Engineering Disulfide Cross-Links in RNA Using Thiol-Disulfide Interchange Chemistry

This unit presents methods for incorporating disulfide cross-links within RNA structures using thiol-disulfide interchange chemistry (Fig. 5.1.1). The alkyl phenyl disulfide **S.1** and the alkyl thiol **S.2** are incorporated at specific ribose 2′ positions within RNA, and then react through thiol-disulfide interchange to form the more stable dialkyl disulfide (see Basic Protocol 4). Such disulfide cross-linking can be used to prepare a simple covalent conjugate of two RNA molecules or, in more complex systems, to exert a specific conformational constraint to a dynamic RNA molecule. Compared to the approach previously used to obtain disulfide cross-linking of RNA—oxidation of two thiols (Goodwin et al., 1996; Sigurdsson et al., 1995)—thiol-disulfide interchange has the advantages that it proceeds under mild conditions without an oxidative catalyst and can be kinetically characterized.

Preparation of disulfide cross-linking precursors **S.1** and **S.2** begins with a site-specific incorporation of a 2′-amino-2′-deoxy residue within each of the two RNA species that are to be cross-linked, achieved through standard solid-phase RNA synthesis with a protected 2′-amino-2′-deoxy nucleotide phosphoramidite (see Basic Protocol 1). After deprotection of the synthetic RNA, the 2′ amine is modified with *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3**), affording the alkyl pyridyl disulfide **S.4** (Fig. 5.1.2). Thiol-disulfide interchange of **S.4** with thiophenol affords the alkyl phenyl disulfide **S.1** (see Basic Protocol 2); reduction of **S.4** with dithiothreitol yields the alkyl thiol **S.2** (Fig. 5.1.2; see Basic Protocol 3).

The RNA system to be cross-linked by thiol-disulfide interchange is limited to a two-piece system. Because both cross-linking precursors arise from the same intermediate (**S.4**, Fig. 5.1.2), **S.1** and **S.2** cannot be differentiated synthetically on the same RNA molecule.

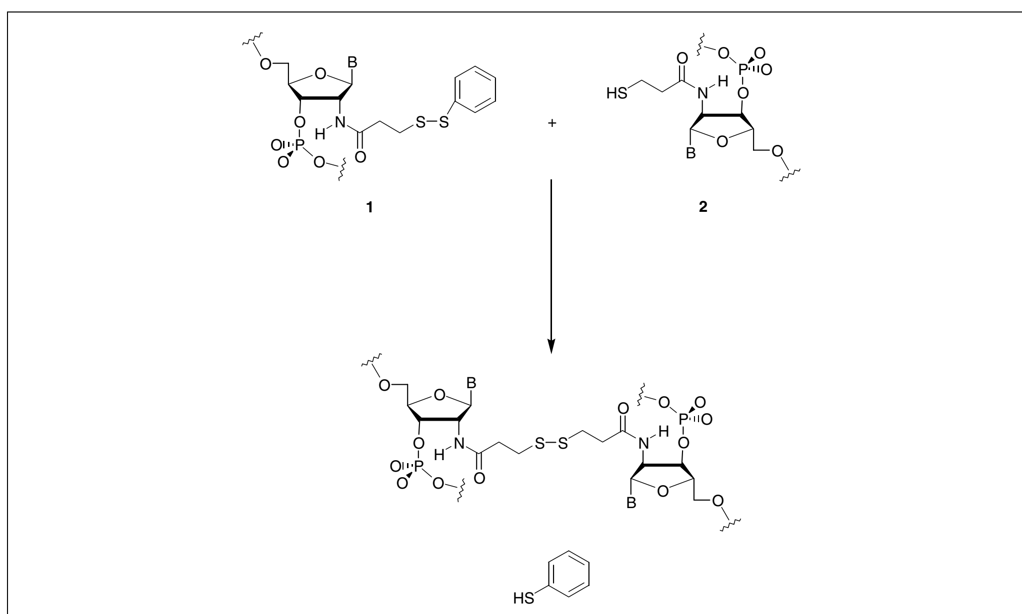

Figure 5.1.1 Disulfide cross-linking of RNA through thiol-disulfide interchange. B, nitrogenous base.

