

Thiol-Containing RNA for the Study of Structure and Function of Ribozymes

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RNA performs multiple functions in cellular environments, such as transferring genetic information, catalyzing chemical reactions, and providing an integral component of ribonucleo-protein complexes involved in mRNA processing and translation. Many of these functions are poorly understood, mainly due to the lack of structural information. Because limited information has been obtained by physical and biophysical techniques, chemical and biochemical methods have been extensively used for studying RNA structure. This article outlines one such method which relies on site-specific incorporation of thiols into RNA. A brief overview of the methods for incorporation of thiols into RNA is followed by a detailed description of a procedure which utilizes postsynthetic modification of 2'-amino-containing RNA for incorporation of thiols. The use of thiol-containing RNA to form disulfide cross-links for the study of the structure and dynamics of ribozymes is subsequently described. © 1999 Academic Press

The conjugation of reporter groups and reactive groups to oligonucleotides represents a useful approach for studying the structure and function of nucleic acids (for reviews see 1–3). The simplest approach for derivatizing oligonucleotides is to attach these groups at either the 3'- or the 5'-terminus, for which a variety of methods are available (3). However, it is often desirable to introduce probes at internal positions in nucleic acids for investigating their tertiary structure or to probe interactions with other macromolecules, such as proteins.

Conjugation of chemical probes to internal positions of nucleic acids can be to either the nucleotide bases or the sugar-phosphate backbone. There are

numerous examples of conjugation through the nucleotide base, most notably by the convertible nucleoside approach of Verdine and co-workers (4, 5). In this approach, a nucleoside which contains a displaceable group on the base moiety is incorporated into the oligonucleotide by solid-phase synthesis. After oligomer synthesis, the solid support is treated with an amine which displaces the leaving group on the convertible nucleotide. This allows conjugation to the exocyclic amino groups of the bases of adenosine, cytosine, and guanosine in RNA (Fig. 1), directing functional groups to either the minor or the major groove of the oligomer (5). This approach has been used to introduce two thiol groups into an RNA sequence, followed by oxidation to form an intramolecular disulfide cross-link which stabilized a short RNA hairpin and shown to be a substrate for the ricin A-chain, a protein which catalyzes hydrolytic depurination of an adenosine nucleotide (6). The hairpin was a better substrate than the un-cross-linked oligomer, presumably due to a change in the concentration-dependent single strand/hairpin/dimer equilibrium.

Conjugation to the 5-position of pyrimidine bases of DNA has been a popular approach for two reasons. First, nucleosides containing a displaceable group in the 5-position are easily obtained, such as 5-iodouridine (7), allowing conjugation of a variety of different groups to the nucleoside base. Second, substituents at this position are projected into the major groove of the oligomer where they do not interfere with Watson-Crick base pairing and minimally perturb the nucleic acid structure. Thiols have been incorporated in the 5-position of pyrimidines in DNA (8) and phosphoramidites have been synthesized for incorporation of thiols, with different tether lengths, into RNA (9).

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Thiols have been tethered to the N-3 position of pyrimidine bases in RNA (10) but this approach has the disadvantage that Watson–Crick base pairing is disrupted. This fact limits the application to the study of RNA structure and function as the thiols can only be incorporated at bases which are not involved in base-pairing interactions, such as at the ends and in some instances in internal bulges or loops.

Fewer examples are available for conjugation to the sugar–phosphate backbone. Modification of the sugar–phosphate backbone minimizes possible interference with base pairing and places the label on the edge of the helix, rather than placing it directly within the groove of the oligomer. Thus, conjugation to the sugar–phosphate backbone increases the prospects of projecting the probe away from the helix, thus probing *interhelical* (or *intermolecular*) rather than *intrahelical* interactions.

