, black) binds to the internal guide sequence (red) through base pairing to form the six base pair helix **P1**. Arrow labeled G_{OH} shows the position of ribozyme-catalyzed cleavage. The shaded purple box denotes the binding site for the guanosine nucleophile. The sequence of the 3′-terminal 41 nucleotides spanning the P3−P8 domain is shown, beginning from the point of ligation to the transcribed RNA for construction of the semisynthetic ribozymes. Boxed nucleotides indicate the positions of modification with **1** (substrate) and **2** (ribozyme). (b) Michel−Westhof model for the architecture of the catalytic core.[18] Helical domains are colored as described in (a). Blue arrow denotes position of substrate cleavage. (c) Positions of modification for disulfide cross-linking between P1 and the P3−P8 domain, designated by blue spheres. The outer blue line represents the approximate radial extension of the thiol or disulfide modification. The substrate is modified at the 3′ (upper) or 5′ (lower) end, highlighted with a red 'S'. Each ribozyme is modified at one of five positions along the P3−P8 domain, spanning a distance of ∼60 Å.

formation of numerous tertiary interactions between the ribozyme core and 2′-hydroxyls of P1 and also the exocyclic amine of the conserved G·U wobble pair within P1.[16]

The secondary structure of the ribozyme core is composed of two distinct quasi-helical domains, the P4-P6 domain (Figure 2a, green) and the P3−P8 domain (Figure 2a, purple).[17] On the basis of conserved sequence homology of 87 group I introns, a model for the three-dimensional architecture of the catalytic core was proposed by Michel and Westhof (Figure 2b),[18] consisting of the two helical domains aligned roughly parallel, forming a cleft into which the P1 helix can dock. The quasi-helical nature of the P4-P6 domain is now supported by the determination of its X-ray crystal structure,[19] and the connection between the domains has been clarified by identification of an interdomain base triplet.[20] In addition, photo-cross-linking and affinity cleavage studies have provided approximate alignment between P1 and the two core domains.[21] Thus, the model is supported experimentally to a resolution of approximately 10 Å and probably better in some regions.

The Michel−Westhof model serves as the structural basis for this study. Specifically, this work examines the relative

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Table 1. Catalytic Activity of Semisynthetic Ribozymes[a]

ribozyme	$(k_{cat}/K_m)^S$ (10^7 M^{-1} min^{-1})	$(k_{cat}/K_m)^G$ (10^5 M^{-1} min^{-1})
unmodified (T7 Pol.)	6.8 ± 1.5	2.7 ± 0.5
unmodified (T4 Lig.)	4.4 ± 1.0	2.6 ± 0.5
U-297	2.0 ± 0.5	1.9 ± 0.4
U-300	2.2 ± 0.5	1.4 ± 0.3
U-307	2.3 ± 0.5	1.4 ± 0.3
C-311	4.6 ± 1.0	0.9 ± 0.2
U-317	4.1 ± 1.0	2.0 ± 0.4

[a] Reactions conducted at 30 °C under single-turnover conditions: Hepes buffer (100 mM, pH 7.2); $MgCl_2$ (10 mM); NaCl (100 mM); ^{32}P-Substrate (≤1 nM); for $(k_{cat}/K_m)^S$, 1 mM GTP and 2.5 nM ribozyme; for $(k_{cat}/K_m)^G$, 0.5 μM GTP and 100 nM ribozyme. Each value is the mean ± range of two independent determinations.

thermal motions between the P1 helix and the P3−P8 helical domain (Figure 2c). The disulfide **1** is incorporated within P1 at positions along the substrate. The thiol **2** is placed at each of five positions along the P3−P8 domain spanning a distance of ∼60 Å, such that locations of disulfide cross-links on P3−P8 with respect to P1 reveal the magnitude of motions that occur, while the relative rates of cross-linking indicate the relative frequencies of such motions.

Construction and Characterization of the Ribozyme System

The first step in preparing the semisynthetic ribozymes containing the thiol **2** was incorporation of a unique 2′-amine into a 41-mer oligonucleotide prepared by solid-phase synthesis (Figure 2a). The remainder of the ribozyme was prepared by in vitro transcription and was then ligated to the synthetic RNA with T4 DNA ligase as described by Moore and Sharp (see Experimental Section).[22] Once a unique 2′-amine was incorporated at each of the five positions within the full-length RNA, modification with **3** and subsequent reduction afforded **2**, as described earlier (Scheme 2). The oligonucleotide substrates (11- or 12-mers), each containing a unique 2′-amine, were prepared by solid phase synthesis and modified to give the disulfide **1** (Scheme 2). The alkyl-phenyl disulfide is stable in aqueous buffer over a pH range of at least pH 4.5−9 and is also stable under electrophoresis conditions.

A detailed mechanistic framework for the ribozyme based on quantitative evaluation of kinetic and thermodynamic parameters has been developed[13] and serves as an assay for the correctly folded tertiary structure.[23] The modified ribozymes were assayed for kinetic activity (eq 1, Table 1) relative to the unmodified ribozyme, prepared by two independent methods: first, by in vitro transcription with T7 RNA polymerase and, second, using an unmodified 41-mer in the two-piece ligation procedure, prepared in parallel with the modified ribozymes. By varying the relative concentrations of ribozyme and guanosine nucleophile, different elements of the reaction pathway can be examined. The second-order rate constant $(k_{cat}/K_m)^S$ is rate-limited by substrate binding, while $(k_{cat}/K_m)^G$ reflects the chemical step.[13] The kinetic competence of the ribozymes (Table 1) supports the conclusion that the thiol modifications did not induce significant structural perturbations.

Modified substrates were tested for binding to the unmodified ribozyme (Table 2). Because the ribozyme is capable of catalyzing hydrolytic cleavage of RNA substrates in the absence of a guanosine nucleophile,[13] a 2′-deoxy residue was placed at the cleavage site to inhibit cleavage of the substrates during the cross-linking assay. It has been established that the 2′-hydroxyl at the cleavage site contributes almost exclusively to transition-state stabilization; any contribution to ground-state stabilization is negligible.[24] Equilibrium dissociation constants (K_d) for binding of modified substrates **B** and **C** were indistinguishable from the K_d of unmodified substrate **A** (Table 2), as were the kinetic dissociation constants (k_{off}), which reflect P1-docking into the active site (poor docking increases k_{off}).[15b,16b] The modified oligonucleotides were active as substrates for ribozyme-catalyzed cleavage (k_{obs}, Table 2), yielding the products corresponding to cleavage exclusively at the proper site (eq 1).

Table 2. Binding and Ribozyme-Catalyzed Cleavage of Cross-Linking Substrates

substrate	structure[a]	K_d (nM)	k_{off} (10^{-2} min^{-1})	k_{obs} (h^{-1})
A	5′-CCCUC*d*UAAACA-3′	0.4 ± 0.1	1.7 ± 0.3	0.36 ± 0.1
B	5′-CCCUC*d*UAAA**C**A-3′	0.5 ± 0.1	1.7 ± 0.3	0.31 ± 0.1
C	5′-**C**CCCUC*d*UAAACA-3′	0.4 ± 0.1	1.8 ± 0.3	0.31 ± 0.1
D	5′-CCCUC*m*UAAA**C**A-3′	4.2 ± 0.5	nd	nd

[a] **C**, the position of the phenyl disulfide (see Scheme 1); *d*, 2′-deoxy; *m*, 2′-OCH_3; nd, not determined. Each value is the mean ± range of two independent determinations.