Contributed by Scott B. Cohen and Thomas R. Cech
Current Protocols in Nucleic Acid Chemistry (2000) 5.1.1-5.1.10
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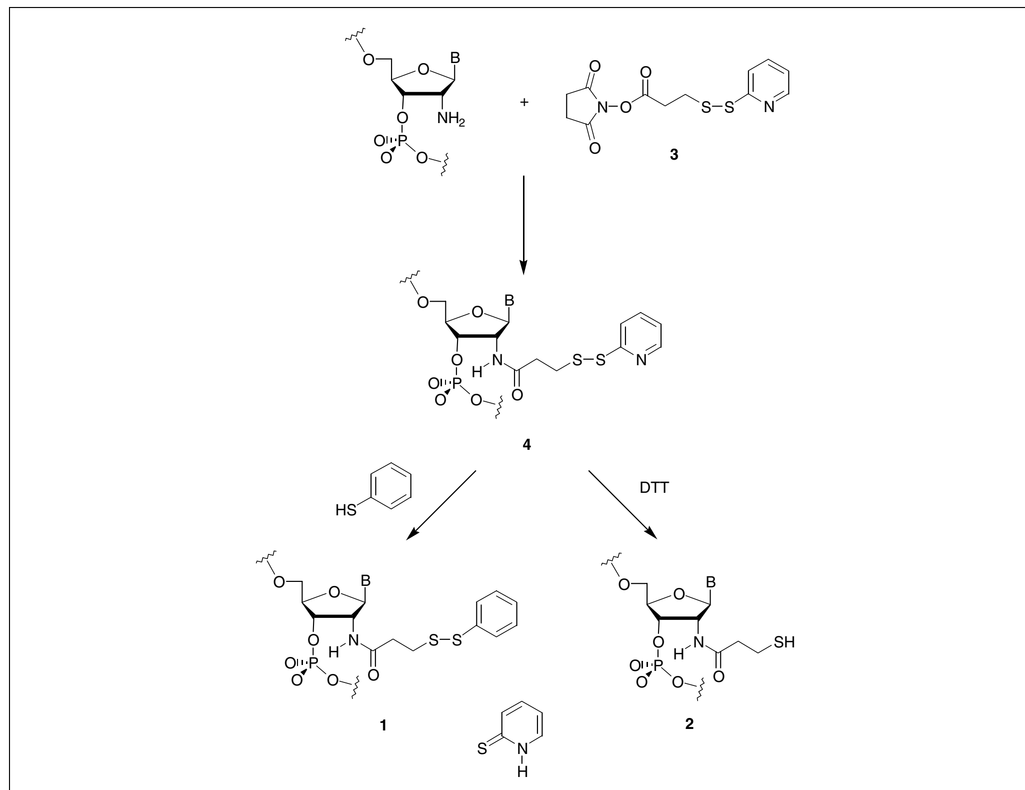

Figure 5.1.2 Preparation of the alkyl phenyl disulfide **S.1** and the alkyl thiol **S.2** as precursors for thiol-disulfide interchange. B, nitrogenous base.

Therefore, the cross-linking system must be designed such that two components of the RNA molecule (two complementary strands, a ribozyme-substrate complex, etc.), containing either **S.1** or **S.2** separately, can be associated through base pairing or other noncovalent interaction. After the full RNA molecule is associated, initiation of cross-linking and the rate at which it proceeds are controlled by manipulating the pH of the reaction mixture. The RNA component containing the alkyl thiol **S.2** is present in saturating excess over the RNA component containing the alkyl phenyl disulfide **S.1** to discourage spurious cross-linking resulting from the presence of unassociated **S.1**.

