Site-Specific Incorporation of Nitroxide Spin-Labels into Internal Sites of the TAR RNA; Structure-Dependent Dynamics of RNA by EPR Spectroscopy

Thomas E. Edwards, Tamara M. Okonogi, Bruce H. Robinson, and Snorri Th. Sigurdsson*

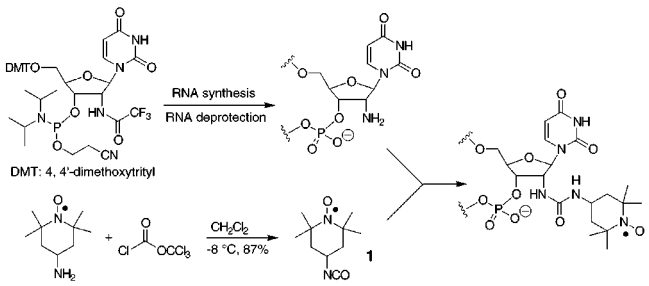
Department of Chemistry, University of Washington Seattle, Washington 98195-1700.

Received September 27, 2000
Revised Manuscript Received December 12, 2000

RNA can catalyze chemical reactions[1] and interacts in specific ways with other macromolecules, such as proteins. The mechanistic understanding of RNA function relies on structural information; however, solving crystal structures of complex RNA molecules remains challenging.[2] Therefore, other biophysical techniques have been used to determine the position of RNA secondary structure elements (i.e., helices, stem-loops, etc.) in three-dimensional space.[3] For example, fluorescence resonance energy transfer (FRET) can be used to measure long-range distances (35−85 Å). Techniques that allow measurement of intermediate distances would be valuable for improving the resolution of RNA structural models that are derived from FRET[4] and other biophysical techniques.

Electron paramagnetic resonance (EPR) spectroscopy has been used to study protein structure and folding,[5] and an EPR "spectroscopic ruler" has been described for measurement of distances between two unpaired electrons separated by 8−25 Å.[6] Moreover, EPR has been used to determine the solvent accessibility of spin-labels,[7] making EPR analysis of RNA containing a single unpaired electron a viable tool for the study of RNA tertiary structure. EPR is also a sensitive probe of dynamics over a wide range of motions (ps−ms) and has, for example, been used to determine the sequence-dependent motion of DNA duplexes.[8] EPR may, thus, yield information about the role of dynamics in RNA function, for which there is limited data available.[9] Furthermore, EPR active probes of dynamics have been used to elucidate structural differences in local regions within macromolecules.[10] The application of EPR for the study of RNA structure and dynamics requires incorporation of a stable free radical. We describe here the incorporation of a nitroxide spin-label into specific internal, base-paired sites of RNA and analysis of the spin-labeled RNA by EPR.

Scheme 1. Preparation of Spin-Labeled RNA



Nitroxide spin-labels have been incorporated into DNA, by conjugation to either the nucleoside base[11] or the sugar−phosphate backbone.[12] However, the currently available methods for the incorporation of spin-labels into RNA are restricted to either an unpaired uridine[13] or the 5′-end.[14] Both of these strategies are somewhat limited because the spin-label cannot be conjugated to internal, base-paired nucleotides. A variety of molecules have been conjugated to the 2′-position of base-paired nucleotides in RNA.[15] Particularly attractive is the use of a 2′-amino-modified RNA, which can be prepared by automated chemical synthesis using commercially available phosphoramidites (Scheme 1). The 2′-amino group can be reacted with electrophiles, such as aliphatic isocyanates.[16]

An isocyanate derivative of tetramethylpiperidyl-*N*-oxy (TEMPO) (**1**) was prepared in one step from the commercially available 4-amino-TEMPO (Scheme 1).[17] Subsequent reaction of isocyanate **1** with 2′-amino-modified RNA gave spin-labeled RNA in >90% yield (Supporting Information). Electrospray MS and enzymatic digestion of the modified oligomer verified incorporation of the spin-label into RNA. HPLC analysis of the enzymatic digest revealed the absence of a 2′-amino nucleoside and the presence of a strongly retained substance that was shown to coelute with the expected spin-labeled nucleoside, prepared by chemical synthesis (Supporting Information). A nucleoside containing a reduced spin-label was shown not to be present in the enzymatic digests, demonstrating that the nitroxide was not reduced during preparation of **1**[18] or incorporation into RNA. Finally, EPR spectroscopy of the spin-labeled RNA revealed the presence of a free radical, as described below.

