3. Add 200 μl of 2% lithium perchlorate in acetone. Cap the tube, vortex, and centrifuge at 14,000 rpm for 15 min at 4°.
4. Remove the liquid carefully, wash the precipitate with acetone (200 μl), and continue as described earlier for imidazole ladders.

STOCK SOLUTIONS. For Phen-Im, dissolve 5.1 mg of the compound in 1 ml water to produce a 10 m*M* stock solution. Store at ambient temperature in the dark. The 5× stock of Bis–Tris buffer is 50 m*M* Bis–Tris–propane–HCl, pH 7.0, 200 m*M* NaCl, 50 m*M* $MgCl_2$, and 1 m*M* EDTA.

Particular Case of $tRNA^{Asp}$ Cleavage with Sp-Im

1. Mix 1 μl of labeled tRNA solution, 1 μl of the carrier RNA solution (1 mg/ml), 5 μl water, and 2 μl of stock Im buffer. Incubate for 20 min at 20°.
2. Add 1 μl of 25 m*M* Sp-Im solution. Incubate for 7 hr at 37°.
3. Add 200 μl of 2% lithium perchlorate in acetone. Cap the tube, vortex, and centrifuge at 14,000 rpm for 15 min at 4°.
4. Remove the liquid carefully, wash the precipitate with acetone (200 μl), and continue as described earlier for imidazole ladders.

SP-IM SOLUTION. Dissolve 3.4 mg of Sp-Im in 0.4 ml water to produce a 25 m*M* stock solution. Store at ambient temperature in the dark.

Analysis of Cleaved RNA and Quantitation of Data

RNA samples are dissolved in 7 *M* urea containing 0.02% bromphenol blue and 0.02% xylene cyanol and are subjected to electrophoresis through a denaturing polyacrylamide gel (12% acrylamide, 7 *M* urea, 30 × 40 × 0.04 cm^3). The gel contains 8 *M* urea in 1× TBE (100 m*M* Tris, 100 m*M* borate, and 2.8 m*M* EDTA). Gels are electrophoresed in 1× TBE running buffer. For calibration of cleavage patterns, end-labeled RNA is cleaved statistically at G residues by digestion with RNase T1 or cleaved by imidazole at 90°, as described earlier. Gels must be run at a temperature of 45–50° to keep RNA denatured. After separation, the gels are transferred onto Whatman (Clifton, NJ) 3MM filter paper, covered with Saran wrap, and dried. Dried gels are exposed to X-ray film (Kodak X-Omat, Rochester, NY) either at room temperature or at −70° with intensifying screens.

Cleavage patterns are quantitated using a Bio-Imaging Analyzer, e.g., the FUJIX BioImaging Analyzer BAS 2000 system. Photostimulatable imaging plates (type BAS-III from Fuji Photo Film Co., Ltd., Japan) are pressed on gels and exposed at room temperature for 30 min. Imaging plates are analyzed by performing volume integrations of specific cleavage sites and reference blocks using the FUJIX BAS 2000 workstation software (version 1.1).

Acknowledgments

We thank J.-P. Behr and G. Zuber who designed and prepared the Sp-Im conjugate, M. Podyminogin who synthesized and first tested the Phen-Im conjugate, and N. Zjdan and D. Konevetz who prepared the Lys-Im and D-Im conjugates. We are indebted to A. Vlassov, I. Kuznetsova, M. Helm, and N. Beloglazova who did the original work with $tRNA^{Asp}$, $tRNA^{Phe}$, and the minihelices; we also thank C. Florentz for helpful discussions and constant interest and A. Théobald-Dietrich for help in tRNA purification. This work was supported by INTAS Grant 96-1418, RFBR Grants 95-03-323691 and 96-15-97732, CNRS, and University Louis Pasteur, Strasbourg.