NOTE: Experiments involving RNA require careful precautions to prevent contamination and RNA degradation; see *APPENDIX 2A* (do not use DEPC; this should be unnecessary, and is inadvisable with 2′ amine chemistry).

BASIC PROTOCOL 1

PREPARATION OF RNA OLIGONUCLEOTIDES CONTAINING A SITE-SPECIFIC 2′ AMINE GROUP

Solid-phase synthesis of the two RNA oligonucleotides containing a unique 2′ amine employs the same procedures used for standard RNA phosphoramidites with the inclusion of a 2′-amino-2′-deoxy nucleotide phosphoramidite. The 2′-amino-2′-deoxy C-, U-, and G-phosphoramidites are synthesized according to the procedures of Verheyden et al. (1971), Imazawa and Eckstein (1979), and Benseler et al. (1992) and are produced commercially by Nexstar Pharmaceuticals. The following protocol describes deprotection and purification procedures for the solid support–bound product. Treatment with ammonium hydroxide to remove the exocyclic and 2′ amine protecting groups remaining from the synthesis procedure is followed by removal of the 2′-*O*-silyl protecting groups with fluoride to liberate the 2′ hydroxyl. One RNA will then be radiolabeled and modified with

an alkyl phenyl disulfide group (**S.1**) as described in Basic Protocol 2. The other RNA will be modified to contain an alkyl thiol group (**S.2**; Basic Protocol 3).

For a general overview of oligonucleotide synthesis, see *APPENDIX 3C.*

Materials

Solid support–bound product of automated RNA synthesis (1-µmol synthesis scale)
3:1 (v/v) concentrated ammonium hydroxide (NH_4OH)/absolute ethanol
24:46:30 (v/v/v) triethylamine/1-methyl-2-pyrrolidinone/triethylamine trihydrofluoride (see recipe)
TE buffer, pH 7.5 (*APPENDIX 2A*)
NAP-25 Sephadex column (Amersham Pharmacia Biotech)
1 M sodium chloride
Absolute ethanol
TBE buffer (*APPENDIX 2A*)
80% formamide/TBE solution (see recipe)
Denaturing polyacrylamide gel: 20% polyacrylamide/8 M urea in TBE buffer, dimensions 20 cm long × 26 cm wide × 0.3 cm thick (see *APPENDIX 3B*, *CPMB UNIT 7.6* or Sambrook et al., 1989)
TEN buffer (see recipe)

4-mL screw-cap vial
Teflon tape
Water baths, 55° and 65°C
Rotary drying system for microcentrifuge tubes (Savant)
40-mL Oak Ridge centrifuge tube
Preparative centrifuge (Sorvall or Beckman)
UV lamp (hand held)
50-mL polypropylene centrifuge tube
0.45-µm cellulose acetate filter

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis and UV shadowing (*APPENDIX 3B*, *CPMB UNIT 7.6* or Sambrook et al., 1989)

Remove the RNA protecting groups

1. Transfer the solid support–bound RNA product into a 4-mL screw-cap vial.

2. Add 1.5 mL of 3:1 (v/v) NH_4OH/ethanol. Seal the vial with Teflon tape and incubate 8 hr at 55°C.

3. Cool the vial to –20°C and decant the supernatant into a 1.5-mL microcentrifuge tube. Concentrate the supernatant under vacuum with a rotary drying system.

4. Dissolve the dry residue in 400 µL of 24:46:30 (v/v/v) triethylamine/1-methyl-2-pyrrolidinone/triethylamine trihydrofluoride. Incubate 1.5 hr at 65°C.

   Heating at 65°C (as suggested by Wincott et al., 1995) will help dissolve the dry residue. For RNAs shorter than 10 nt, reduce the amount of fluoride solution to 100 µL and skip step 5.

Purify the crude RNA

5. Transfer the solution to 5 mL TE buffer, pH 7.5, and mix thoroughly.

6. Load 2.5 mL of the resulting solution onto each of two NAP-25 Sephadex columns. Elute the RNA from each column with 3.5 mL water, collecting the first 3.5 mL of eluate from each (7 mL total product solution).