To investigate the effect of the spin-label on the stability of RNA secondary structure, we incorporated the spin-label into the structurally well-characterized trans-activation responsive region

*Corresponding author. E-mail: sigurdsson@chem.washington.edu

(1) For a recent review, see: *The RNA World*, 2nd ed.; Gesteland, R. F., Cech, T. R., Atkins, J. F., Eds.; Cold Spring Harbor: 1999; Vol. 37. Doherty, E. A.; Doudna, J. A. *Annu. Rev. Biochem.* **2000**, *69*, 597−615.

(2) For recent reviews, see: Doherty, E. A.; Doudna, J. A. *Annu. Rev. Biochem.* **2000**, *69*, 597−615. Holbrook, S. R.; Kim, S. H. *Biopolymers* **1997**, *44*, 3-21.

(3) For a recent review, see: Kjems, J.; Egebjerg, J. *Curr. Opin. Biotechnol.* **1998**, *9*, 59−65.

(4) Tuschl, T.; Gohlke, C.; Jovin, T. M.; Westhof, E.; Eckstein, F. *Science* **1994**, *266*, 785−788. Walter, N. G.; Hampel, K. J.; Brown, K. M.; Burke, J. M. *EMBO J.* **1998**, *17*, 2378−2391. Walter, F.; Murchie, A. I. H.; Lilley, D. M. J. *Biochemistry* **1998**, *37*, 17629−17636.

(5) For a recent review, see: Hubbell, W. L.; Cafiso, D. S.; Altenbach, C. *Nat. Struct. Biol.* **2000**, *7*, 735−739.

(6) Rabenstein, M. D.; Shin, Y. K. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8239−8243. Hustedt, E. J.; Smirnov, A. I.; Laub, C. F.; Cobb, C. E.; Berth, A. H. *Biophys. J.* **1997**, *72*, 1861−1877.

(7) Lin, Y.; Nielsen, R.; Murray, D.; Hubbell, W. L.; Mailer, C.; Robinson, B. H.; Gelb, M. H. *Science* **1998**, *279*, 1925−1929. Ball, A.; Nielsen, R.; Gelb, M. H.; Robinson, B. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6637−6642. Oh, K. J.; Altenbach, C.; Collier, R. J.; Hubbell, W. L. *Methods Mol. Biol.* **2000**, *145*, 147−169.

(8) Okonogi, T. M.; Alley, S. C.; Reese, A. W.; Hopkins, P. B.; Robinson, B. H. *Biophys. J.* **2000**, *78*, 2560−2571.

(9) For a recent review see: Hagerman, P. J. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 139−156.

(10) Spaltenstein, A.; Robinson, B. H.; Hopkins, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 2303−2305.

(11) Spaltenstein, A.; Robinson, B. H.; Hopkins, P. B. *Biochemistry* **1989**, *28*, 9484−9495. Strobel, O. K.; Kryak, D. D.; Bobst, E. V.; Bobst, A. M. *Bioconjugate Chem.* **1991**, *2*, 89−95. Miller, T. R.; Alley, S. C.; Reese, A. W.; Solomon, M. S.; McCallister, W. V.; Mailer, C.; Robinson, B. H.; Hopkins, P. B. *J. Am. Chem. Soc.* **1995**, *117*, 9377−9378.

(12) Nagahara, S.; Murakami, A.; Makino, K. *Nucleosides Nucleotides* **1992**, *11*, 889−901.

(13) Ramos, A.; Varani, G. *J. Am. Chem. Soc.* **1998**, *120*, 10992−10993. Hara, H.; Horiuchi, T.; Saneyoshi, M.; Nishimura, S. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 305−311.

(14) Macosko, J. C.; Pio, M. S.; Tinoco, I. Jr.; Shin, Y. K. *RNA* **1999**, *5*, 1158−1166.