[12] Site-Specific Sulfhydryl Groups for Study of RNA Conformation via Disulfide Cross-Linking

By SNORRI TH. SIGURDSSON

The hammerhead ribozyme is an RNA motif, which catalyzes the site-specific cleavage of RNA and is utilized by a number of small pathogenic plant viroid and satellite RNAs for processing during rolling cycle replication.[1] The hammerhead consists of three helices (Fig. 1, see color plate), connected by single-stranded regions. The nucleotide sequence in the helical regions has minimal effect on the catalytic activity as long as base pairing is maintained. Substitutions, however, of nucleotides in the single-stranded regions of the catalytic core reduce activity dramatically. Advances in the chemical synthesis of oligoribonucleotides have enabled the incorporation of modified nucleotides to investigate the role of specific functional groups and to probe metal ion-binding sites. However, it has been difficult to correlate functional group data with specific catalytic and structural roles in the absence of a three-dimensional structure of the ribozyme.

Two models of the eagerly awaited tertiary structure of the hammerhead ribozyme were published late in 1994. One model, based on single crystal X-ray crystallography, displayed a wishbone shape with helices II and III almost collinear with helix I and II proximal (Fig. 1A, see color plate).[2] The other model was based on fluorescence resonance energy transfer (FRET) measurements in solution, which yielded the relative orientation of the three helices, allowing the central core to be modeled (Fig. 1B, see color plate).[3] Comparison of the two models revealed striking similarities

[1] R. H. Symons, *Annu. Rev. Biochem.* **61,** 641 (1992).
[2] H. W. Pley, K. M. Flaherty, and D. B. McKay, *Nature (London)* **372,** 68 (1994).
[3] T. Tuschl, C. Gohlke, T. M. Jovin, E. Westhof, and F. Eckstein, *Science* **266,** 785 (1994).

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regarding the placement of helices in three-dimensional space. However, the relative orientations of helices I and II in the two models were different. At the same point of reference, the minor grooves of helices I and II face each other in the X-ray structure, whereas the major grooves face each other in the FRET model. Thus, helices I and II need to be rotated simultaneously about 180° around their helical axes to convert one structure to the other.

Which one of these two models represented the global shape of the transition state of the hammerhead-catalyzed reaction? Many would argue in favor of the X-ray structure, given the fact that X-ray crystallography is a well-established technique for determining three-dimensional structures of biomolecules, such as proteins. However, biochemical data indicated that the X-ray structure represented an inactive ground state structure.[4] For example, the required 2′-hydroxyl group at the cleavage site was not positioned for an in-line attack on the scissile phosphorus and no metal ion was found close to the cleavage site. In addition, functional group modification experiments have shown that the exocyclic amino group of G5 is essential for activity. This group, however, was exposed to solvent in the X-ray structure. Thus, the X-ray structure was a ground state structure, requiring a conformational change to reach the transition state. Was the required conformational change local or global? Was the FRET model a better representation of the global shape of the active structure?

Strategy

To determine which one of these structural models represented catalytically active species better, covalent cross-links were used to "lock" each of two ribozymes in the conformation displayed in the two models. Due to the different orientations of helices I and II in the two models, pairs of nucleotides were identified for cross-linking that were proximal in one model and distal in the other. For example, nucleotides L2.4 and 2.6 are separated by about 11 Å in the X-ray structure and by about 32 Å in the FRET model (Fig. 1, see color plate). The catalytic activity of the cross-linked ribozymes would serve as an indicator of cross-link-induced structural perturbations of the active structure. Thus, if the cross-linked ribozyme was as active as a non-cross-linked ribozyme, the model in which the cross-linked nucleotides were proximal would be favored.

Disulfide cross-linking between cysteine residues has been used to study global conformations of proteins.[5] An advantage of using disulfide cross-