Investigating Ribozyme Dynamics with Disulfide Cross-Linking

The cross-linking reactions were conducted with saturating excess of ribozyme (100 nM) over a limiting amount of ^{32}P-labeled substrate to discourage disordered, spurious cross-linking as a result of unbound substrate. Each substrate−ribozyme complex was allowed to associate at pH 4.5, where nucleophilic attack by thiol is very slow. Cross-linking was initiated by addition of Hepes buffer to a final pH of 7.2 and followed over time (Figure 3a). The cross-linking reactions were dependent on Mg^{2+} (Figure 3b, lanes b) and on the sequence of the RNA substrate being complementary to the internal guide sequence (Figure 3b, lanes c), supporting the conclusion that cross-linking occurred through a bound substrate−ribozyme complex. Further support for this conclusion was obtained by establishing that cross-linking, formally a bimolecular reaction, was independent of ribozyme concentration (Figure 4). The mild and specific nature of the cross-linking reaction was demonstrated by quantitative release of the substrate upon treatment of a purified cross-linked substrate−ribozyme complex with DTT (100 mM, pH 8, 1 h, 37 °C); subsequent addition of Mg^{2+} and guanosine led to cleavage of the substrate at a rate nearly identical to that observed for a control reaction containing uncross-linked ribozyme and unmodified substrate **A** (k_{obs} = 0.60 and 0.66 h^{-1}, respectively; data not shown).

Cross-linking with substrate **B**, modified 3′ to the cleavage site near the top of the substrate−ribozyme complex (Figure 2c), occurred at all five positions tested (Figure 5). Even the U-300 and U-297 positions, which had originally been designed as negative controls, underwent cross-linking at easily measurable rates. The profile of cross-linking rates among the five positions on the ribozyme depended on the concentration of Na^+ and Mg^{2+} ions. Under conditions typical of those used for ribozyme activity assays (10 mM Mg^{2+}), cross-linking showed only a factor of three between the fastest and slowest rates of cross-linking (Table 3). The maximum selectivity between positions, a factor of ∼15, was obtained by increasing $[Mg^{2+}]$ from 10 to 100 mM (Figure 5b, Table 3). Significantly, the rates of cross-linking with high Mg^{2+} decreased as the

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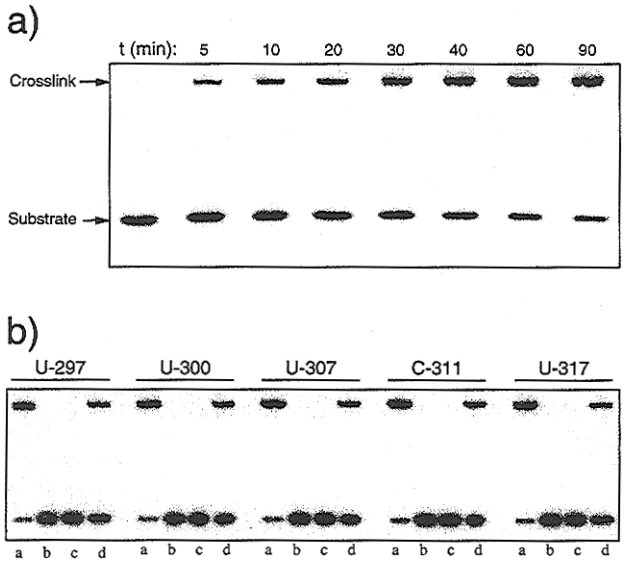

Figure 3. (a) Representative cross-linking assay, conducted by reaction of 5′-^{32}P-substrate **B** (≤1 nM) with ribozyme U-297 (100 nM) at 30 °C in Hepes buffer (100 mM, pH 7.2) with $MgCl_2$ (10 mM) and NaCl (1 M) over a period of 90 min. (b) Cross-linking control reactions (30 °C, 3 h). Lanes a, standard cross-linking reactions performed with substrate **B** in the presence of Mg^{2+} (10 mM); lanes b, cross-linking control reactions performed with substrate **B** in the absence of Mg^{2+}; lanes c, cross-linking control reactions performed with control substrate 5′-UACUA*d*CAAA**C**A-3′ in the presence of Mg^{2+} (10 mM); lane d, cross-linking control reactions performed with a 1/1 mixture of substrate **B** and the control substrate in the presence of Mg^{2+} (10 mM).

distance between the substrate and ribozyme modifications increased, consistent with the Michel−Westhof model (Figure 2).[18] These results reveal unexpectedly large thermal motions of at least 50 Å between the P1 and P3−P8 domains.

To further test the hypothesis that cross-linking occurred with the substrate bound in the proper orientation, the dependence of cross-linking rates on the position of the disulfide **1** was explored. Substrate **C** is modified at the 5′-end, a position roughly centered with respect to the five modifications on the ribozyme (Figure 2c). Cross-linking with substrate **C** in the presence of 100 mM Mg^{2+} occurred at similar rates to all five ribozyme positions (Table 4). The reactions with substrate **C** were also observed to be dependent on Mg^{2+} and independent of ribozyme concentration (data not shown). These results support the structural orientation of the substrate−ribozyme complex, as inferred with substrate **B**, and provide independent support for the conclusion that a significant amount of flexibility exists in this ribozyme system.

Cross-Linking through a Docked vs Undocked P1-Helix

Molar Na^+ resulted in identical rates of cross-linking at all five positions on the ribozyme (Figure 5a) and also decreased ribozyme activity by a factor of ∼25 [$(k_{cat}/K_m)^S$, Table 3]. It is proposed that under these conditions, cross-linking is occurring through a substrate−ribozyme complex that has P1 undocked from the active-site cleft, referred to as an "open" complex, and that the data of Figure 5b (100 mM Mg^{2+}) represent cross-linking of the docked, "closed" complex. This latter complex is a discrete physical state characterized by formation of specific tertiary interactions between the P1 helix and the ribozyme core.[16] In an earlier investigation of the docking equilibrium, Narlikar and Herschlag demonstrated that molar Na^+ prevented docking, presumably by displacing Mg^{2+} ions that mediate tertiary docking interactions, yielding a thermodynamically stable open complex.[25] The decrease in cross-linking selectivity

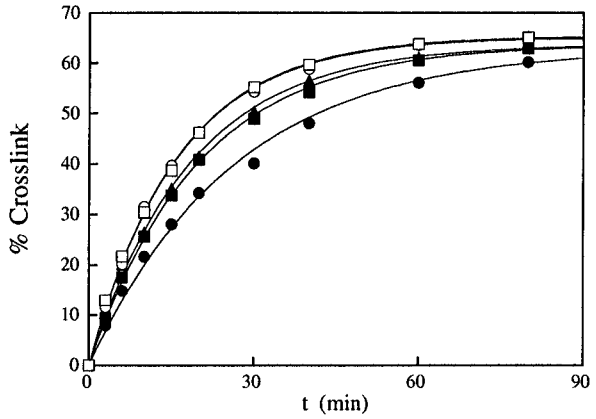

Figure 4. Cross-linking as a function of ribozyme concentration. All reactions contained substrate **B** (≤1 nM), $MgCl_2$ (100 mM), Hepes buffer (100 mM, pH 7.2, 30 °C), and varying concentrations of modified ribozyme C-311: (●) 20 nM, $k = 0.040$ min^{-1}; (■) 50 nM, $k = 0.050$ min^{-1}; (▲) 100 nM, $k = 0.054$ min^{-1}; (○) 250 nM, $k = 0.062$ min^{-1}; (□) 500 nM, $k = 0.062$ min^{-1}.

with molar Na^+ is consistent with an open complex in which P1 has considerable freedom to swing about the periphery of the ribozyme core.