7. Add 1 mL of 1 M sodium chloride to the product solution and transfer to a 40-mL Oak Ridge centrifuge tube.

8. Precipitate the RNA by adding 24 mL absolute ethanol and then centrifuging 30 min at 10,000 × *g*, 2°C. Decant the supernatant and allow the RNA pellet to air dry.

9. Dissolve the RNA pellet in 150 µL TE buffer and 150 µL of 80% formamide/TBE (300 µL total).

10. Purify the RNA to single-nucleotide resolution by denaturing polyacrylamide gel electrophoresis at 25 W until the full-length product has migrated about two-thirds of the way down the gel, as indicated by the dye markers (see *APPENDIX 3B*, *CPMB UNIT 7.6* or Sambrook et al., 1989, for standard procedures).

    A gel 20 cm long × 26 cm wide × 0.3 cm thick is sufficient for three separate RNA oligonucleotide preparations.

Isolate the pure RNA

11. Identify the full-length RNA band by UV shadowing and excise the gel slice.

12. Place the gel slice in a 50-mL polypropylene centrifuge tube, crush thoroughly, and add 15 mL TEN buffer. Place the suspension on a shaker for 24 hr at 2°C.

13. Pellet the polyacrylamide by centrifugation for 10 min at 2000 × *g*, 2°C.

14. Filter the supernatant through a 0.45-µm cellulose acetate filter.

15. Precipitate the RNA by adding 3 vol absolute ethanol and then centrifuging 30 min at 10,000 × *g*, 2°C.

16. Dissolve the RNA in 100 µL TE buffer, pH 7.5. Determine RNA concentration spectrophotometrically by measuring A_{260}, and adjust to 100 µM. Store up to 6 months at −20°C or indefinitely at −80°C.

BASIC PROTOCOL 2

PREPARATION OF ^{32}P-LABELED RNA CONTAINING AN ALKYL PHENYL DISULFIDE GROUP

The RNA component of the cross-linking system containing the alkyl phenyl disulfide **S.1** is prepared as a ^{32}P-labeled reagent to allow monitoring of the cross-linking reaction, indicated by a shift to a product that migrates more slowly under denaturing electrophoresis conditions. Labeling of the RNA at the 5′ end with ^{32}P is followed by chemical modification of the 2′ amine with *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3**), yielding the corresponding amide **S.4**. Thiol-disulfide interchange of the pyridyl disulfide moiety of **S.4** with thiophenol yields the alkyl phenyl disulfide **S.1** (Fig. 5.1.2).

Materials

RNA oligonucleotide with 2′ amine group (100 µM in TE buffer; see Basic Protocol 1)
≥0.1 Ci/µL [γ-^{32}P]ATP (6000 Ci/mmol)
10 U/µL T4 polynucleotide kinase and 10× buffer (New England Biolabs)
1 M sodium chloride
Absolute ethanol
1 M sodium borate buffer, pH 8
500 mM *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3**; Pierce Chemicals) in *N*,*N*-dimethylformamide (prepare solution just before use)
70 mM thiophenol in absolute ethanol
TE buffer, pH 7.5 (*APPENDIX 2A*)

1× TBE buffer (*APPENDIX 2A*)
80% formamide/TBE solution (see recipe)
Denaturing polyacrylamide gel: 20% polyacrylamide/8 M urea in TBE buffer, dimensions 20 cm long × 10 cm wide × 0.05 cm thick (see *APPENDIX 3B, CPMB UNIT 7.6* or Sambrook et al., 1989)
TEN buffer (see recipe)
10 mM sodium acetate buffer, pH 4.5 (*APPENDIX 2A)*

Preparative centrifuge (Sorvall or Beckman)
X-ray film for autoradiography
Water bath, 37°C

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B, CPMB UNIT 7.6* or Sambrook et al., 1989)

CAUTION: Step 8 should be performed in a fume hood because of the stench of thiophenol. Any labware that comes in contact with thiophenol should be soaked in bleach solution.