(15) Manoharan, M. In *Antisense Research and Applications*; Crooke, S. T., Lebleu, B., Eds.; CRC Press: Boca Raton, Ann Arbor, London, Tokyo, 1993; pp 303−349. Douglas, M. E.; Beijer, B.; Sproat, B. S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 995−1000. Sigurdsson, S. T.; Tuschl, T.; Eckstein, F. *RNA* **1995**, *1*, 575−583.

(16) Sigurdsson, S. T.; Eckstein, F. *Nucleic Acids Res.* **1996**, *24*, 3129−3133.

(17) Sigurdsson, S. T.; Seeger, B.; Kutzke, U.; Eckstein, F. *J. Org. Chem.* **1996**, *61*, 3883−3884.

(18) Nitroxides have been shown to disproportionate in the presence of HCl, which is generated during the preparation of **1**. See: Rozantsev, E. G. *Free Nitroxyl Radicals*; Plenum Press: New York, 1970; pp 102−105.

10.1021/ja005649i CCC: $20.00
Published on Web 01/26/2001

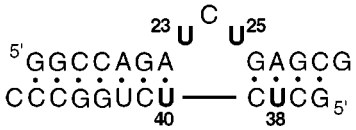


Figure 1. The TAR RNA. Four samples were prepared, each containing one spin-label at position U23, U25, U38 or U40.

Table 1. Thermodynamic Data for Spin-Labeled TAR RNAs[a]

	T_m (°C)	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol·K)
unmodified	50.7 ± 1.1	2.45 ± 0.17	58.2 ± 5.2	180 ± 16
U23	51.6 ± 1.2	2.93 ± 0.24	66.4 ± 8.4	205 ± 26
U25	50.7 ± 0.8	3.12 ± 0.39	73.7 ± 1.8	227 ± 12
U38	47.8 ± 0.8	1.78 ± 0.07	53.5 ± 5.3	167 ± 17
U40	49.1 ± 1.2	2.19 ± 0.12	59.6 ± 2.8	185 ± 10

[a] Optical melting experiments were performed in 50 mM NaCl, 0.05 mM Na_2EDTA, and 5 mM sodium phosphate buffer (pH 7.0).

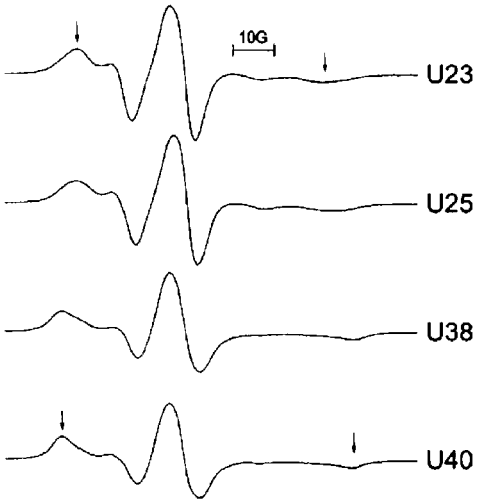


Figure 2. EPR spectra of spin-labeled TAR RNAs at 0 °C in 50% sucrose/100 mM NaCl, 0.1 mM Na_2EDTA, and 10 mM sodium phosphate buffer (pH 7.0). The arrows identify the high and low field extrema of the spectra. The decreased motion of U38/U40 relative to U23/U25 results in a wider spectrum.

(TAR) of HIV RNA (Figure 1),[19] whose interaction with the Tat protein is essential for efficient transcription during replication of the HIV virus.[20] Four TAR RNAs were prepared, each containing a single spin-label in position U23, U25, U38 or U40. Optical melting experiments were used to determine the thermodynamic parameters of duplex formation for the unmodified TAR RNA and the spin-labeled RNAs (Table 1).[21] The melting curves for each sample showed a single cooperative and reversible melting transition. The modified RNAs had melting temperatures within 3 °C of the unmodified RNA. The only modification that destabilized the TAR RNA was that of U38, but only by ~0.7 kcal/mol. Interestingly, modification of both U23 and U25, located in the bulge loop, stabilized the TAR RNA by ~0.5 kcal/mol. These results indicate that the spin-label does not appreciably perturb the secondary structure of RNA.