The more selective cross-linking observed in the presence of 100 mM Mg^{2+} (Figure 5b) is suggestive of the closed substrate−ribozyme complex. To more accurately probe the issue of open vs closed complex, a nondocking substrate was synthesized for comparison with substrate **B** under constant ionic conditions (100 mM Mg^{2+}). Substrate **D** differs from substrate **B** only by the presence of a 2′-methoxy group at the cleavage site, which limits docking due to steric disruption of tertiary docking interactions.[24] Cross-linking with substrate **D**, performed in the exact manner as with substrate **B** (Figure 5b), resulted in a clear decrease in selectivity, from a factor of ∼15 down to ∼3 (Table 4). The reactions with substrate **D** were also observed to be dependent on Mg^{2+} and independent of ribozyme concentration (data not shown). The contrasting behavior between substrate **B** and nondocking substrate **D** under identical conditions supports the proposal that cross-linking with substrate **B** in 100 mM Mg^{2+} proceeds at least in part through the closed substrate−ribozyme complex.

Activity of Cross-Linked Substrate−Ribozyme Complexes

To determine if cross-linking proceeded through an active substrate−ribozyme complex, purified cross-linked substrate−ribozyme complexes prepared by reaction of 5′-^{32}P-substrate **B** with ribozymes modified at U-317, U-307, and U-297 were assayed for their RNA cleavage activity, indicated by release of 5′-^{32}P-CCCUC*d*U-3′. All three cross-linked products displayed activity in the presence of 100 mM Mg^{2+} and guanosine (Figures 6 and 7). The reaction kinetics were biphasic, with the magnitude of initial burst phase dependent on the displacement incurred by the cross-link. The fastest and largest burst was afforded by cross-link U-317 and was just 2−3-fold slower than reaction of uncross-linked ribozyme U-317 with unmodified substrate **A** under identical conditions (Figure 7). The greater burst shown by cross-link U-317 and its near optimal reactivity provide further support for the structural orientation of the substrate−ribozyme complex.

The progression of the reactions to a slower phase is consistent with a subpopulation of the cross-linked ribozyme being rate-limited by a refolding event. Alternatively, the reaction could be reaching an equilibrium if cross-linking enhances the rate of the reverse reaction.[1g] The reaction profiles

Table 3. Rates of Cross-Linking with 5′-CCCUC*d*UAAA**C**A-3′[a]

[NaCl][b]	[$MgCl_2$][b]	U-297	U-300	U-307	C-311	U-317	$(k_{cat}/K_m)^S$
1000	10	5.7 ± 1.0	5.5 ± 1.0	4.5 ± 1.0	5.0 ± 1.0	5.0 ± 1.0	0.2 ± 0.05
0	10	3.0 ± 0.5	3.0 ± 0.5	2.5 ± 0.5	3.5 ± 1.0	8.0 ± 2.0	4.0 ± 1.0
0	100	1.3 ± 0.2	1.4 ± 0.3	2.0 ± 0.4	5.5 ± 1.5	20 ± 4	15 ± 5

[a] Cross-linking rates expressed in units of (10^{-2} min^{-1}). [b] Metal ion concentrations exressed in units of mM. Reactions performed in Hepes buffer (100 mM, pH 7.2) at 30 °C. [c] $(k_{cat}/K_m)^S$ expressed in units of (10^7 M^{-1} min^{-1}). Each value is the mean ± range of three independent determinations.

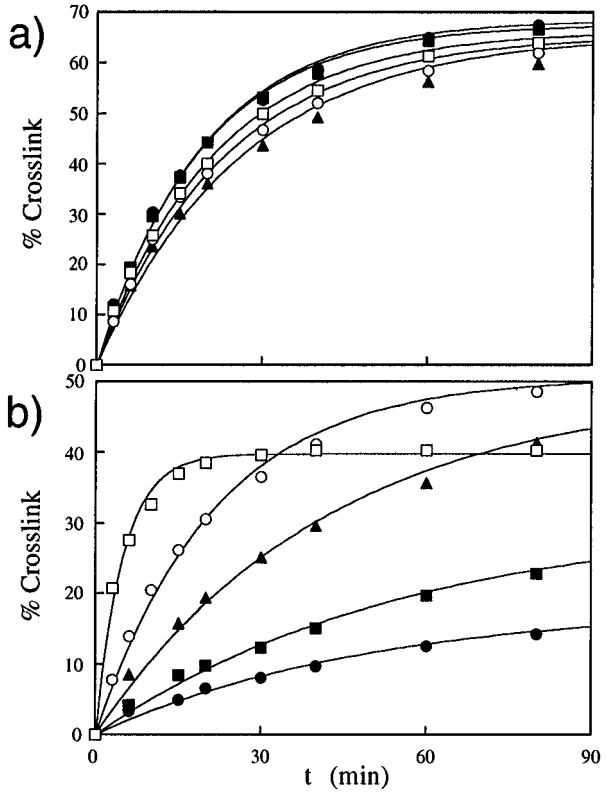

Figure 5. Cross-linking profiles obtained with substrate **B** under different ionic conditions. (a) Substrate **B** (≤1 nM), modified ribozyme (100 nM), $MgCl_2$ (10 mM), NaCl (1 M), Hepes buffer (100 mM, pH 7.2), 30 °C. (b) Substrate **B** (≤1 nM), modified ribozyme (100 nM), $MgCl_2$ (100 mM), Hepes buffer (100 mM, pH 7.2), 30 °C. U-297 (●), U-300 (■), U-307 (▲), C-311 (○), U-317 (□).

were observed to be insensitive to addition of saturating excess uncross-linked ribozyme (100 nM) or addition of saturating excess unlabeled substrate **A** (100 nM), supporting the intramolecular nature of the ribozyme-catalyzed cleavage reactions. The observation of cleavage activity by a variety of cross-linked substrate−ribozyme complexes, representing displacements between P1 and P3−P8 of as much as 50 Å, strongly supports the proposal that cross-linking (Figure 5b) proceeds through a closed and active substrate−ribozyme complex. While it cannot be concluded unequivocally that the free ribozyme engages in such long-range displacements during the actual cleavage event, it can be concluded that such displacements are compatible with ribozyme activity.

Conclusions

In this work, generalizable techniques have been developed for incorporation of thiol **1** and disulfide **2** into large RNAs. These groups react under mild conditions to form disulfide cross-links via thiol−disulfide interchange. When applied to a 310-nucleotide group I ribozyme, an unexpectedly large amount of motion between helical domains was revealed. The cross-linking data support the conclusion that motions between adjacent helical domains of at least 50 Å occur. The observed cleavage activity of the cross-linked substrate−ribozyme complexes suggests that these long-range motions are compatible with ribozyme catalysis.