An example of phosphate modification is the reaction of an internal nonbridging phosphorothioate in RNA with α -haloacetamides (11). Chemical synthesis of oligomers containing phosphorothioates results in a diastereomeric mixture and thus the label is positioned on either of the nonbridging positions of the phosphate (Fig. 2a), although in some instances, the diastereomers can be separated by RP-HPLC. Furthermore, the efficiency of the reaction of the internal phosphorothioate with the alkylating agent is rather low (ca. 50% conversion) and the resulting phosphate triester is susceptible to hydrolysis, especially in RNA where the 2'-hydroxyl group

can displace the thiol substituent in an intramolecular transesterification reaction. Therefore, a 2'-deoxynucleotide is usually incorporated immediately on the 5' side of the trisubstituted phosphate. McLaughlin and co-workers have incorporated a thiol into the sugar–phosphate backbone of DNA by oxidation of an internucleotide H-phosphonate at a unique site in the presence of cystamine to produce an internal phosphoramidate (Fig. 2b) (12). This approach has the same disadvantages as alkylation of phosphorothioates, namely, that a diastereomeric mixture is obtained which also contains a rather unstable N-substituted phosphoramidate.

For modifications of the sugar moiety, the 2'-position has almost exclusively been used for conjugation. Sproat and co-workers have described the synthesis of a modified uridine phosphoramidite which yields, after incorporation into RNA by solid-phase synthesis and deprotection, an oligomer containing a 2'-O-(2-thioethyl) group (Fig. 2c) (13). A cytidine with the same modification has also been incorporated into RNA (14). Both of these approaches require a multistep synthesis for the preparation of the phosphoramidites which does not offer flexibility with regards to the length of the tether between the thiol and the RNA.

A postsynthetic labeling method for the incorporation of thiols is described below. The thiol is conjugated to the 2'-position of the sugar moiety utilizing the efficient reaction of 2'-aminonucleotides with either aromatic isothiocyanates or aliphatic isocyanates (Fig. 3) (15, 16). Thus, the thiol can be tethered to the oligomer with either an aliphatic or an aromatic linker, the latter being rigid and somewhat sterically demanding which may in some instances cause structural perturbations in compact tertiary structures of RNA.

The major advantage of this postsynthetic strategy is that a single phosphoramidite is used to incorporate an amino group at a specific position in the oligomer to which a wide variety of compounds can be linked. This approach allows conjugation of different probes to the oligomer and, in the context of this article, enables incorporation of thiols with different tether lengths. Furthermore, 2'-amino-modified oligonucleotides are easily accessible by chemical synthesis using 2'-aminopyrimidine phosphoramidites which are commercially available (Glen Research). Moreover, aromatic isothiocyanates and aliphatic isocyanates containing a protected thiol are readily prepared from the corresponding amine (17).

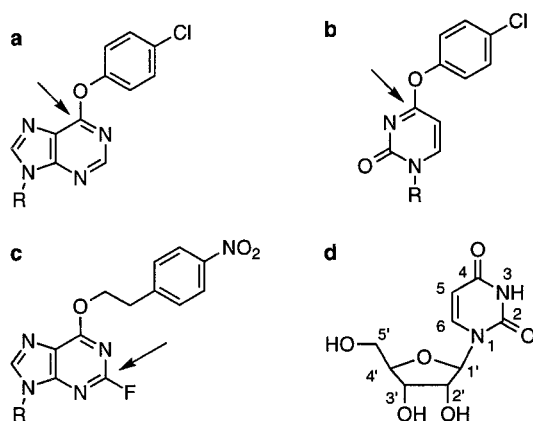


FIG. 1. (a–c) Convertible nucleosides. R, ribose. The arrow shows site of displacement upon reaction with amines to make N-substituted adenosine, cytidine, and guanosine, respectively. (d) Numbering system for pyrimidine nucleosides.