The EPR spectra of the spin-labeled TAR RNAs were recorded in a viscous medium (Figure 2). Under these conditions, the uniform modes of motion for the duplexes are slow enough for the spectra to be sensitive to the internal motion of the spin-label. The spectra for TAR RNA containing spin-labels at U38 and U40 are similar and show the correlation times expected for the uniform modes of the TAR RNA (Supporting Information). These results show that there is limited motion of the spin-label independent of the nucleic acid, a prerequisite for probes of dynamics. This was not a foregone conclusion, because there is, in principle, free rotation possible around two bonds in the tether, namely, those flanking the urea moiety.

The spectra of U23 and U25 show significantly more mobility (~40-fold) than spin-labeled U38 or U40. In fact, the mobility of U23 or U25 is similar to that of a single strand, exhibiting motion on the nanosecond time scale (Supporting Information). The finding that nucleotides in a loop are more dynamic than those located in a duplex region is not unexpected and has been previously observed in EPR studies of DNA using both partially flexible[10] and rigid spin-labels.[22] Relaxation experiments using ^{13}C NMR have also shown increased motion of the bases in the TAR loop.[23] While it is possible that some of the differences in mobility of U23 (or U25) relative to U38 (or U40) are due to motion of the spin-label independent of the nucleic acid, the EPR probe clearly distinguishes between nucleotides constrained in a duplex region and those found in a bulge loop.

To our knowledge, this is the first EPR study of nucleic acid dynamics using spin-labels that are tethered to the sugar-moiety of nucleotides. In contrast to previous EPR studies of nucleic acid dynamics, this approach does not require a multistep synthesis for the site-specific incorporation of the spin-label.[11] The spin-labeling method is applicable to RNA prepared by solid-phase synthesis (ca. 40−50 nt long). However, spin-labeled RNA can be ligated to a much longer RNA that has, for example, been prepared by in vitro transcription.[24] Although this ligation strategy is not very efficient, it is feasible because small quantities (nmol) are required for EPR studies.

In conclusion, we have shown that nitroxide spin-labels can be site-specifically incorporated into internal sites of RNA utilizing readily available materials. Incorporation of the spin-label into the TAR RNA does not appreciably affect the stability of the duplex, indicating that the spin-labels are structurally nonperturbing. EPR analyses of the spin-labeled TAR RNAs revealed dramatic structure-dependent mobility differences among the spin-labeled sites. The changes in mobility of these sites upon binding to the Tat protein are currently under investigation and will be reported in due course.

Acknowledgment. We thank NIH (GM56947 and GM55963) and NIEHS for financial support. T.E.E. was supported by the NIH National Research Service Award 5 T32 GM08268. We thank members of the Sigurdsson group and T. Madden for critical reading of the manuscript.

Note Added after ASAP. The version of this article posted ASAP on 1/26/01 contained incorrect data for T_m and $-\Delta G°_{37}$ in Table 1 that was introduced during the production process. The correct version was posted on 2/14/01.

Supporting Information Available: (1) Synthetic procedures for compound **1** and spin-labeled nucleoside **2**, derived from reaction of **1** with 2′-amino-2′-deoxyuridine, and their spectra (^1H NMR, MS and IR); (2) protocols for preparation and purification of spin-labeled RNA; (3) HPLC analyses of spin-labeling reactions and enzymatic digestions of spin-labeled RNA; (4) EPR spectra of **2**, spin-labeled TAR RNA, single strand and duplex controls; and (5) EPR simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

JA005649I

(19) Ippolito, J. A.; Steitz, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9819−9824.
(20) For recent reviews, see: Karn, J. *J. Mol. Biol.* **1999**, *293*, 235−254. Rana, T. M.; Jeang, K. T. *Arch. Biochem. Biophys.* **1999**, *365*, 175−185.
(21) Breslauer, K. J. *Methods Mol. Biol.* **1994**, *26*, 347−372.
(22) Okonogi, T. M.; Robinson, B. H. Manuscript in preparation.
(23) King, G. C.; Harper, J. W.; Xi, Z. J. *Methods Enzymol.*; **1995**; *261*, 436−450.
(24) Moore, M. J.; Sharp, P. A. *Science* **1992**, *256*, 992−997.