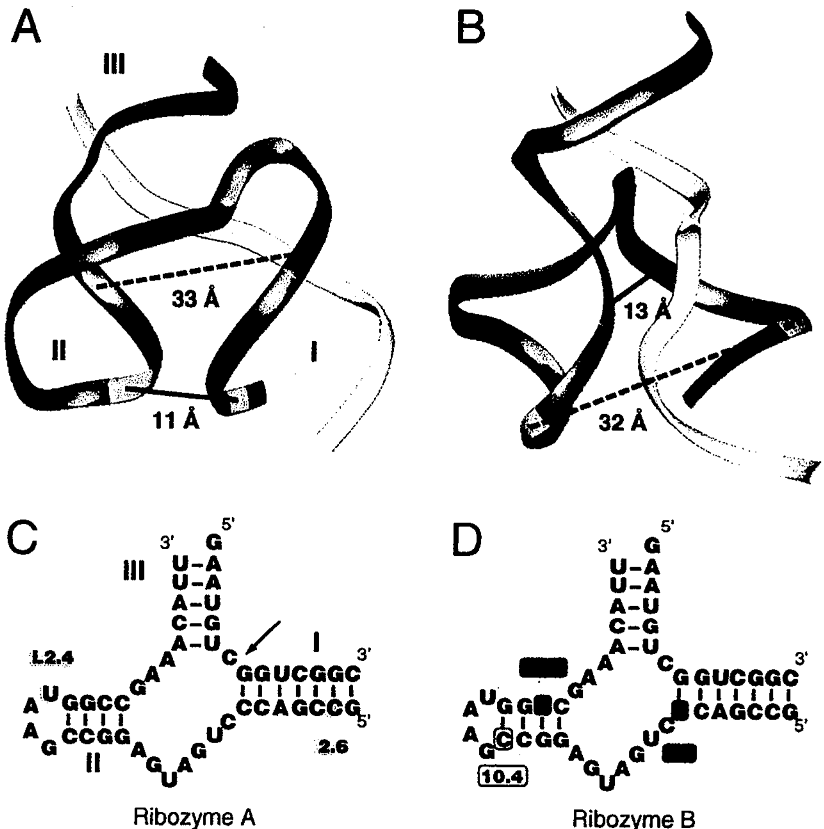

FIG. 1. Ribbon representation of the tertiary structures of the hammerhead ribozyme based on X-ray crystallography (A) and FRET solution measurements (B). The ribozymes and substrates are colored blue and yellow, respectively. Nucleotides that are close in space according to the X-ray structure and the FRET model (green and red, respectively) are connected by solid lines, whereas broken lines represent the corresponding long distances in the other model. The distances between the 2′-hydroxyl groups of connected residues are given in angstroms (Å). Roman numerals indicate the number of the helices. (C and D) RNA constructs chemically synthesized for interhelical cross-linking experiments. The colored residues contain a 2′-amino functionality. An arrow indicates the site of cleavage. Residue 10.4, also utilized for cross-linking to test the FRET model (see text), is boxed. Adapted from S. Th. Sigurdsson, T. Tuschl, and F. Eckstein, *RNA* **1,** 575 (1995), with permission from Cambridge University Press.

[4] D. M. McKay, *RNA* **2,** 395 (1996).
[5] J. J. Falke and D. E. Koshland, Jr., *Science* **237,** 1596 (1987).

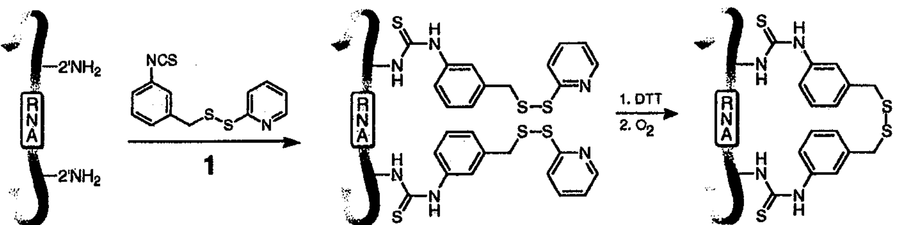

FIG. 2. Formation of an intramolecular disulfide cross-link by chemical modification of a 2′-amino-containing ribozyme. Adapted from S. Th. Sigurdsson *et al., RNA* **1,** 575 (1995), with permission from Cambridge University Press.

links is that they form in high yield between specific functional groups (thiols) and that the cross-linking can be reversed with the addition of thiols. Therefore, a method was sought that enabled the site-specific incorporation of thiols into the hammerhead ribozyme. Thiols can, in principle, be linked to either RNA nucleotide bases[6,7] or the sugar–phosphate backbone. However, to minimize the disruption of base pairing, cross-linking through the sugar–phosphate backbone was preferred. Furthermore, distances between the backbones in helices I and II were shorter than those between bases and allowed the use of a short tether.