Label the RNA

1. Mix the following in a 1.5-mL microcentrifuge tube (9 µL total volume):

   1 µL 100 µM RNA oligonucleotide with 2′ amine
   5 µL ≥0.1 Ci/µL [γ-^{32}P]ATP (6000 Ci/mmol; ≥0.5 mCi total)
   1 µL 10× T4 polynucleotide kinase buffer
   2 µL H_2O.

   Warm the solution to 37°C.

2. Add 1 µL of 10 U/µL T4 polynucleotide kinase and incubate 30 min at 37°C.

3. Add 200 µL water and 50 µL of 1 M sodium chloride; mix well.

4. Precipitate the labeled RNA by adding 900 µL absolute ethanol and centrifuging 20 min at 16,000 × *g*, 2°C.

5. Decant the supernatant and allow the RNA pellet to air dry.

Modify the 2′ amine

6. Dissolve the labeled RNA in:

   140 µL water
   20 µL 1 M sodium borate buffer, pH 8
   20 µL 1 M sodium chloride.

   Warm the solution to 37°C.

7. Add 20 µL freshly dissolved 500 mM *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3**) in *N*,*N*-dimethylformamide and mix well. Incubate 20 min at 37°C.

   *Upon addition of **S.3**, the solution will become cloudy because of the compound's limited water solubility but will clear during the course of the reaction as a result of hydrolysis.*

8. In a fume hood, add 200 µL of 70 mM thiophenol in ethanol. Incubate 2 min at 23°C.

   The reaction should display a light yellow color from formation of pyridine-2-thione.

9. Precipitate the RNA by adding 700 µL absolute ethanol and centrifuging 20 min at 16,000 × *g*, 2°C.

10. Decant the supernatant and allow the RNA pellet to air dry.

Purify the modified RNA

11. Dissolve the crude product in 10 µL TE buffer and 10 µL of 80% formamide/TBE solution (20 µL total).

12. Purify the RNA by denaturing polyacrylamide gel electrophoresis at 15 W (see APPENDIX 3B, CPMB UNIT 7.6 or Sambrook et al., 1989, for standard procedures).

    A gel 20 cm long is sufficient to provide clean separation from the faster-migrating RNA that remains unmodified after treatment with ***S.3*** *(~25%).*

13. Identify the band by autoradiography and excise the gel slice.

14. Crush the gel slice thoroughly in a 1.5-mL microcentrifuge tube and add 500 µL TEN buffer. Incubate the suspension 10 min on ice, vortexing occasionally.

15. Pellet the polyacrylamide by centrifugation for 2 min at 16,000 × *g*, 2°C, and carefully withdraw the supernatant into a fresh 1.5-mL microcentrifuge tube.

16. Precipitate the RNA by adding 4 vol absolute ethanol and centrifuging 30 min at 16,000 × *g*, 2°C.

17. Dissolve the modified RNA in 100 µL 10 mM sodium acetate buffer, pH 4.5. Determine radioactivity level to confirm that it is $\geq 10^5$ cpm/µL. Store in 20-µL aliquots at −80°C.

BASIC PROTOCOL 3

PREPARATION OF RNA CONTAINING ALKYL THIOL GROUP

The RNA component of the cross-linking system containing the alkyl thiol **S.2** is prepared using similar chemistry as for **S.1**. The 2′ amine is modified with *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3**), yielding the corresponding amide **S.4**. Reduction of the pyridyl disulfide moiety of **S.4** with dithiothreitol yields the alkyl thiol **S.2** (Fig. 5.1.2).

