

Long-Range Distance Measurements to the Phosphodiester Backbone of Solid Nucleic Acids Using ^{31}P – ^{19}F REDOR NMR

Matthew E. Merritt, Snorri Th. Sigurdsson*, and Gary P. Drobny*

Department of Chemistry, University of Washington
Seattle, Washington 98195

Received December 4, 1998

Revised Manuscript Received May 13, 1999

With the increasing recognition of the importance of the phosphate backbone in nucleic acid structure and function,^{1–6} we became interested in applying solid-state NMR to the study of this region. Rotational-echo double-resonance (REDOR) NMR measures the heteronuclear dipolar coupling in the solid state, without any dependence on spectral densities.^{7,8} Since the static dipolar coupling is measured in REDOR, there is the potential to measure distances 2 to 3 times as long as those measured by liquid-state NOE experiments. For example, ^{31}P – ^{19}F REDOR has recently been used to measure distances in the 12–16 Å range in an enzyme–substrate complex.⁹

However, the application of ^{31}P –X REDOR to nucleic acids is limited by the fact that, except in a few cases, the solid-state ^{31}P NMR spectrum shows no resolution of distinct sites on the backbone. To circumvent the lack of resolution in the ^{31}P spectrum, phosphorothioates can be site-specifically introduced into DNA and RNA. Due to the ease of incorporation of phosphorothioates into nucleic acids and their interesting metal-binding properties, they have been used in elucidating the mechanism of restriction enzymes and ribozymes.^{10–13} In this paper we present the use of phosphorothioates and fluorinated nucleotides in conjunction with REDOR NMR to measure distances from the bases and sugars of DNA to the phosphodiester backbone.

Eckstein and Jovin were the first to use phosphorothioates to assign resonances in the phosphodiester backbone of DNA.¹⁴ The substitution of a single nonbridging oxygen atom with a sulfur atom shifts the phosphorus resonance downfield by approximately 55 ppm (Figure 1). Since the sulfur is only slightly larger than an oxygen and does not change the charge of the phosphate group, it is much less perturbing than methyl phosphonate or other substitutions that neutralize the backbone charge.^{1,3} Indeed, X-ray crystallography of a DNA hexamer containing three phosphorothioates indicates that the phosphate linkages are “conformationally equivalent” to the phosphorothioates in the structure.¹⁵

* Corresponding authors.

(1) Tomky, L. A.; Strauss-Soukup, J. K.; Maher, L. J., III. *Nucleic Acids Res.* **1998**, *26*, 2298–2305.

(2) Thorogood, H.; Grasby, J. A.; Connolly, B. A. *J. Biol. Chemistry* **1996**, *271*, 8855–8862.

(3) Strauss, J. K.; Maher, L. J., III. *Science* **1994**, *266*, 1829–1834.

(4) Puglisi, J. D.; Chen, L.; Frankel, A. D.; Williamson, J. R. *Science* **1993**, *90*, 3680–3684.

(5) Ferre-D'Amare, A. R.; Zhou, K.; Doudna, J. *Nature* **1998**, *395*, 567–574.

(6) Calnan, B. J.; Tidor, B.; Biancalana, S.; Hudson, D.; Frankel, A. D. *Science* **1991**, *252*, 1167–1171.

(7) Gullion, T.; Schaefer, J. *J. Magn. Reson.* **1989**, *81*, 196.

(8) Gullion, T.; Schaefer, J. *Adv. Magn. Reson.* **1989**, *13*, 55.

(9) Studelska, D. R.; Klug, C. A.; Beusen, D. D.; McDowell, L. M.; Schaefer, J. *J. Am. Chem. Soc.* **1996**, *118*, 5476–5477.

(10) Milligan, J. F.; Uhlenbeck, O. C. *Biochemistry* **1989**, *28*, 2849–2855.

(11) Eckstein, F. *Annu. Rev. Biochem.* **1985**, *54*, 367–402.

(12) Eckstein, F.; Thomson, J. B. *Methods Enzymol.* **1995**, *262*, 189–202.