The ability to site specifically incorporate 2′-amino nucleotides into RNA and selectively modify the 2′-amino groups with aromatic isothiocyanates was utilized for the incorporation of thiols into the hammerhead ribozyme.[8] Because an aromatic isothiocyanate containing a protected thiol functionality was not available commercially, compound **1** was prepared. Reaction of **1** with the 2′-amino groups in RNA yielded 2′-thiourea-substituted RNA (Fig. 2). Subsequent deprotection and oxidation of the thiols resulted in the formation of disulfide cross-links. It should be noted that although this article only describes the use of aromatic isothiocyanate **1**, reactions of 2′-amino groups with an aliphatic isocyanate containing the same protected thiol attached to a more flexible and less sterically hindered aliphatic tether have also been described.[9–11]

[6] C. R. Allerson and G. L. Verdine, *Chem. Biol.* **2,** 667 (1995).
[7] C. R. Allerson, S. L. Chen, and G. L. Verdine, *J. Am. Chem. Soc.* **119,** 7423 (1997).
[8] S. Th. Sigurdsson, T. Tuschl, and F. Eckstein, *RNA* **1,** 575 (1995).
[9] S. Th. Sigurdsson and F. Eckstein, *Nucleic Acids Res.* **24,** 3129 (1996).
[10] D. J. Earnshaw, B. Masquida, S. Mueller, S. Th. Sigurdsson, F. E. Eckstein, E. Westhof, and M. J. Gait, *J. Mol. Biol.* **274,** 197 (1997).
[11] S. Th. Sigurdsson, METHODS: A Companion to Methods in Enzymology **18,** 71 (1999).

Procedure

Preparation of Ribozymes and Substrates

Oligoribonucleotides are prepared by automated chemical synthesis on an Applied Biosystems (Foster City, CA) 380B DNA synthesizer on a 1-μmol scale using phosphoramidites from MilliGen/Biosearch with the exception of phosphoramidites used for the incorporation of 2′-amino nucleotides. These are now available commercially from Glen Research. The oligoribonucleotides are deprotected by incubation in 3 ml of concentrated aqueous ammonia (32%)/ethanol (3:1) for 16 hr at 55° in a screw-top vial. The solvent is removed completely on a Speed-Vac evaporator, and the residue is treated with 0.5 ml of 1.0 *M* tetrabutylammonium fluoride in tetrahydrofuran (THF) (Aldrich, Milwaukee, WI) at 25° for 16 hr to remove silyl-protecting groups. Next, 0.5 ml of 1.8 *M* sodium acetate (pH 5.8) is added and the solution is concentrated to a volume of about 0.5 ml and extracted twice with 0.8 ml of ethyl acetate. After precipitation of the RNA by the addition of 1.6 ml of cold absolute ethanol, the mixture is centrifuged and the supernatant discarded. The pellet is dissolved in water and the RNA is purified by 20% denaturing polyacrylamide gel electrophoresis (DPAGE). RNA is detected in the gel by UV shadowing, eluted from the excised gel slices by electroelution in Tris–borate–EDTA buffer, and desalted with a Sep-Pak C_{18} cartridge. The cartridges are washed sequentially with acetonitrile (10 ml) and 100 m*M* triethylammonium bicarbonate, pH 7.5 (10 ml). The sample is loaded, washed with 4% acetonitrile in 100 m*M* triethylammonium bicarbonate (20 ml), and eluted with acetonitrile/methanol/water (35:35:30, v/v). Next, the solvent is removed in a Speed-Vac, and methanol is added (0.2 ml) and evaporated to remove traces of triethylamine. Finally, the RNA is dissolved in water (0.2 ml) and the concentration is determined using the molar extinction coefficient of 6600 M^{-1} cm^{-1} per nucleotide.