The long-range motions within the ribozyme were observed under constant conditions and are interpreted as distinct from the specific conformational changes that can be induced by changes in experimental conditions ($[Na^+]$)[25] or by binding of substrate.[21b] Data such as Figure 5b suggest a continuum of conformations rather than discrete states of the ribozyme. Thus, the cross-linking data are interpreted as reflecting thermal displacements from a preferred ground-state structure. The unexpected characteristic of the ribozyme is that such extreme displacements from the ground-state are so readily accessed.

The long-range displacements observed in the current work were not anticipated on the basis of previous biochemical studies with the ribozyme. Photochemical cross-linking experiments performed with a substrate bound to a ribozyme equipped with an azidophenacyl moiety attached to the internal guide sequence afforded only one major site of cross-linking, localized in the ribozyme core.[21b] The photochemical cross-link likely reflects the predominant equilibrium state of the molecule, whereas the thiol−disulfide interchange can identify less frequent long-range motions. Even cross-linking to position U-317 (Figure 5b) may require motions not reflective of the predominant equilibrium state of the ribozyme; that this rate was observed to be much slower than cross-linking in helix **a** (Figure 1) suggests that some motion between P1 and P3−P8 is required.

A rigid ribozyme structure was proposed from activity assays performed with modified substrates, which found that substrate binding and cleavage were sensitive to tertiary interactions from numerous 2′-hydroxyl groups and that the ribozyme could discriminate between changes at the single functional group level, such as changing a 2′-hydroxyl to a 2′-amine.[16b,c] Under the ionic conditions used in these activity assays (10 mM Mg^{2+}), cross-linking to U-297 was slower than cross-linking to U-317 by a factor of only three (Table 3). The contrasting conclusions between these activity assays and the dynamics studied here can be reconciled by considering the proposal that not all tertiary interactions occur simultaneously in all conformations sampled by the ribozyme. Because the tertiary interactions were observed to be energetically independent—disruption of one interaction did not adversely affect another[16b,c]—it is possible that a dynamic ribozyme may form only a subset of all possible tertiary interactions at one time.

Given that different experimental approaches are sensitive to different scales and frequencies of thermal motions, ribozyme and protein dynamics will be compared on the basis of relative disulfide trapping rates within a given system.[26] In studies of the galactose/glucose binding protein of *E. coli*, the rates of cross-linking between adjacent α-helices decreased by approximately three orders of magnitude over a distance of 15 Å.[2] In contrast, we find that relative cross-linking rates in the

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(26) Because absolute rates of disulfide trapping depend not only on encounter frequency but also on chemical parameters such as leaving group stability, pH, and the presence of a catalyst, the analysis is restricted to relative rates within a given system.

Table 4. Cross-Linking as a Function of Substrate[a]

substrate	U-297	U-300	U-307	C-311	U-317
5′-CCCUC*d*UAAA**C**A-3′	1.3 ± 0.2	1.4 ± 0.3	2.0 ± 0.4	5.5 ± 1.5	20 ± 4
5′-**C**CCCUC*d*UAAA**C**A-3′	5.5 ± 1.0	5.2 ± 1.0	3.5 ± 0.5	3.8 ± 1.0	5.0 ± 1.0
5′-CCCUC*m*UAAA**C**A-3′	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	2.3 ± 0.5	4.2 ± 1.0

[a] Cross-linking rates expressed in units of (10^{-2} min^{-1}). All reactions performed in Hepes buffer (100 mM, pH 7.2) at 30 °C with $MgCl_2$ (100 mM). Each value is the mean ± range of two independent determinations.

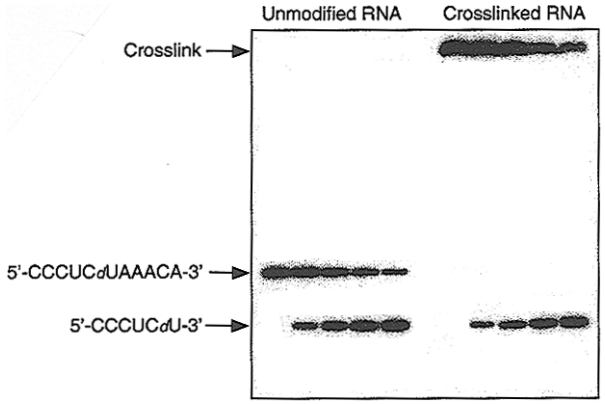

Figure 6. Products of oligonucleotide cleavage catalyzed by unmodified and cross-linked ribozymes. Unmodified RNA, reaction with 5′-^{32}P-CCCUC*d*UAAACA-3′ (≤1 nM), *Nhe I* ribozyme (100 nM), $MgCl_2$ (100 mM), GTP (1 mM), Hepes buffer (100 mM, pH 7.5), time course of 0, 5, 15, 30, 120 min at 30 °C. Cross-linked RNA, reaction of purified ^{32}P-cross-linked product (∼10 nM, prepared from reaction of 5′-^{32}P-substrate **B** with ribozyme U-317), $MgCl_2$ (100 mM), GTP (1 mM), Hepes buffer (100 mM, pH 7.5), time course of 0, 15, 60, 240, 720 min at 30 °C.

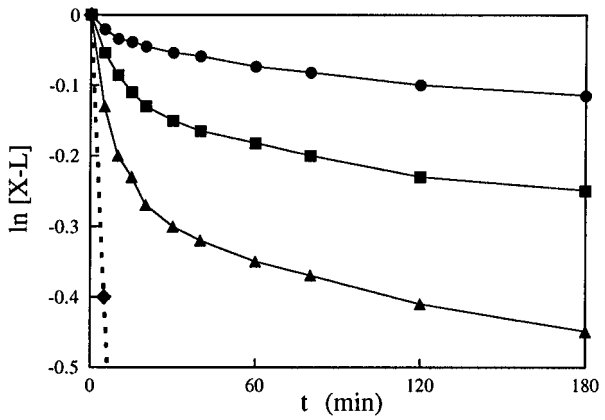

Figure 7. Rates of oligonucleotide cleavage catalyzed by unmodified and cross-linked ribozymes. Reactions performed under conditions described in Figure 6. U-297 (●), U-307 (■), U-317 (▲), unmodified RNA (♦).

ribozyme decrease only slowly with distance, a factor of ∼15 over a 50 Å range under the ionic conditions that provide greatest selectivity. Thus, it appears that large folded RNA molecules can exhibit flexibility much greater than that of proteins, although more examples of each type of macromolecule must be analyzed before a generalization can be established.

