A UNIQUELY MODIFIED RNA: INTRODUCTION OF A SINGLE RNA CLEAVAGE AGENT INTO THE M1 RIBOZYME

Paul L. Richardson, Margaret L. Gross, Karen J. Light-Wahl, Richard D. Smith & Alanna Schepartz

Department of Chemistry
Yale University
New Haven, Connecticut 06511

Chemical Science Department
Pacific Northwest laboratory
Richland, WA 99352

Abstract: We describe an efficient, four-step synthesis of an 5'-modified cytidine phosphoramidite reagent (1) that permits nonnative functionality to be introduced into a synthetic oligoribonucleotide. This reagent was used to prepare a 377-nt E. coli M1 ribozyme equipped with a single RNA cleavage agent at nucleotide 11.

Many RNAs adopt discrete three-dimensional structures that are essential for their biological function. Unfortunately, our ability to decipher the structural basis of RNA function is hindered by a lack of tools to elucidate RNA tertiary folding patterns. Multi-dimensional NMR methods and X-ray crystallography are revolutionizing the analysis of small elements of RNA structure but have not yet been applied generally to RNAs containing more than 50 nucleotides. In the absence of high resolution techniques, alternative methods are needed to aid the study of both higher-order RNA structure and the process of acquiring structure (the RNA folding problem).

Recently we demonstrated that metal-catalyzed self-cleavage reactions performed under physiological conditions can detect alternative conformational states of proteins in solution. The protein cleaving agent EDTA-Fe was joined through a disulfide bond to a unique cysteine thiol in staphylococcal nuclease and a self-cleavage reaction was initiated with ascorbate ion. Changes in structure between the native and non-native states were characterized by comparing the self-cleavage sites observed under native and non-native conditions.

Extension of this idea to the study of RNA tertiary structure and folding requires incorporation of a single cleavage agent at a precisely defined position within a large, structured RNA. The recent rediscovery that RNA fragments can be joined by the action of DNA ligase and a complementary DNA template simplifies this problem to the synthesis of a reagent that allows an RNA cleavage agent to be introduced into a synthetic oligonucleotide. Here we present an efficient, four-step synthesis of 5'-[(N-(9-fluorenylmethoxycarbonyl)-amino)propyl]-5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-cytidine-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite (1, Fig. 2A). This modified phosphoramidite reagent bears a masked primary amine that
Figure 1. Nucleotides 1-377 of an *E. coli* M1 ribozyme modified site-specifically at nucleotide 11 (labeled X in figure). Nucleotides derived from M1_{1-2}^{11M}, M1_{1-2}^{11EDTA} or M1_{1-2}^{11DTPA} are depicted in black boxes. Nucleotides derived from M1_{1-2}_{22}^{377} are shown in normal type.
A uniquely modified RNA is unmasked post-oligonucleotide synthesis\textsuperscript{17-24} and may be acylated subsequently with a variety of suitably activated reporter groups. Unlike most reagents available for introducing modified bases into RNA, \textsuperscript{1} introduces a ribonucleotide, complete with 2'-OH group.\textsuperscript{16,25} The 2'-OH is important for RNA stability\textsuperscript{26,27} and may contribute to RNA tertiary complexity by providing additional points of hydrogen bonding and metal binding sites.\textsuperscript{28-36} Here we apply this reagent to generate a modified version of the 377-nucleotide (nt) M1 ribozyme of \textit{E. coli}\textsuperscript{37} containing a single DTPA- or EDTA-Fe RNA cleavage agent at position 11, M1\textsubscript{1-377}\textsuperscript{11DTPA} and M1\textsubscript{1-377}\textsuperscript{11EDTA} (Fig. 1).

Figure 2. (A) Synthesis of phosphoramidite \textsuperscript{1}. (B) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating reaction of 83 nM \textsuperscript{32}P M1\textsubscript{1-21} or M1\textsubscript{1-21}\textsuperscript{11M} with 28 mM DTPADA in DMSO:TEA:H\textsubscript{2}O (75:5:20) or 6.5 mM EDTADA in 167 mM Na\textsubscript{2}CO\textsubscript{3} (pH 8.0).