DESCRIPTION OF THE METHOD

Preparation of Aromatic Isothiocyanate **3**

In our work on the structure and function of the hammerhead ribozyme, we were interested in the incorporation of thiols at specific positions in the RNA. We utilized the fact that 2'-amino-modified nucleotides could be incorporated into oligonucleotides (18, 19) and that 2'-amino groups react selectively with aromatic isothiocyanates to form 2'-thiourea-modified oligomers (Fig. 4) (20, 21). Because an aromatic isothiocyanate containing a thiol functionality was not commercially available, compound **3** was synthesized in three steps (15). The synthesis of this aromatic isothiocyanate entailed the conversion of a primary alcohol to a thiol, protection of the thiol as a disulfide, and subsequent conversion of the amino group to an isothiocyanate. It was necessary to protect the thiol due to the high reactivity of thiols with isothiocyanates. After incorporation into the oligomer, the disulfide was reduced to yield the free thiol.

Synthesis of Isothiocyanate **3**

3-Aminobenzylmercaptan. The hydroxyl group of **1** was converted to a thiol in the following manner: A solution of 3-aminobenzylalcohol (1.00 g, 8.12 mmol) and thiourea (0.68 g, 8.93 mmol) in 48% aqueous HBr (3.0 mL) was heated at 100°C for 17 h. To 1.5 mL of this solution, whose total volume was 4 mL, was added NaOH (1.42 g, 35.5 mmol) in H₂O (14 mL) at 25°C, followed by heating at 100°C for 2 h. After cooling the solution to 25°C, NH₄Cl (1.0 M aq, 60 mL) was added. The mixture was extracted with CH₂Cl₂ (40 mL + 2 × 20 mL), the combined organic phases were dried (Na₂SO₄), and the solvent was removed *in vacuo* to give 3-aminobenzylmercaptan (0.38 g, 89%) which was used in the next step without further purification.

2-Pyridyl-3-aminobenzyl disulfide (2). Before converting the amino group to an isothiocyanate, the thiol group was protected as a disulfide: 3-Aminobenzylmercaptan (9.46 g, 68.0 mmol) in ethanol (41 mL) was added to a solution of 2,2'-dipyridyl disulfide (30.0 g, 136 mmol) and glacial acetic acid (3.2 mL, 55.8 mmol) in ethanol (82 mL) and the solution was stirred for 2.5 h at 25°C. The solvent was removed *in vacuo* and the residue was dissolved in CH₂Cl₂ (400 mL) and washed with NaOH (2 M aq, 2 × 200 mL). The combined aqueous phases were extracted with CH₂Cl₂ (200 mL) and the organic phases were combined and dried (Na₂SO₄). The solvent was removed *in vacuo* and the product was purified by flash column chromatography (a gradient of 5–50% EtOAc in *n*-hexane) and afforded **2** (10.6 g, 63%) as a pale yellow oil.

2-Pyridyl-3-isothiocyanatobenzyl disulfide (3). The final step in this synthesis was the conversion of the amine to an isothiocyanate: A solution of **3** (8.20 g, 33.0 mmol) in chloroform (250 mL) was added dropwise to a solution of thiophosgene (4.17 g, 36.3 mmol) in chloroform (50 mL) over 10 min. After stirring for 1 h at 25°C, the mixture was diluted with CH₂Cl₂ (330 mL) and washed with NaOH (1 M aq, 165 mL). The aqueous phase was extracted with CH₂Cl₂ (40 mL) and the organic phases were combined and dried (Na₂SO₄). The solvent was removed *in vacuo* and the product was purified by flash column chromatography (CH₂Cl₂), giving **3** (8.80 g, 92%) as a light brown oil.

Preparation of Aliphatic Isocyanate **5**

Isothiocyanate **3** served its purpose in our study of the hammerhead ribozyme (15), described below. However, there were some potential drawbacks to using this compound when studying compact RNA structures. The aromatic ring severely restricts the rotational freedom of the linker yielding an inflexi-