The frequencies of the large RNA motions within the ribozyme that give rise to the disulfide cross-links remain to be rigorously established. To obtain a rough approximation of the frequencies, the second-order rate constant for thiol−disulfide interchange can be used as a scaling factor for the first-order cross-linking rates observed in the ribozyme system. A rate constant on the order of 10^2 M^{-1} min^{-1} was measured for the reaction of **1** with **2** on separate noncomplementary 11-mer oligonucleotides (pH 7.2, 30 °C), implying disulfide bond formation occurs once per ∼10^9 encounters (∼10^{11} M^{-1} min^{-1} is taken as the diffusion controlled limit[27]). This simplified analysis suggests that the cross-linking halftimes measured in the ribozyme (Figure 5b) represent thermal motions on the microsecond time scale. This is much faster than the time scale for the chemical step of ribozyme-catalyzed cleavage (k_{chem} ∼ 1 s^{-1} at 30 °C).[28]

It is quite plausible that different kinds of motions within the ribozyme account for the large displacements observed. The contrasting cross-linking results obtained in the preliminary investigation with helices **a** and **b** (Figure 1) suggest that bending of paired regions does not contribute significantly to ribozyme flexibility. A more probable source of flexibility comes from the single-stranded regions, mismatches, and bulges that occur between the paired regions. Mills et al. showed that two or more single-stranded nucleotides placed between adjoining DNA helices induced significant flexibility relative to a continuous helix.[29] Translational and rotational motions between RNA domains are also likely to contribute to the cross-linking observed in the present study. Previous electron microscopic observations suggested significant interdomainal flexibility in the ribozyme, more so than in the single-domain tRNA.[30]

Undocking of P1 from the active site provides another potential source of displacement relative to the P3−P8 domain. Although results obtained with docking and nondocking substrates (**B** and **D**, respectively) demonstrated that cross-linking must be occurring at least in part through the closed substrate−ribozyme complex, a baseline level of cross-linking from occasional undocking of P1 cannot be ruled out. However, the possibility that these large displacements can indeed occur through the closed complex is supported by the cleavage activity of the cross-linked substrate−ribozyme complexes.

It will be of interest to determine if the large magnitude of thermal motions observed in the *Nhe I* ribozyme is a general feature of catalytic RNAs from the class of group I introns, or if other systems are significantly more rigid. The group I intron from the bacterium *Azoarcus* exhibits remarkable thermal stability due to a G-C rich secondary structure and two GAAA tetraloop interactions;[31] these structural features may contribute to a more rigid ribozyme structure. Some group I introns require binding of a protein splicing factor for activity;[32] the dynamics of the free RNA may differ greatly from that of the active RNA−protein complex. The chemistry developed here should be broadly applicable to studying the dynamics of RNA structures.

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Experimental Section

General Methods. 2′-Trifluoroacetylamino-5′-dimethoxytrityl-N4-benzoyl cytidine-3′-cyanoethyl phosphoramidite and 2′-trifluoroacetylamino-5′-dimethoxytrityl uridine-3′-cyanoethyl phosphoramidite were kindly provided by Dr. Wolfgang Pieken of Nexstar Pharmaceuticals (Boulder, CO). A-, C-, G-, and U-ribonucleotide phosphoramidites were obtained from Glen Research. *N*,*N*-Dimethylformamide, thiophenol, dithiothreitol (DTT), triethylamine, 1-methyl-2-pyrrolidinone, triethylamine trihydrofluoride, and spermidine trihydrochloride were from Aldrich. *N*-Succinimidyl-3-(2-pyridyldithio) propionate (**3**) was from Pierce. (γ-^{32}P)-ATP and (α-^{32}P)-cordycepin (cordycepin = 3′-deoxyadenosine triphosphate) were obtained from New England Nuclear at a specific activity of 6000 Ci/mmol. Unlabeled nucleoside triphosphates were from Pharmacia. A 40% aqueous solution of acrylamide:bisacrylamide (29:1) was obtained from Fisher. T4 polynucleotide kinase (10 units/μL), restriction endonuclease *Nhe I* (5 units/μL), and restriction endonuclease *Ear I* (15 units/μL) were from New England Biolabs. Yeast poly-(A)-polymerase (600 units/μL) was from United States Biochemical. Cloned T7 RNA polymerase and T4 DNA ligase were purified from *E. coli* and obtained at concentrations of ~50 and ~2500 units/μL, respectively. "TE" refers to an aqueous solution of tris-HCl buffer (10 mM, pH 7.5) and disodium EDTA (1 mM); "TEN" refers to an aqueous solution of tris-HCl buffer (10 mM, pH 7.5), disodium EDTA (1 mM), and sodium chloride (250 mM); "TBE" refers to a buffer composition of tris base (90 mM), boric acid (90 mM), and disodium EDTA (1 mM). Hepes buffer was from Boehringer Mannheim; aqueous Hepes buffers were prepared as the sodium salt. Analytical polyacrylamide gels were affixed on Whatman chromatography paper and quantitated with a Molecular Dynamics 445 SI Phosphorimager.

RNA Oligonucleotide Preparation. All synthetic RNA oligonucleotides were prepared on an Applied Biosystems 394 RNA synthesizer (1 μmol synthesis scale) using standard phosphoramidite chemistry; deprotection and purification were performed as follows. The solid support-bound product was suspended in concentrated ammonium hydroxide/ethanol solution (3/1 v/v, 1.5 mL) and incubated at 55 °C for 8 h in a sealed vial. After cooling to −20 °C, the supernatant was decanted and removed in vacuo. The residue was dissolved in a mixture of triethylamine/1-methyl-2-pyrrolidinone/triethylamine trihydrofluoride (24/46/30 v/v/v, 400 μL),[33] incubated at 65 °C for 1.5 h, and then transferred immediately to TE buffer (5 mL). The resulting solution was desalted over two NAP-25 sephadex columns (Pharmacia). To the aqueous product solution (7 mL) was added sodium chloride solution (1 M, 1 mL). The product was precipitated by addition of absolute ethanol (24 mL) followed by centrifugation (10 000g, 2 °C, 30 min). The RNA pellet dissolved in TE buffer (150 μL) and 80% aqueous formamide solution (150 μL) containing TBE, bromophenol blue (0.05%), and xylene cyanol (0.05%). The oligonucleotide was purified to single-nucleotide resolution by polyacrylamide gel electrophoresis (20% polyacrylamide and 8 M urea in TBE, gel dimensions $20_h \times 26_w \times 0.3$ cm, 25 W, three oligonucleotide preparations per gel). The product band was visualized by UV shadow and excised. The gel slice was crushed thoroughly and suspended in TEN buffer (10 mL) at 2 °C for 24 h. The supernatant was filtered (0.45 μm cellulose acetate) and the oligonucleotide precipitated by the addition of three volumes absolute ethanol followed by centrifugation (10 000g, 2 °C, 30 min). The purified RNA oligonucleotide was stored in TE buffer at −20 °C.