Chemical Modification and Cross-Linking of 2′-Amino Ribozymes A and B

1. The ribozymes are radiolabeled at either the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP or the 3′ end using T4 RNA ligase and 5′-^{32}P-labeled cytidine 3′,5′-diphosphate. The buffers are supplied by the manufacturer (Amersham, Piscataway, NJ). After a 15-min reaction time for the 5′-labeling reaction, nonradiolabeled ATP (final concentration of 1.6 m*M*) is added to "chase" the reaction to completion. Most of the experiments are carried out with the 5′-labeled ribozyme. Analysis of the cross-linked ribozymes by limited alkaline hydrolysis utilizes both 3′- and 5′-labeled ribozymes.
2. Isothiocyanate **1** is reacted with radiolabeled ribozyme for 28 hr at 37° [50 m*M* **1**; 1 m*M* ribozyme; 50 m*M* borate buffer, pH 8.6; 50% dimethylformamide (DMF); final volume 10 μl].
3. The reaction mixture is diluted and the noncleavable substrate 5′-GAAUGUdCGGUCGGC is added (10 μM ribozyme; 20 μM substrate; 50 m*M* sodium cacodylate, pH 7.5; 50 m*M* NaCl; final volume 1270 μl). The substrate is annealed to the ribozyme by heating the solution to 90° for 3 min followed by slow cooling to 25° over a period of 2.5 hr.
4. Dithiothreitol (DTT) (1 *M* aqueous solution, 127 μl) is added to the solution containing the ribozyme–substrate complex and incubated for 2 hr at 25° to remove thiol-protecting groups.
5. The oligomers are precipitated at −20° after the sequential addition of sodium acetate (3.0 *M* aqueous solution, pH 5.2; 1140 μl) and ethanol (9120 μl). The pellet is collected by centrifugation and redissolved (10 μM ribozyme; 50 m*M* sodium cacodylate, pH 7.5; 50 m*M* NaCl; 20 m*M* $MgCl_2$; final volume 1710 μl).
6. For cross-linking, the solution is divided into six microfuge tubes and an equal volume of DMSO is added to each tube. The solutions are incubated for 30 hr at 25° under an atmosphere of oxygen and the RNA is precipitated as described earlier. It should be noted that the cross-linking conditions are not optimized; DMSO is used for cross-linking because it has been shown to increase the rate of cross-linking but gives only slightly better results than those obtained without DMSO.

Analysis of Cross-Linking Procedure and Purification of Cross-Linked Ribozymes

The chemical modification of the 5′-radiolabeled, 2′-amino containing ribozymes was monitored by 20% DPAGE (Fig. 3). As can be seen, reactions with isothiocyanate **1** were close to quantitative (Fig. 3, lanes 2), and a major product with different electrophoretic mobility was observed after cross-linking (Fig. 3, lanes 3): The minor products that had strongly retarded electrophoretic mobility were presumably intermolecular cross-links.

It is noteworthy that the electrophoretic mobilities of the putative cross-linked ribozymes were very different, despite the fact that they had the same charge and molecular weight. Cross-linked ribozyme A had electrophoretic mobility similar to that of the non-cross-linked sample. This was of concern because even a minor contamination of non-cross-linked ribozyme could

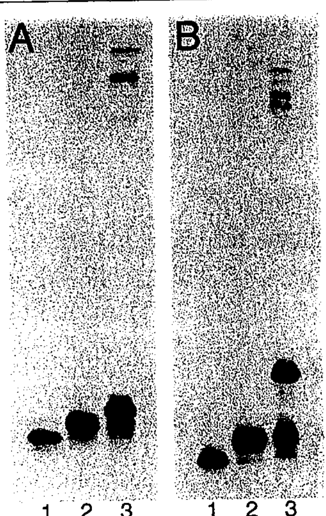

FIG. 3. Analysis of the cross-linking reactions of ribozymes A (**A**) and B (**B**) by 20% DPAGE. Lanes 1, 2′-amino-modified ribozymes; lanes 2, reaction mixtures after treatment with DTT; and lanes 3, crude cross-linking reaction mixtures. Reprinted from S. Th. Sigurdsson *et al., RNA* **1,** 575 (1995), with permission from Cambridge University Press.

affect the measurement of the catalytic activity of the cross-linked ribozyme. This would in turn interfere with the assessment of structural perturbations caused by the cross-link.