Materials

20 nmol RNA oligonucleotide with 2′ amine group (see Basic Protocol 1)
1 M sodium borate buffer, pH 8
1 M sodium chloride
500 mM *N*-succinimidyl-3-(2-pyridyldithio) propionate (**S.3;** Pierce Chemicals) in *N*,*N*-dimethylformamide (prepare just before use)
Absolute ethanol
1 M dithiothreitol (DTT) in water (APPENDIX 2A)
TEN buffer (see recipe)
TE buffer, pH 7.5 (APPENDIX 2A)
Water bath, 37°C

Modify the 2′ amine

1. In a 1.5-mL microcentrifuge tube, precipitate 20 nmol of the RNA to be modified by mixing 200 µL of the 100 µM 2′-amine-modified RNA with 50 µL of 1 M sodium chloride followed by 750 µL absolute ethanol, then centrifuging 20 min at 16,000 × *g*, 2°C.

2. Dissolve the RNA pellet in:

    120 µL H_2O
    40 µL 1 M sodium borate buffer, pH 8
    20 µL 1 M sodium chloride.

    Warm the solution to 37°C.

3. Add 20 µL freshly dissolved 500 mM **S.3** in *N*,*N*-dimethylformamide and mix well. Incubate 20 min at 37°C.

   This reaction may be scaled up as needed, maintaining the following final concentrations: 200 mM $NaB(OH)_3$*, 100 mM NaCl, 100 µM RNA, and 50 mM* ***S.3****.*

4. Precipitate the RNA by adding 600 µL absolute ethanol and centrifuging 20 min at 16,000 × *g*, 2°C.

5. Repeat steps 2 to 4.

   Double treatment with ***S.3*** *should modify ≥95% of the 2′ amines.*

Liberate the thiol

6. Dissolve the RNA pellet in 140 µL water and 40 µL of 1 M sodium borate buffer, pH 8. Liberate the thiol by adding 20 µL of 1 M DTT. Incubate 30 min at 37°C.

7. Precipitate the RNA by adding 50 µL of 1 M sodium chloride and 750 µL absolute ethanol, and centrifuging 20 min at 16,000 × *g*, 2°C.

8. Dissolve the modified RNA in 300 µL TEN buffer. Precipitate the RNA by adding 900 µL absolute ethanol and then centrifuging again as in step 7.

9. Dissolve the modified RNA in 100 µL TE buffer. Determine RNA concentration spectrophotometrically by measuring A_{260}, and adjust to 100 µM. Store up to 6 months at −20°C or indefinitely at –80°C.

CROSS-LINKING OF RNA THROUGH THIOL-DISULFIDE INTERCHANGE

BASIC PROTOCOL 4

The two RNA components containing **S.1** and **S.2** are associated at pH 4.5, where the nucleophilicity of the alkyl thiol **S.2** is attenuated such that cross-linking before association is discouraged. Cross-linking is then initiated by increasing the pH, usually to the range of pH 7 to 8. The cross-linking reaction is conducted with the thiol component **S.2** in saturating excess (up to 10 times the equilibrium dissociation constant) over the phenyl disulfide component **S.1** to discourage spurious cross-linking resulting from the presence of unassociated **S.1**.

The following protocol is from original ribozyme cross-linking experiments by Cohen and Cech (1997). The concentrations of RNA components, cross-linking pH, and other experimental conditions can be adjusted to accommodate other experimental systems.

Materials

^{32}P-labeled RNA modified with phenyl disulfide **S.1** (≥10^5 cpm/µL in 10 mM sodium acetate buffer, pH 4.5; see Basic Protocol 2)
RNA modified with alkyl thiol **S.2** (1 µM in TE buffer; see Basic Protocol 3)
100 mM sodium acetate buffer, pH 4.5 (*APPENDIX 2A*)
100 mM magnesium chloride
5 M sodium chloride
Formamide quenching solution (see recipe)
1 M sodium HEPES buffer, pH 7.5 (*APPENDIX 2A*)

0.65-mL microcentrifuge tube
Water bath, 30°C

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B, CPMB UNIT 7.6* or Sambrook et al., 1989)

1. In a 0.65-mL microcentrifuge tube, mix (90 µL total volume):

    10 µL 1 µM **S.2**-modified RNA
    20 µL 100 mM sodium acetate buffer, pH 4.5
    10 µL 100 mM magnesium chloride
    20 µL 5 M sodium chloride
    30 µL H_2O.