The synthesis of \textsuperscript{1} is illustrated in Fig. 2A. Reaction of 2',3',5'-tri-O-acetyluridine with 2-mesitylene-sulfonyl chloride followed by displacement with 2,4,6-trimethyl phenol provided mesitylate \textsuperscript{2} in 95% yield.\textsuperscript{38} Treatment of \textsuperscript{2} with an ammonia-saturated methanol solution,\textsuperscript{38} followed by dimethoxytrityl chloride,\textsuperscript{39} generated the 2',3'-diol \textsuperscript{3} in 73% yield. Reaction of \textsuperscript{3} with tert-butylimethylsilyl chloride and silver nitrate provided a 2:1 mixture of 2' and 3'-silyl isomers.\textsuperscript{39} Treatment of the mixture with neat 1,3-diaminopropane followed by benzotriazolyl-9-fluorenylmethyl carbonate\textsuperscript{40} afforded \textsuperscript{4} in a three-step yield of 30%. Subsequent reaction of \textsuperscript{4} with cyanethyl N,N-diisopropyl chlorophosphoramidite provided \textsuperscript{1}.\textsuperscript{20,41} Phosphoramidite \textsuperscript{1} was introduced into position 11 of an oligoribonucleotide (GAAGCUGACCMGACAGUCGCC, M1\textsubscript{1-21}11M) comprising the first 21 nucleotides of the M1 ribozyme using standard solid-phase procedures. M1\textsubscript{1-21}11M was cleaved from the resin, deprotected, and gel-purified. Autoradiography of purified \textsuperscript{5}\textsuperscript{32}P end-labeled material indicated a homogeneous product of >95% purity. The presence of the modified base was verified by nuclease digestion of M1\textsubscript{1-21}11M followed by HPLC analysis.\textsuperscript{42} The chemical composition of M1\textsubscript{1-21}11M was confirmed by ESI-MS.\textsuperscript{43}

Treatment of M1\textsubscript{1-21}11M with ethylenediaminetetraacetic dihydride (EDTADA) or diethylenetriamine-pentaacetic dihydride (DTPADA) produced products with lower electrophoretic mobility on denaturing polyacrylamide gels, whereas M1\textsubscript{1-21}, containing adenosine at position 11, showed no reaction (Fig. 2B). Reaction
Fig. 3. (A) Autoradiogram of a 10% denaturing polyacrylamide gel illustrating ligation of 5' 32P M11-211M, M11-211EDTA, or M11-211DTPA (0.5 μM) to M122-377 (0.5 μM) in the presence of template DNA (0.5 μM) and T4 DNA ligase.13 Lanes labeled M1 contain 32P M11-377 prepared by transcription. Ligation yields (lanes 7-9) approach 60%. (B) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating cleavage of 5' 32P CCCCCGAGGGAGGCGAGGCGAGGCGAGGCGACTTCGTCGCTCAGCTTC by M11-211M, M11-211EDTA, or M11-211DTPA in the presence of 1 μM Fe(III), 1 μM Cu(II), or 100 μM Zn(II) in a buffer containing 20 mM potassium phosphate, 20 mM KCl (pH 8.0) and 10 mM DTT. Histograms correspond to data from lane 6. The cleavage seen in lane 5 is likely due chelation of adventitious iron by M11-211EDTA since it is suppressed by excess Zn(II). (C) Autoradiogram illustrating that EDTA (lane 6) and DTPA (lane 9) are tethered uniquely to position 11 in M11-377 RNAs prepared by ligation. 5' end-labeled ligation products were subjected to base-catalyzed hydrolysis (pH 9.5, labeled OH) and G-specific sequencing (T1 endonuclease, labeled G) reactions and fractionated on a 20% denaturing gel. Cleavage products with 10 nucleotides or fewer (position a or lower) comigrate independent of the base at position 11, whereas those with 11 nucleotides or more do not (position b and above). Both EDTA (lanes 4-6) and DTPA (lanes 7-9) retard the mobilities of cleavage products greater than 10 nucleotides in length.
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with EDTADA proceeded readily in 167 mM Na₂CO₃ (pH 8.0), whereas reaction with DTPADA was most efficient in DMSO:TEA:H₂O (75:5:20). The reactions were performed on a preparative scale (5 nmol) and the gel-purified products identified as M₁₂₁EDTA (52% yield) and M₁₂₁DTPA (69% yield) by ESI-MS and by a base-specific sequencing assay (not shown). Both M₁₂₁EDTA and M₁₂₁DTPA promote cleavage of a complementary DNA oligonucleotide upon incubation with Fe(III) or Cu(II) (but not excess Zn(II)) and DTT (20 mM potassium phosphate pH = 8.0, 20 mM KCl, 25 °C, 14h) (Fig. 3A). Maximal cleavage occurs over a 7 base range centered about the complement of the modified base (★). Cleavage is more efficient with the EDTA derivative than with the DTPA derivative.⁶