Preparation of Unmodified *Nhe I* Ribozyme. Transcription of the plasmid pT7L−21[34] was performed by dissolving the DNA template (200 μg, linearized with *Nhe I*) in a solution (10 mL) containing tris-HCl buffer (50 mM, pH 8.2), $MgCl_2$ (10 mM), spermidine trihydrochloride (10 mM), DTT (10 mM), A-, C-, G-, and U-nucleoside triphosphates (1 mM each), and Triton X-100 (0.05%) (20 μg/mL DNA template). Transcription was initiated at 37 °C by addition of T7 RNA polymerase (~50 units/μL, 500 μL). The reaction was incubated at 37 °C for 18 h, after which time the cloudy precipitate (magnesium pyrophosphate) was removed from suspension by centrifugation (2000g, 23 °C, 10 min). Disodium EDTA solution (500 mM, 0.5 mL) was added. The transcription product was precipitated by addition of sodium chloride solution (1 M, 1 mL) and absolute ethanol (20 mL), followed by centrifugation (10 000g, 2 °C, 30 min). The RNA pellet was dissolved in TE buffer (200 μL) and 80% aqueous formamide solution (800 μL) containing TBE, bromophenol blue (0.05%), and xylene cyanol (0.05%). The RNA was purified to ≥95% homogeneity by polyacrylamide gel electrophoresis (8% polyacrylamide and 8 M urea in TBE, gel dimensions $20_h \times 26_w \times 0.3$ cm, 25 W, ~6 h). The product band was visualized by UV shadow and excised. The gel slice was crushed thoroughly and suspended in TEN buffer (25 mL) at 2 °C for 24 h. The supernatant was filtered (0.45 μm cellulose acetate) and the ribozyme precipitated by addition of three volumes absolute ethanol followed by centrifugation (10 000g, 2 °C, 30 min). The purified *Nhe I* ribozyme (~10 nmol) was stored in TE buffer at −80 °C at a concentration of 5 μM ($\epsilon = 2.7 \times 10^6$ M^{-1} cm^{-1}).

Construction of Semisynthetic *Nhe I* Ribozymes. The semisynthetic ribozymes were constructed using a two-piece ligation between a 3′-hydroxyl of the transcribed RNA (nucleotides 22−290) and a 5′-monophosphate of a synthetic 41-mer RNA oligonucleotide (nucleotides 291−331) containing a unique 2′-amine (see Figure 2a). The ligations were mediated by a DNA oligonucleotide "splint" complementary to the 3′- and 5′- RNA termini and effected with T4 DNA ligase.[22] For the following procedures, "10× kinase/ligase buffer" refers to an aqueous solution containing tris-HCl buffer (500 mM, pH 8.2), $MgCl_2$ (100 mM), DTT (100 mM), ATP (10 mM), and Triton X-100 (0.5%). The transcribed RNA was prepared by transcription of a plasmid derived from pT7L−21,[34] modified with an *Ear I* restriction site for linearization of the plasmid after nucleotide 290. Transcription was performed exactly as just described for the unmodified *Nhe I* ribozyme. The transcribed product was obtained in ~90% purity and used in the subsequent ligation without gel purification ($\epsilon = 2.2 \times 10^6$ M^{-1} cm^{-1}). A 41-mer RNA oligonucleotide (10 nmol) was dissolved in water (900 μL) and 10× kinase buffer (100 μL), and the solution warmed to 37 °C. Phosphorylation of the 5-hydroxyl was initiated by addition of T4 polynucleotide kinase (10 units/μL, 20 μL), thus affording the following concentrations: RNA oligonucleotide, 10 μM; tris-HCl buffer, 50 mM; $MgCl_2$, 10 mM; DTT, 10 mM; ATP, 1 mM; Triton X-100, 0.05%; T4 polynucleotide kinase, 0.2 units/μL. The reaction was incubated at 37 °C for 30 min and was then quenched by addition of disodium EDTA solution (500 mM, 50 μL). To the resulting solution was added the transcribed RNA (12 nmol, 1.2 equiv) and a DNA oligonucleotide (10 nmol, 1.0 equiv) complementary to RNA nucleotides 251−310 (the long stretch of complementarity to the transcribed RNA serves to disrupt folding of the ribozyme core, thus enhancing ligation efficiency). The RNA/DNA mixture was precipitated by the addition of three volumes absolute ethanol followed by centrifugation (16 000g, 2 °C, 20 min). The RNA/DNA pellet was dissolved in TE buffer (400 μL), affording the following concentrations: transcribed RNA, 30 μM; synthetic RNA, 25 μM; DNA oligonucleotide, 25 μM; tris-HCl buffer, 10 mM; EDTA, 1 mM. The solution was heated at 90 °C for 3 min and then allowed to cool slowly over a period of 30 min to 30 °C. After the temperature had reached 30 °C, the solution was chilled on ice for 5 min. 10× ligase buffer (50 μL) was added, and the solution warmed to 37 °C. The ligation was initiated at 37 °C by addition of T4 DNA ligase (2500 units/μL, 50 μL), thus affording the following concentrations: transcribed RNA, 24 μM; synthetic RNA, 20 μM; DNA oligonucleotide, 20 μM; tris-HCl buffer, 60 mM; $MgCl_2$, 10 mM; DTT, 10 mM; ATP, 1 mM; Triton X-100, 0.05%; T4 DNA ligase, 250 units/μL. The reaction was incubated at 37 °C for 6 h and achieved a ligation efficiency of ~70%. The reaction was quenched by addition of disodium EDTA solution (500 mM, 25 μL). The crude mixture was precipitated by addition of sodium chloride solution (1 M, 50 μL) and absolute ethanol (1 mL), followed by centrifugation (16 000g, 2 °C, 15 min). To modify the 2′-amine, the RNA/DNA pellet was dissolved in water (120 μL), sodium borate buffer (1 M, pH 8, 40 μL), and sodium chloride solution (1 M, 20 μL). The solution was warmed to 37 °C and treated with a freshly prepared solution of **3** (500 mM in DMF, 20 μL, 37 °C, 15 min), thus affording the following concentrations: 2′-NH_2 RNA, 50 μM; **3**, 50 mM; sodium borate buffer, 200 mM; NaCl, 100 mM; DMF, 10%. The reaction was diluted with

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water (200 μL), and the product precipitated by addition of absolute ethanol (1 mL) followed by centrifugation (16 000g, 2 °C, 15 min). The amine modification procedure was repeated once more to afford ≥90% modification at all positions on the ribozyme. The RNA/DNA pellet was dissolved in TE buffer (50 μL) and 80% aqueous formamide solution (250 μL) containing TBE, bromophenol blue (0.05%) and xylene cyanol (0.05%). The modified ribozyme was purified to ≥95% homogeneity by polyacrylamide gel electrophoresis (8% polyacrylamide and 8 M urea in TBE, gel dimensions $20_h \times 26_w \times 0.3$ cm, 25 W, ∼8 h, three ribozyme preparations per gel). The ligated product band was visualized by UV shadow and excised. The gel slice was crushed thoroughly and suspended in TEN buffer (15 mL) at 2 °C for 24 h. The supernatant was filtered (0.45 μm cellulose acetate), and the ribozyme precipitated by the addition of three volumes absolute ethanol followed by centrifugation (10 000g, 2 °C, 30 min). The ribozyme was dissolved in water (140 μL) and sodium borate buffer (1 M, pH 8, 40 μL). The thiol was liberated by treatment with DTT (1 M, 20 μL, 37 °C, 30 min). The reaction was diluted with water (200 μL) and sodium chloride solution (1 M, 50 μL) and then precipitated by addition of absolute ethanol (1 mL) followed by centrifugation (16 000g, 2 °C, 20 min). The thiol-modified ribozyme (∼3 nmol) was stored in TE buffer at −80 °C at a concentration of 5 μM.