    Warm the solution to 30°C.

2. Add 10 µL of ≥10^5 cpm/µL ^{32}P-labeled **S.1**-modified RNA. Incubate 30 min at 30°C.

3. Remove 5 µL of the reaction and transfer to a fresh microcentrifuge tube containing 25 µL formamide quenching solution. Store frozen on dry ice.

    This aliquot serves to measure the small amount of cross-linking (if any) that occurs during the association incubation at pH 4.5.

4. Initiate cross-linking by adding 10 µL of 1 M sodium HEPES buffer, pH 7.5, to attain a final reaction pH of ~7.2.

    The absolute rates of cross-linking can be controlled by manipulating the pH of the cross-linking reaction (Δlog k/ΔpH ~1 in the pH range of 4.5 to 8.0).

5. At the desired time intervals, transfer 5 µL of the reaction to separate tubes containing 25 µL formamide quenching solution. Store the time aliquots frozen on dry ice until analysis by denaturing polyacrylamide gel electrophoresis (see *APPENDIX 3B, CPMB UNIT 7.6* or Sambrook et al., 1989).

    For example, in the original ribozyme cross-linking experiments, 3- to 5-min intervals were used.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Formamide, 80%, in TBE

To 40 mL formamide add 25 mg bromphenol blue and 25 mg xylene cyanol; mix well. Add 10 mL of 5× TBE buffer (*APPENDIX 2A*). Store up to 6 months at 23°C.

The resulting 50 mL contains 80% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, 90 mM Tris-borate, and 1 mM EDTA.

Formamide quenching solution

800 µL formamide
0.5 mg bromphenol blue
0.5 mg xylene cyanol
100 µL 1 M sodium acetate buffer, pH 4.5
100 µL 0.5 M disodium EDTA
Prepare fresh

This must be prepared fresh on the day of use because EDTA will precipitate during extended storage at pH 4.5.

The resulting 1.0 mL contains 80% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, 100 mM sodium acetate buffer (pH 4.5), and 50 mM EDTA.

TEN buffer

10 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
1 mM disodium EDTA (*APPENDIX 2A*)
250 mM sodium chloride
Store indefinitely at room temperature (e.g., 23°C).

Triethylamine/1-methyl-2-pyrrolidinone/triethylamine trihydrofluoride, 24/46/30 (v/v/v)

To a stirred solution of 12 mL triethylamine and 23 mL 1-methyl-2-pyrrolidinone (Aldrich), add dropwise 15 mL triethylamine trihydrofluoride (Aldrich). Continue stirring until the solution is homogeneous (1.4 M fluoride ion). Store in 1-mL aliquots up to 6 months at −20°C.

CAUTION: *Wear gloves when working with fluoride solutions.*

This solution is derived from Wincott et al. (1995).

COMMENTARY

Background Information

The authors' goal was to use site-specific disulfide cross-linking to measure long-range conformational dynamics within a large (>300-nt) catalytic RNA molecule (ribozyme). Existing methods of forming disulfide bonds had relied on chemical 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, are effective oxidants (Patai, 1974) but are incompatible with RNA because they promote oxidative cleavage of the ribose backbone (Chen and Sigman, 1988). In addition, large structured RNAs often contain binding sites for multivalent metal ions, further complicating analysis. Sulfoxide catalysis has been used to form disulfides in RNA (Sigurdsson et al., 1995), but the experimental conditions required (≥50% dimethyl sulfoxide) are not compatible with a folded and active ribozyme structure.