To complete the modified ribozyme syntheses, M₁₂₁M and M₁₂₁EDTA and M₁₂₁DTPA were ligated to M₁₂₂-₃₇₇, which contains the remaining 355 nucleotides of M₁. M₁₂₂-₃₇₇ was prepared by T₇ RNA polymerase catalyzed transcription of pPR22A-T7. Addition of GMP to the transcription reaction produced RNA transcripts containing a 5'-monophosphate and avoided the capricious two-step procedure necessary to convert the 5'-triphosphate obtained in GTP primed transcriptions into the 5'-monophosphate required by DNA ligase.⁴⁵ Ligation were performed in the presence of T₄ DNA ligase and a DNA template complementary to the 5' 45 nucleotides of M₁ RNA (Fig. 3B lanes 7-9) and proceeded with efficiencies that approached 60%. No ligation was observed in the absence of template or ligase (lanes 4-9), and neither modified base had a significant effect on the ligation yield. A base-specific digestion assay was used to verify that the full length ribozyme carried a site-specific modification at position 11 (Fig. 3C). Ligation reactions were performed with either M₁₂₂-₃₇₇ or the oligonucleotide as the labeled component; however, ligation of 5' 32P oligonucleotides to M₁₂₂-₃₇₇ yields modified 5' 32P M₁ RNA of high specific activity suitable for further study. Experiments to determine the self-cleavage sites of M₁-₃₇₇EDTA are in progress.

Summary We describe an efficient synthesis of a modified phosphoramidite reagent that permits nonnative functionality to be introduced into a synthetic oligoribonucleotide. The modified base described here differs little from cytosine and causes minimal destabilization within a model RNA hairpin (ΔTm = 0.3 kcal/mol⁻¹). Thus, it may be positioned within both single-stranded and duplex regions and should participate in many tertiary interactions. We note that a variety of different reporter groups can be joined to a single oligonucleotide containing this base with subsequent ligation to another RNA molecule. This method represents an alternative to that of Tor and Dervan,⁴⁶ which requires synthesis of a deoxyribonucleoside phosphoramidite and a ribonucleotide triphosphate to direct the enzymatic incorporation of an isomeric base into an RNA oligomer, and to that of Nolan et al.⁴⁷ which places the modification at the 5' end of a circularly permuted RNA.

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References

(43) Smith, R. D. et al., Anal. Chem. 1992, 62, 882-889. ESI-MS data was obtained with 0.5 5g/mL RNA solutions in doubly distilled deionized H2O at flow rates of 0.2 mL/min with a low volume sheathless ESI source (D.C. Gale and R.D. Smith, manuscript in preparation) and a coaxial flow of SF6, using a Sciex TAGA 6000ES mass spectrometer. MW expected for Mlt.21notation: 6766; found, 6767±2; Mlt.21notation**DTP**K: 7104; found, 7102±2; Mlt.21notation**DTP**K: 7180; found, 7181±2.