It was found that the relative mobility of cross-linked to non-cross-linked material varied greatly with the percentage of acrylamide in the denaturing gels. In a systematic study, ribozymes A and B, as well as the corresponding non-cross-linked ribozymes (obtained by the reduction of the cross-linked samples with dithiothreitol), were subjected to DPAGE containing 8, 12, 16, 20, and 24% acrylamide (0.04 × 20 × 40 cm gel; acrylamide : bisacrylamide, 19:1).[12] The distance that the cross-linked and non-cross-linked material migrated in the gels was subsequently measured. To facilitate a comparison of results from all the gel analyses, the mobilities of ribozyme A and B were normalized, assuming that the non-cross-linked samples had migrated 30 cm in all the gels. The results are presented in the simulated gel shown in Fig. 4, which reveals a dramatic difference in the relative mobility of the cross-linked to the non-cross-linked material. This phenomenon has also been observed for "lariat" intermediates in

[12] S. Th. Sigurdsson and F. Eckstein, *Anal. Biochem.* **235,** 241 (1996).

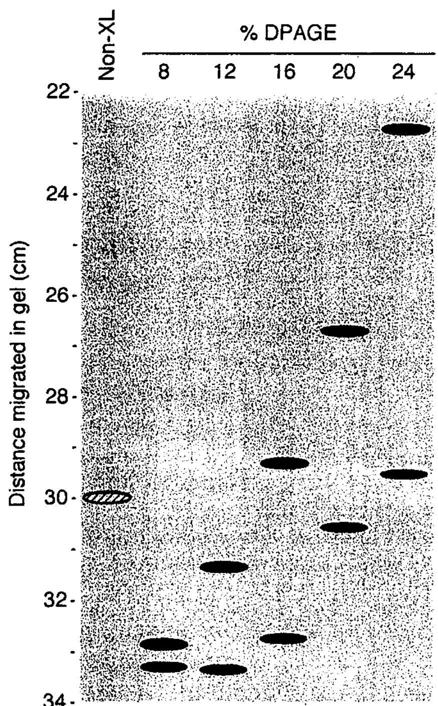

FIG. 4. A normalized representation of the distances migrated by cross-linked ribozymes during DPAGE as a function of percentage acrylamide, assuming that the non-cross-linked (non-XL) material migrated 30 cm under all conditions. The gray and black bands represent ribozymes A and B, respectively.

messenger RNA splicing.[13] It is also striking that in low percentage gels, the cross-linked ribozymes run faster than non-cross-linked ribozymes, whereas the opposite is true in high percentage gels. These results underscore the fact that careful choice of percentage acrylamide in DPAGE gels is important for the optimal separation of RNA containing intramolecular cross-links from non-cross-linked material. Furthermore, these results indicate that DPAGE is a general method for analysis and isolation of nucleic acids containing intramolecular cross-links.

Thus, the major products of the cross-linking reactions of ribozymes A and B were purified by 12 and 20% DPAGE, respectively. The cross-linked material was extracted from the gel slices by a crush and soak procedure

[13] P. J. Grabowski, R. A. Padgett, and P. A. Sharp, *Cell* **37,** 415 (1984).