The new disulfide cross-linking procedure (Cohen and Cech, 1997) that is presented in this unit can be applied to large RNAs and allows kinetic characterization of the cross-linking reaction. Incorporation of cross-linking precursors **S.1** and **S.2** within RNA relies on the presence of a 2′ amine, incorporated by use of the corresponding phosphoramidite during solid-phase RNA synthesis (Verheyden et al., 1971; Imazawa and Eckstein, 1979; Benseler et al., 1992). Modification of the 2′ amine is then performed after the RNA is deprotected, labeled with ^{32}P, or otherwise manipulated. For example, a 2′ amine is compatible with T4 DNA ligase; ligation of a 41-nt synthetic RNA containing a 2′ amine to a 269-nt transcribed RNA as described by Moore and Sharp (1992) provided a route for preparing a 310-nt ribozyme containing the alkyl thiol **S.2** (Cohen and Cech, 1997).

Thiol-disulfide interchange chemistry (Fig. 5.1.1) proceeds under mild conditions (aqueous solution, pH ~7) without an oxidative catalyst and has been used to measure conformational dynamics between helical domains within a 310-nt ribozyme (Cohen and Cech, 1997). Association of the substrate domain containing **S.1** with a set of ribozymes, each containing **S.2** at a different position, afforded substrate-ribozyme cross-linking representing interhelical displacements of at least 50 Å. The kinetic profile of the cross-linking revealed the distribution of motions between the two domains. Cross-linking was achieved under a variety of experimental conditions (temperature 30° to 50°C; pH 6 to 8; NaCl concentration 0 to 1.0 M; $MgCl_2$ concentration 0 to 100 mM) and allowed preparation of a series of conformationally constrained substrate-ribozyme complexes.

Critical Parameters

Optimization of the modification reaction of the 2′ amine with **S.3** revealed a strong temperature dependence: the efficiency of the reaction was significantly lower at 23°C than at 37°C. Allow ample time for the solution to warm to 37°C before addition of **S.3**.

The absolute rates of cross-linking can be controlled by manipulating the pH of the cross-linking reaction ($\Delta\log k/\Delta pH$ ~1). The pH may require optimization. For example, if the cross-linking precursors **S.1** and **S.2** are in close proximity within the associated RNA molecule, then a reaction pH of 8 may result in cross-linking that is too fast to measure (<1 min). Lowering the pH from 8 to 7 will slow the reaction.

Careful purification of the ^{32}P-labeled RNA containing **S.1** will enhance the yield of the cross-linking reaction. In the preparation of **S.1**, the concentration of RNA in the modification reaction with **S.3** is relatively low (~1 µM, compared to 100 µM when preparing **S.2**); as a consequence, the reaction is less efficient. With careful electrophoresis, it is possible to

isolate the RNA containing the **S.1** modification as a distinct band because it will migrate more slowly than the RNA left with a free 2′ amine. Complete separation has been achieved for RNAs up to 30 nt.

Anticipated Results

The yield of cross-link is defined as the percentage of ^{32}P-labeled RNA containing **S.1** that becomes cross-linked to the RNA modified with **S.2**. Typically, cross-linking yields are ~70%; yields as low as 40% and as high as 90% have been observed. Factors that may affect the yield of cross-link include the temperature (experiments at 2°C afforded lower yields than those at 30°C), the proximity of **S.1** and **S.2** within the RNA molecule (which influences the reaction rate), and the purity of the RNA components, particularly **S.1**.

Time Considerations

Preparation of the RNA oligonucleotide containing a 2′ amine will take 2 to 3 days. Preparation of RNA modified with **S.1** or **S.2** can usually be done within a single day for each. The length of time required for cross-linking will depend on the experimental system; cross-linking reactions have been observed to be complete within minutes to several hours.

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Key References

Cohen and Cech, 1997. See above.

Describes the initial development of thiol-disulfide interchange chemistry and its application to measuring conformational dynamics within a 310-nt catalytic RNA.

Sigurdsson et al., 1995. See above.

Presents the first example of the use of chemistry derived from a 2′ amine to tag the RNA backbone in a sequence-specific fashion.

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