Preparation of Cross-Linking Substrates B, D, and Control. The RNA substrate (100 pmol) containing a unique 2′-amine was 5′-labeled with γ-^{32}P-ATP (0.5 mCi) and T4 polynucleotide kinase in a volume of 10 μL following standard procedures.[35] The reaction was diluted with water (200 μL) and sodium chloride solution (1 M, 50 μL). The RNA was precipitated by addition of absolute ethanol (900 μL) followed by centrifugation (16 000g, 2 °C, 20 min). The labeled product was dissolved in water (140 μL), sodium borate buffer (1 M, pH 8, 20 μL), and sodium chloride solution (1 M, 20 μL). The solution was warmed to 37 °C and treated with a freshly prepared solution of **3** (500 mM in DMF, 20 μL, 37 °C, 15 min), thus affording the following concentrations: RNA substrate, ∼0.5 μM; **3**, 50 mM; sodium borate buffer, 100 mM; NaCl, 100 mM; DMF, 10%. The solution was cooled to 23 °C and treated with thiophenol (70 mM in ethanol, 200 μL, 23 °C, 2 min). The modified RNA was precipitated by addition of absolute ethanol (700 μL) followed by centrifugation (16 000g, 2 °C, 20 min). The product was dissolved in TE buffer (10 μL) and 80% aqueous formamide solution (10 μL) containing TBE, bromophenol blue (0.05%), and xylene cyanol (0.05%). The RNA was purified to ≥95% homogeneity by polyacrylamide gel electrophoresis (20% polyacrylamide and 8 M urea in TBE, gel dimensions $10_w \times 20_h \times 0.05$ cm, 10 W, ∼3 h). This provided clean separation from the faster-migrating RNA that remained unmodified after treatment with **3** (∼10%). The product band was identified by autoradiography and excised. The gel slice was crushed thoroughly and suspended in TEN buffer (400 μL, 2 °C, 10 min). The product was precipitated by addition of absolute ethanol (2 mL) followed by centrifugation (16 000g, 2 °C, 20 min). The product was stored in aqueous sodium acetate buffer (10 mM, pH 4.5, 200 μL) at −80 °C in 20-μL aliquots.

Preparation of Cross-Linking Substrate C. The RNA substrate (100 pmol) containing a unique 2′-amine was 3′-labeled with α-^{32}P-cordycepin (0.5 mCi) and yeast poly-(A)-polymerase in a volume of 50 μL following standard procedures.[36] The reaction was diluted with water (200 μL) and sodium chloride solution (1 M, 50 μL). The RNA was precipitated by addition of absolute ethanol (1 mL) followed by centrifugation (16 000g, 2 °C, 20 min). From this point in the procedure, modification of the 2′-amine and substrate purification was performed as described for the other cross-linking substrates.

Ribozyme Activity Assays: $(k_{cat}/K_m)^S$ and $(k_{cat}/K_m)^G$. Reactions were conducted at 30 °C under single-turnover conditions ([ribozyme]/[substrate] ≥ 5). For $(k_{cat}/K_m)^S$, a 5-μL aliquot of ribozyme (50 nM in TE buffer) was mixed with water (45 μL), Hepes buffer (1 M, pH 7.2, 10 μL), $MgCl_2$ (100 mM, 10 μL), NaCl (1 M, 10 μL), and GTP (10 mM, 10 μL). The solution was incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. The cleavage reaction was initiated at 30 °C by addition of 5′-^{32}P-CCCUCUAAACA-3′ (∼10^5 cpm in TE buffer, 10 μL), thus affording the following concentrations: ribozyme, 2.5 nM; GTP, 1 mM; $MgCl_2$, 10 mM; NaCl, 100 mM; Hepes buffer, 100 mM; ^{32}P-substrate, ≤1 nM. At least eight 5-μL aliquots were removed over a 10 min time course and transferred to an 80% aqueous formamide solution (20 μL) containing TBE, disodium EDTA (50 mM), bromophenol blue (0.05%), and xylene cyanol (0.05%). Reaction products were separated by polyacrylamide gel electrophoresis (20% polyacrylamide and 8 M urea in TBE). For $(k_{cat}/K_m)^G$, a 10-μL aliquot of ribozyme (1 μM in TE buffer) was mixed with water (45 μL), Hepes buffer (1 M, pH 7.2, 10 μL), $MgCl_2$ (100 mM, 10 μL), NaCl (1 M, 10 μL), and GTP (10 μM, 5 μL). The solution was incubated at 50 °C for 10 min, and the reaction conducted at 30 °C as described for $(k_{cat}/K_m)^S$, thus affording the following concentrations: ribozyme, 100 nM; GTP, 0.5 μM; $MgCl_2$, 10 mM; NaCl, 100 mM; Hepes buffer, 100 mM; ^{32}P-substrate, ≤1 nM. Cleavage of the substrate proceeded to ≥90% efficiency. The five modified ribozymes were assayed in parallel with the two unmodified ribozymes. Data were fit to the eq $y = mx + b$, where y is ln([S]/([S] + [P])) and m is the observed first-order rate constant (k_{obs}). $(k_{cat}/K_m)^S$ was obtained from (k_{obs})/[ribozyme]; $(k_{cat}/K_m)^G$ was obtained from (k_{obs})/[GTP].

Equilibrium Dissociation Constants (K_d) for Cross-Linking Substrates. To solutions of Hepes buffer (100 mM, pH 7.2, 10 μL), $MgCl_2$ (100 mM, 5 μL), and glycerol (1.5 μL, with 0.1% xylene cyanol) were added varying amounts of unmodified *Nhe I* ribozyme, followed by water to attain a final volume of 40 μL. The solutions were incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. The equilibrations were initiated at 30 °C by addition of ^{32}P-substrate (∼10^4 cpm in TE buffer, 10 μL), thus affording the following concentrations: Hepes buffer, 20 mM; $MgCl_2$, 10 mM; glycerol, 3%; ^{32}P-substrate, ≤50 pM; ribozyme, 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.8, and 2.0 nM. The solutions were equilibrated at 30 °C for 1 and 2 h, after which time a 5-μL aliquot of each was electrophoresed at 30 °C over a native polyacrylamide gel [10% polyacrylamide in tris base (33 mM), Hepes (66 mM), $MgCl_2$ (10 mM), and disodium EDTA (0.1 mM), 10 W]. Analysis after 1 and 2 h afforded the same result, indicating that the systems had attained equilibrium within 1 h. The modified substrates were assayed in parallel with the unmodified substrate. Data were fit to the eq $\theta = [E]/([E] + K_d)$, where θ is the fraction substrate bound and [E] is the ribozyme concentration.