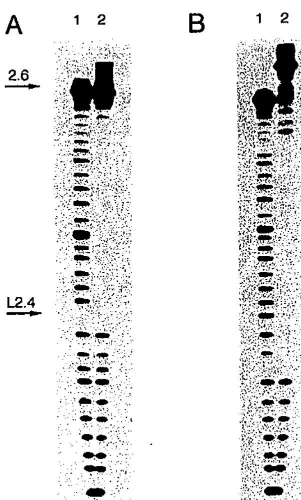

FIG. 5. Limited alkaline hydrolysis of cross-linked ribozymes A (**A**) and B (**B**). Lanes 1, ribozymes A and B, and lanes 2, cross-linked ribozymes A and B. Arrows indicate positions of the 2′-amino-modified residues in ribozymes A and B, where no cleavage occurs. Reprinted from S. Th. Sigurdsson *et al., RNA* **1,** 575 (1995), with permission from Cambridge University Press.

using sodium acetate (1.0 *M* aqueous solution, pH 5.2) from which the products were subsequently precipitated by the addition of ethanol. This resulted in an approximately 15% overall yield of cross-linked material, based on the starting 2′-amino oligomers.

Analysis of Cross-Linked Ribozymes

Limited Alkaline Hydrolysis. To verify that the isolated products did indeed contain an intramolecular cross-link and to verify the location of the cross-link, the putative cross-linked ribozymes were subjected to limited alkaline hydrolysis. 3′-^{32}P-labeled ribozymes A and B, and the corresponding cross-linked ribozymes, were subjected to limited alkaline hydrolysis (50 m*M* $NaHCO_3$; final volume 20 μl; 100°; 5 min), followed by analysis by 20% DPAGE (Fig. 5). This procedure was also performed with 5′-radiolabeled ribozymes (data not shown). As expected for ribozymes containing intramolecular cross-links, there was a virtual footprint in the ladder of bands. This was due to the cleavage between cross-linked nucleotides yielding products that have the same molecular weight and charge as the uncleaved oligomer and thus have similar electrophoretic mobility.

Procedure for Obtaining Kinetic Parameters

1. Non-cross-linked ribozymes are obtained by reduction of the cross-linked ribozymes with DTT (5 m*M*; 50 m*M* Tris–HCl, pH 7.5) for 4 hr at 25° prior to the heat shock protocol (step 2).
2. Solutions of ribozyme and substrate RNA (50 m*M* Tris–HCl, pH 7.5) are preheated separately at 90° for 1 min and cooled to 25° over a 15-min period.
3. $MgCl_2$ is added (final concentration, 10 m*M*; 50 m*M* Tris–HCl, pH 7.5), and the solutions are incubated for 15 min at 25°.
4. The solutions of ribozymes and substrates are combined in such a way that a trace amount of 5′-^{32}P-labeled substrate ($<$1 n*M*) is incubated in the presence of the ribozymes at varying concentrations (20–300 n*M*) (50 m*M* Tris–HCl, pH 7.5; final volume 50 μl; 10 m*M* $MgCl_2$) at 25°.
5. Aliquots (8 μl) are withdrawn at appropriate time intervals and added to 16 μl of urea stop mix (3.5 *M* urea, 25 m*M* EDTA, 0.02% bromphenol blue, 0.02% xylene cyanol) and subsequently subjected to 20% DPAGE.
6. The rates of cleavage, at different concentrations of ribozymes (20–300 n*M*), are obtained by plotting the remaining fraction of uncleaved substrate to a single exponential decay as a function of time. The rates reflect time points where less than 30% of the substrate is cleaved because a fraction of the substrate remains uncleaved, even after incubation for a few hours. However, it has been pointed out that the cleavage rate can be determined more accurately by accounting for the uncleaved fraction during curve fitting.[14] The k'_{cat} and K'_m parameters are determined from Eadie–Hofstee plots.

Analysis of Kinetic Data

Activities of the cross-linked ribozymes were determined under single turnover conditions (Table I) to monitor the rates of chemical cleavage rather than product release. Non-cross-linked ribozymes yielded activities similar to that of the original 2′-amino-modified ribozymes, demonstrating that the structures were not perturbed by the chemical modifications. However, the activity of the two cross-linked ribozymes differed by about 300-fold: ribozyme A had a cleavage efficiency similar to that of non-cross-linked species, whereas the activity of ribozyme B was reduced dramatically. In fact, the activity of cross-linked ribozyme B was accounted for by a contamination of less than 1% of the non-cross-linked material.