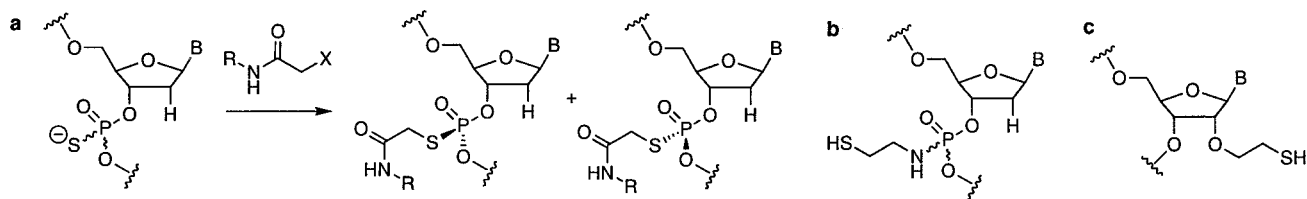


FIG. 2. (a) Reaction of α -haloacetamides with a diastereomeric mixture of phosphorothioates. X is Cl, Br, or I. (b) A diastereomeric mixture of N-substituted phosphoramidates. (c) A 2'-O-(2-thioethyl)-substituted nucleotide.

ble, sterically demanding tether which could cause structural perturbations. In addition, the length of the linker cannot be easily changed due to limited availability of the required starting materials for the synthesis of an isothiocyanate as outlined above.

To address these concerns, methods were sought for conjugation of aliphatic compounds to 2'-amino groups in oligomers which would afford more flexibility, both literally and figuratively speaking. The reaction of commercially available succinimidyl esters or aliphatic isothiocyanates was met with limited success (unpublished results). However, it was found that aliphatic isocyanates react efficiently with 2'-amino groups in oligomers. Most of the initial methods for the preparation of aliphatic isocyanates which were attempted to convert **4** to **5** yielded complex mixtures, presumably due to side reactions with the pyridyl disulfide functionality. Therefore, we developed a mild and efficient procedure for conversion of aliphatic amines to isocyanates and used it to prepare **5** (Fig. 5) (17).

Synthesis of Isocyanate **5**

S-(2-Pyridyldithio)cysteamine hydrochloride (**22**) (0.100 g; 0.449 mmol) was partitioned between CH_2Cl_2 (1.5 mL) and 1 M NaOH (1 mL). The organic phase was separated, dried (Na_2SO_4) (note that the free amine **4** decomposes upon concentration of the solution), and added dropwise to a stirred solution of trichloromethyl chloroformate (0.022 g; 0.11 mmol) in CH_2Cl_2 (1 mL) at 0°C over 1 min. The suspension was stirred for 2 min at 0°C and then partitioned between 1 M HCl (5 mL) and CH_2Cl_2 (10 mL). The organic phase was washed successively with 1 M HCl (1 mL) and 1 M NaOH (1 mL) and dried (Na_2SO_4). The solvent was removed *in vacuo* to yield **5** as a pale yellow oil (0.024 g; 25% based on the starting amine). This material was sufficiently pure (ca. 98% as determined by ^1H NMR analysis) for use

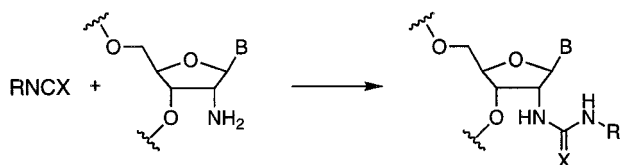


FIG. 3. Conjugation of either aromatic isothiocyanates (R, an aromatic ring; X = S) or aliphatic isocyanates (R, aliphatic; X = O) to 2'-aminonucleotides within RNA oligomers to form the corresponding 2'-(thio)urea-containing nucleotides.

in reactions with 2'-amino oligomers without further purification. It is recommended to store **5** desiccated at -20°C in dilute solutions of dry CH_2Cl_2 (for example 1 mg in 100 μL). The solvent is removed *in vacuo* prior to use in reactions with the oligomers.