Kinetic Dissociation Constants (k_{off}) for Cross-Linking Substrates. A 10-μL aliquot of unmodified *Nhe I* ribozyme (1 μM in TE buffer) was mixed with water (62 μL), Hepes buffer (1 M, pH 7.2, 5 μL), $MgCl_2$ (100 mM, 10 μL), and glycerol (3 μL, with 0.1% xylene cyanol). The solution was incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. Trace ^{32}P-substrate (∼10^5 cpm in TE buffer, 10 μL) was added, and the solution incubated at 30 °C for 30 min, thus affording the following concentrations: ribozyme, 100 nM; Hepes buffer, 50 mM; $MgCl_2$, 10 mM; glycerol, 3%; ^{32}P-substrate, ≤1 nM. Excess unlabeled unmodified substrate (100 μM, 3 μL) was added at 30 °C. Immediately following addition of unlabeled substrate and at 15 min intervals thereafter (2 h), a 5-μL aliquot was electrophoresed at 30 °C over a native polyacrylamide gel [10% polyacrylamide in tris base (33 mM), Hepes (66 mM), $MgCl_2$ (10 mM), and disodium EDTA (0.1 mM), 10 W]. The modified substrates were assayed in parallel with a labeled unmodified substrate. Data were fit to the eq $y = mx + b$, where y is ln[E•S] and m is the observed first-order rate constant (k_{off}).

Kinetic Assay for Cross-Linking of Substrate−Ribozyme Complexes. For cross-linking under the conditions of Figure 4a, a 10-μL aliquot of thiol-modified ribozyme (1 μM in TE buffer) was mixed with water (20 μL), aqueous sodium acetate buffer (100 mM, pH 4.5, 20 μL), $MgCl_2$ (100 mM, 10 μL), and NaCl (5 M, 20 μL). The resulting solution was incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. The modified ^{32}P-substrate [∼10^5 cpm in sodium acetate buffer (10 mM, pH 4.5, 10 μL)] was added, and the solution incubated at 30 °C for 30 min. A 4-μL aliquot was removed, transferred to a 90% aqueous formamide solution (25 μL) containing sodium acetate buffer (100 mM,

(35) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(36) Lingner, J.; Keller, W. *Nucleic Acids Res.* **1993**, *21*, 2917−2920.

pH 4.5), disodium EDTA (50 mM), bromophenol blue (0.05%) and xylene cyanol (0.05%), and then stored frozen on dry ice until gel loading. Cross-linking was initiated at 30 °C by addition of Hepes buffer (1 M, pH 7.5, 10 μL), thus affording the following concentrations: ribozyme, 100 nM; $MgCl_2$, 10 mM; NaCl, 1 M; sodium acetate, 20 mM; Hepes buffer, 100 mM (final pH 7.2); ^{32}P-substrate, ≤1 nM. At least eight 5-μL aliquots were removed per reaction, transferred to a 90% aqueous formamide solution (25 μL) containing sodium acetate buffer (100 mM, pH 4.5), disodium EDTA (50 mM), bromophenol blue (0.05%), and xylene cyanol (0.05%), and then stored frozen on dry ice until gel loading. Reaction products were separated by polyacrylamide gel electrophoresis (8% polyacrylamide and 8 M urea in TBE). For cross-linking under the conditions of Figure 4b, a 10-μL aliquot of thiol-modified ribozyme (1 μM in TE buffer) was mixed with water (40 μL), aqueous sodium acetate buffer (100 mM, pH 4.5, 20 μL), and $MgCl_2$ (1 M, 10 μL). The resulting solution was incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. The modified ^{32}P-substrate [$\sim 10^5$ cpm in sodium acetate buffer (10 mM, pH 4.5, 10 μL)] was added, and the reaction conducted as just described, thus affording the following concentrations: ribozyme, 100 nM; $MgCl_2$, 100 mM; sodium acetate, 20 mM; Hepes buffer, 100 mM (final pH 7.2); ^{32}P-substrate, ≤1 nM. Typically, 3−5% cross-link was observed during the 30-min preincubation at pH 4.5; this small amount of cross-link was subtracted from cross-link occurring after addition of Hepes buffer. The resulting data were fit to the eq $y = 1 - [(1-m)e^{-k}]$ where y is the fraction cross-linked product, m is the final yield of cross-link at infinite time, and k is the first-order rate constant.

Preparation of Cross-Linked Substrate−Ribozyme Complexes. A 50-μL aliquot of modified ribozyme U-317, U-307, or U-297 (5 μM in TE buffer) was mixed with water (200 μL), Hepes buffer (1 M, pH 7.2, 50 μL), $MgCl_2$ (100 mM, 50 μL), and NaCl (5 M, 100 μL). The resulting solution was incubated at 50 °C for 10 min to obtain a homogeneously folded population of ribozyme and then incubated at 30 °C for 5 min. The cross-linking reaction was initiated at 30 °C by addition of modified ^{32}P-substrate **B** ($\sim 10^7$ cpm in TE buffer, 50 μL), thus affording the following concentrations: ribozyme, 500 nM; $MgCl_2$, 10 mM; NaCl, 1 M; Hepes buffer, 100 mM; ^{32}P-substrate, ≤100 nM. The reaction was incubated at 30 °C for 3 h and then quenched by addition of disodium EDTA (500 mM, 25 μL). The product was precipitated by addition of absolute ethanol (1 mL) followed by centrifugation (16 000g, 2 °C, 20 min). The product was dissolved in TE buffer (10 μL) and 80% aqueous formamide solution (10 μL) containing TBE, bromophenol blue (0.05%), and xylene cyanol (0.05%). The cross-linked RNA was purified to ≥95% homogeneity by polyacrylamide gel electrophoresis (8% polyacrylamide and 8 M urea in TBE, gel dimensions $10_w \times 20_h \times 0.05$ cm, 20 W, ∼1 h). The product band was identified by autoradiography and excised. The gel slice was crushed thoroughly and suspended in TEN buffer (400 μL, 2 °C, 10 min). The purified cross-link was stored in TEN buffer at −80 °C in 20-μL aliquots.

Activity of Cross-Linked Substrate−Ribozyme Complexes. To a solution of water (40 μL), sodium acetate buffer (100 mM, pH 4.5, 20 μL), and $MgCl_2$ (1 M, 10 μL) was added the ^{32}P-cross-linked complex ($\sim 10^5$ cpm in TEN buffer, 10 μL). The resulting solution was incubated at 50 °C for 10 min and then at 30 °C for 5 min. The cleavage reaction was initiated at 30 °C by simultaneous addition of GTP (10 mM, 10 μL) and Hepes buffer (1 M, pH 8, 10 μL), thus affording the following concentrations: ^{32}P-cross-linked complex, estimated 5−10 nM; $MgCl_2$, 100 mM; GTP, 1 mM; Hepes buffer, 100 mM (final pH 7.5); NaCl, 25 mM (from TEN buffer). At least ten 5-μL aliquots were removed over a 3 h time course and transferred to an 80% aqueous formamide solution (20 μL) containing TBE, disodium EDTA (50 mM), bromophenol blue (0.05%), and xylene cyanol (0.05%). Reaction products were separated by polyacrylamide gel electrophoresis (20% polyacrylamide and 8 M urea in TBE).

Acknowledgment. We would like to acknowledge Dr. Wolfgang Pieken of Nexstar Pharmaceuticals (Boulder, CO) for a generous supply of the 2′-amino-2′-deoxyribose pyrimidine phosphoramidites and for suggesting the use of thiol−disulfide interchange and Professor Fritz Eckstein (Max-Planck Institute, Göttingen) for sharing his results on disulfide cross-linking before publication. Thanks also to Anne Gooding for oligonucleotide synthesis. T.R.C. is an Investigator and S.B.C. is an Associate of the Howard Hughes Medical Institute.

JA9707421