[14] T. K. Stage-Zimmermann and O. C. Uhlenbeck, *RNA* **4,** 875 (1998).

TABLE I
KINETIC PARAMETERS FOR CROSS-LINKED AND NON-CROSS-LINKED RIBOZYMES A AND B[a]

Ribozyme (Rz)	k'_{cat} (min^{-1})	K'_m (nM)	k_{cat}'/K_m' (μM^{-1} min^{-1})
Rz A	0.72	19	37
Rz A XL	0.60	35	17
Rz A non-XL	0.58	25	23
Rz B	0.49	20	24
Rz B XL	0.002[b]	nd	nd
Rz B non-XL	0.67	46	15

[a] XL, cross-linked; nd, not determined.
[b] Observed rate constant at 200 nM ribozyme.

It might be argued that cross-linked ribozyme B was inactive because of reasons other than distance constraints. For example, there is a possible steric interaction between the cross-linker and nucleotides in the catalytic core. Therefore, a ribozyme containing a cross-link between nucleotides 2.1 and 10.4 was prepared and characterized kinetically (Fig. 1, see color plate). The 2′-amino groups on those nucleotides are separated by 13 Å in the FRET model and would place the cross-link in a different position than in ribozyme B. The results are very similar to those obtained for cross-linked ribozyme 2; the observed rate constant was 0.005 min^{-1} at 500 nM ribozyme concentration and the catalytic efficiency for the non-cross-linked species was 30 μM^{-1} min^{-1}. The combined results from the kinetic measurements strongly support the X-ray structure as a good representative of the global shape of the transition state structure of the hammerhead ribozyme.

Concluding Remarks

The first step toward understanding the fascinating function of RNAs, such as ribozymes, is to obtain a three-dimensional structure. X-ray crystallography of RNA molecules is still in its early stages, with only a handful of structures having been obtained of complex RNAs. Thus, other physical, biophysical, biochemical, and chemical approaches are being used extensively for obtaining structural information. The cross-linking approach described in this article is one such useful tool to study RNA tertiary structure and has been applied to other ribozymes. For example, it has been used to obtain distance constraints for the generation of a three-dimensional model of the hairpin ribozyme,[10] and an extension of this method has allowed the assessment of the dynamics of helical elements within the *Tetrahymena* group I ribozyme by following the kinetics of cross-linking.[15]

This cross-linking method is advantageous for testing a three-dimensional model: nucleotides that are close spatially can be cross-linked and the activity of the cross-linked species yields structural information. This method will be valuable in conjunction with other techniques, such as FRET, which can be used to generate structural models to be tested by cross-linking experiments. However, this approach becomes increasingly more difficult to apply to three-dimensional structure determination as the complexity of the RNA increases or where there is limited structural data available from other techniques. In addition, the flexibility of some ribozymes may make the interpretation of such cross-linking experiments difficult. For example, the *Tetrahymena* group I ribozyme containing cross-links between nucleotides that were separated by about 50 Å in the current three-dimensional model still retained some catalytic activity.[15] However, this method will be valuable for the study of RNA structure as long as these potential limitations are kept in mind.

Acknowledgments

I am grateful to Professor F. Eckstein, in whose laboratory this work was carried out. I thank Dr. T. Tuschl and members of my research group for critical reading of the manuscript.

[15] S. B. Cohen and T. R. Cech, *J. Am. Chem. Soc.* **119,** 6259 (1997).

[13] Directed Hydroxyl Radical Probing Using Iron(II) Tethered to RNA

By SIMPSON JOSEPH and HARRY F. NOLLER

Introduction

Directed hydroxyl radical probing using tethered Fe(II)-EDTA is a powerful tool for studying the tertiary structure of RNA at low resolution.[1,2] In the presence of reducing agents, Fe(II)-EDTA generates neutral, reac-

[1] J. F. Wang and T. R. Cech, *Science* **256,** 526 (1992).
[2] H. Han and P. B. Dervan, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4955 (1994).

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