Modification of 2'-Amino-Containing Oligomers with Iso(thio)cyanates

2'-Amino groups in oligoribonucleotides can be selectively modified with either aromatic isothiocyanates (**15**) or aliphatic isocyanates (**16**) using different reaction conditions. The less reactive aromatic isothiocyanates require incubation at 37°C for several hours while the aliphatic isocyanates react at 4°C over a shorter period of time. The higher reactivity of aliphatic isocyanates, relative to the aromatic isothiocyanates, requires more careful control of the reaction conditions to minimize competing hydrolysis of the isocyanate and to avoid reactions at the exocyclic amino groups of the nucleotide bases.

Reactions of 2'-amino-containing oligomers (up to ca. 30–40 nucleotides in length) with these reagents can be monitored by reverse-phase HPLC (**16**) or denaturing polyacrylamide gel electrophoresis (DPAGE) (**15**). As these reactions convert greater than 95% of the oligomer to the desired product, rigorous purification may not be necessary for most applications. The excess reagent can be extracted from the solution with an organic solvent. Purification by precipitation is usually sufficient but HPLC and DPAGE can be used to purify the modified oligomers to homogeneity.

Reactions with Aromatic Isothiocyanates

The oligomers 5'UCGCAUCGCU and 5'UCGCA(2'- NH_2)CGCU were each incubated with **3** for 28 h at 37°C [50 mM **3**; 1 mM RNA; 50 mM borate buffer, pH 8.6; 50% DMF; final volume 10 μL]. After

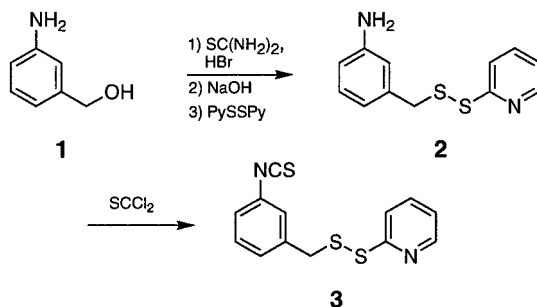


FIG. 4. Synthesis of aromatic isothiocyanate **3**.

26 h, aliquots of the reaction mixtures were withdrawn for HPLC analysis. Solvent gradients for analytical HPLC analysis were run at 1 mL/min, using a linear gradient of 50 mM triethylammonium acetate, pH 7.0, containing from 0 to 18% CH₃CN after 15 min with a subsequent increase to 70% CH₃CN in 5 min and returning to original conditions after 10 min. The retention times for the oligomers before the reaction were 10.0 and 9.7 min, respectively. After incubation with the isothiocyanate the 2'-amino oligoribonucleotide was converted (>95%) to a product which had a retention time of 15.3 min. On the other hand, the retention time of the unmodified oligoribonucleotide was unchanged, indicating that it did not react with the isothiocyanate.

Reactions with Aliphatic Isocyanates

Reactions of isocyanates with oligoribonucleotides on an analytical scale were carried out in an ice bath in a cold room (6°C). Solutions of the oligomers 5'-GCCGACCAU and 5'-AGCGA(2'-NH₂U)GCGA (2 mM; 70 mM borate buffer, pH 8.6; 2.5 μL) were independently treated sequentially with DMF (2.0 μL) and **5** in DMF (150 mM; 0.5 μL) and incubated for 3.5 h. For HPLC analyses, 1.5 μL aliquots of reaction mixture were diluted into 20 μL triethylammonium acetate buffer (100 mM, pH 7.0) and extracted with CH₂Cl₂ (2 × 150 μL) to remove the organic material. The HPLC analyses were carried out using the same conditions as described above. The retention times for the oligomers before the reaction were 10.7 and 11.0 min, respectively. After reaction with the isocyanate more than 95% of the 2'-amino oligomer was converted to a new product which had a retention time of 14.5 min. Conversely, the retention time of the unmodified oligomer remained unchanged, indicating no reaction with the isocyanate.

Large-scale reaction of oligomer 5'-AGCGA(2'-NH₂U)GCGA with **5** was carried out in an ice bath. To a solution of the oligomer (2 mM; 70 mM borate buffer, pH 8.6; 233 μL) was added **5** in DMF (30 mM; 233 μL), followed by purification of the modified oligomer by HPLC. This reaction resulted in 78% conversion to the product but a second addition of the isocyanate or

carrying out the reactions at -8°C resulted in yields comparable to the analytical reactions.

Characterization of 2'-Urea-Containing Oligomers

The incorporation of the new functionality into the oligomer was verified by mass spectrometry and by enzymatic digestion. HPLC analysis of the enzymatic digest revealed the absence of the 2'-aminonucleoside and the presence of a compound having a longer retention time. The new compound was determined to be the corresponding 2'-urea nucleoside by comparison to an authentic sample, prepared by organic synthesis (16).

Does the introduction of a 2'-(thio)urea linkage perturb the structure of the modified RNA? Ultimately, the answer to this question will have to wait for high-resolution structural information, such as from X-ray crystallography or NMR spectroscopy. However, the effects of 2'-urea linkages, obtained from the reaction of 2'-amino-containing oligomer with isocyanate **5**, on RNA duplex stability have been studied. It has previously been determined that the replacement of a 2'-hydroxyl with a 2'-amino group in RNA results in a moderate duplex destabilization, as indicated by the decrease in duplex melting temperature of ca. 4°C, corresponding to the energy of a hydrogen bond (21). Subsequent modification of the 2'-amino group with isocyanate **5** results in a further decrease of only 1.3°C (16). These destabilizing effects are of similar magnitude as those reported for other internal labels at either the sugar or base moieties of oligonucleotides (21, 23, 24) with the exception of intercalating agents which can stabilize duplex structure (25).

APPLICATIONS

This method was originally developed for the incorporation of two thiols into the hammerhead ribozyme (Fig. 6) to investigate its tertiary structure by forming intramolecular disulfide cross-links (15). The study was inspired by the publication of two three-dimensional structures of the hammerhead which appeared in the span of a few days, late in 1994. One was an X-ray structure (26) and the other was based on fluorescence resonance energy transfer (FRET) measurements in solution (27). The two models had a similar global structure, a wishbone shape with helices II and III nearly collinear and helix I close to helix II. However, the orientation of helices I and II to each other were different in the two structures.

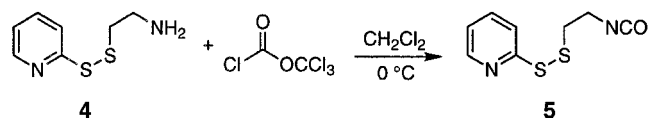


FIG. 5. Synthesis of aliphatic isocyanate **5**.

It was not clear which one of these structures resembled more closely the catalytically active species. Although X-ray crystallography of proteins is a well-established method, the same cannot be said about RNA. The crystal structure of the hammerhead ribozyme was the second structure of a complex RNA molecule, the other being tRNA (28, 29). The crystal structure displayed an inactive ground state structure as the conformation at the cleavage site did not allow for the required in-line attack (26). The question remained if the ribozyme was required to undergo a local or a global conformational change to reach the transition state of the reaction. Thus, it was possible that the FRET structure represented the catalytically active conformation in solution, under physiological conditions.

Three ribozymes containing 2'-amino group modifications were prepared and modified with isothiocyanate **3** as described above (15, 30). Two of these ribozymes contained 2'-amino modifications in positions which were proximal according to the FRET model and one ribozyme contained modifications which were close in the X-ray structure (Fig. 6). The thiols were deprotected and subsequent oxidation yielded ribozymes containing intramolecular cross-links which served to "lock" the ribozymes in a conformation displayed by one of the two models. Measurement of the catalytic activity of these three cross-linked ribozymes revealed that two were essentially inactive whereas one was fully active. The active ribozyme had nucleotides cross-linked which

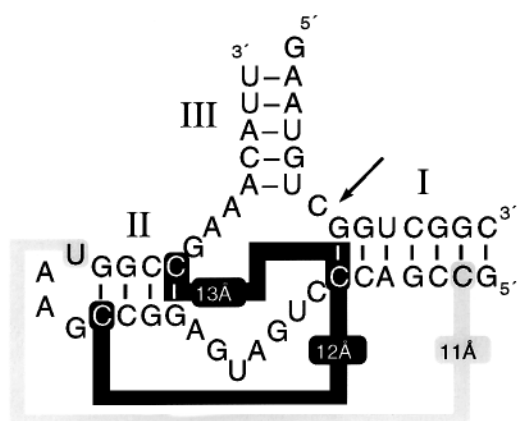


FIG. 6. The primary sequence and secondary structure of the three hammerhead ribozyme constructs used in this study. The black and gray lines connect three pairs of 2'-aminonucleotides that are close in space according to the FRET model and the X-ray structure, respectively, showing the distances between the 2'-amino groups.

were proximal in the X-ray structure, suggesting that the *global* conformation displayed in the X-ray structure resembles that of the transition state.

The aforementioned cross-linking experiments represent the first example of engineered *interhelical* disulfide cross-links in RNA. This approach, developed to study the hammerhead ribozyme, has subsequently been used to study both structure and dynamics of other ribozymes. Earnshaw *et al.* have utilized both isothiocyanate **3** and isocyanate **5** for probing the tertiary structure of the hairpin ribozyme (31), for which a crystal structure is not available. The hairpin ribozyme consists of two arms, or domains, which have been shown to interact in the catalytically active structure. A series of ribozymes was prepared, each containing unique cross-links between these two domains. The data from kinetic characterization of these cross-linked ribozymes were used to guide modeling of the interactions between the two domains which resulted in the first three-dimensional model of the hairpin ribozyme (31).

This cross-linking strategy has also been used to study the dynamics of a large ribozyme, a group I intron from *Tetrahymena thermophila* (32). The cross-linking sites were selected based on a three-dimensional model of the ribozyme (33) and the 2'-aminonucleotides to be cross-linked were separated by a range of distances up to ca. 50 Å. These cross-links were *intermolecular*, linking the substrate to the P3-P8 domain of the ribozyme. This enabled incorporation of a thiol into one strand and an activated disulfide into the other strand; disulfide cross-links were formed by a disulfide-exchange reaction (Fig. 7) which allowed convenient measurement of the rates of cross-linking. There was a clear correlation between the rate of cross-linking and the distance between the cross-linking sites but, quite unexpectedly, cross-links were formed between nucleotides separated by ca. 50 Å at rates only 3–15 times slower than between proximal nucleotides. Furthermore, the ribozyme-substrate complex containing these long-range cross-links was shown to be catalytically active. It was concluded that the folded

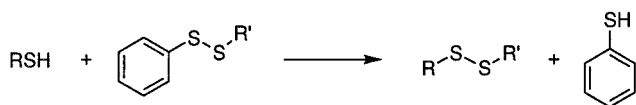


FIG. 7. A schematic representation of disulfide cross-linking of the substrate of group I intron from *Tetrahymena thermophila* to the P3-P8 domain of the ribozyme via disulfide exchange.

ribozyme displayed large thermal motions between different domains.

CONCLUDING REMARKS

The approach described above enables conjugation of thiols to 2'-aminonucleotides at specific positions within RNA. These 2'-amino-containing oligoribonucleotides are prepared by chemical synthesis using commercially available phosphoramidites; RNAs which are too large for chemical synthesis can be prepared by ligation of synthetic 2'-amino oligoribonucleotides to larger RNA fragments. The 2'-amino groups are selectively modified with aromatic isothiocyanates or aliphatic isocyanates, containing a protected thiol, in near quantitative yields. The thiol groups incorporated in this manner can be used to tether a variety of different structural probes and reporter groups to RNA. Alternatively, incorporation of two thiols into RNA, followed by oxidation, forms disulfide cross-links at specific positions within the RNA. Disulfide cross-links engineered into RNA have provided valuable information about the structure and function of ribozymes. In addition to ribozyme studies, this disulfide cross-linking approach should also be applicable toward the study of RNA-protein interactions.

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