(13) Sigurdsson, S. T.; Thomson, J. B.; Eckstein, F. In *RNA Structure and Function*; Simons, R. W., Ed.; Cold Spring Harbor Laboratory Press: New York, 1998; pp 339–375.

(14) Eckstein, F.; Jovin, T. M. *Biochemistry* **1983**, *22*, 4546–4550.

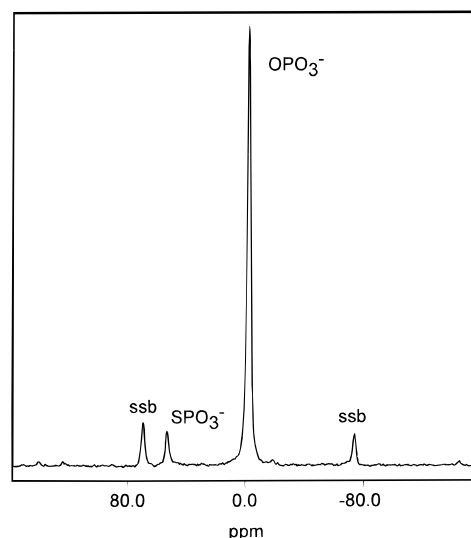


Figure 1. REDOR S_0 (full echo) spectrum with eight rotor cycles of evolution for the $[5'\text{-d}(\text{CGCGAA}\text{-}(\text{pS})\text{-T}^{59}\text{FUCGCG})_2]$ where ^{59}F U denotes 2'-deoxy-5-fluorouridine and (pS) denotes the position of the phosphorothioate. Magic-angle spinning was controlled at 5988 ± 1 Hz. 4096 scans acquired. ssb signifies a spinning side band.

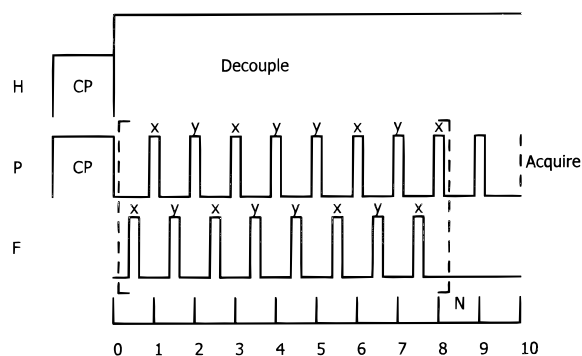


Figure 2. XY8-REDOR pulse sequence for measuring ^{31}P – ^{19}F dipolar couplings. Following cross-polarization from ^1H to ^{31}P , the phosphorus signal is refocused by a series of 180° pulses following the XY8 phase cycling. On alternate scans, ^{19}F pulses were used to recouple the fluorine to the phosphorus nuclei.

With the incorporation of fluorinated nucleotides into DNA and RNA, it is possible to introduce a unique ^{31}P – ^{19}F spin pair into a nucleic acid. Furthermore, since the phosphorothioate is chemically shifted from the backbone, it is possible to perform the REDOR experiment by observing the ^{31}P and dephasing with ^{19}F , without any background correction for the phosphate backbone.

Figure 2 shows the XY8-REDOR pulse sequence as implemented in our experiments. Since the ultimate goal is measurement of weak dipolar couplings, we choose to apply the XY8 phase cycling scheme on both channels.¹⁶ This ensures the maximum amplitude for the refocused phosphorus signal. The experiment is acquired in two parts, one with (S) and one without (S_0) the ^{19}F pulses. The ratio S/S_0 gives a dephasing curve which can be fit by simulation to give the dipolar coupling and hence the distance between the two nuclei.¹⁷ Our measurements were carried out on hydrated amorphous solids at a magnetic field of 4.7 T, with a homebuilt spectrometer and probe.^{18,19} All experiments were carried out at a temperature of -15°C .

(15) Cruse, W. B. T.; Salisbury, S. A.; Brown, T.; Cosstick, R.; Eckstein, F.; Kennard, O. *J. Mol. Biol.* **1986**, *192*, 891–905.

(16) Gullion, T.; Baker, D.; Conradi, M. S. *J. Magn. Reson.* **1990**, *89*, 479.

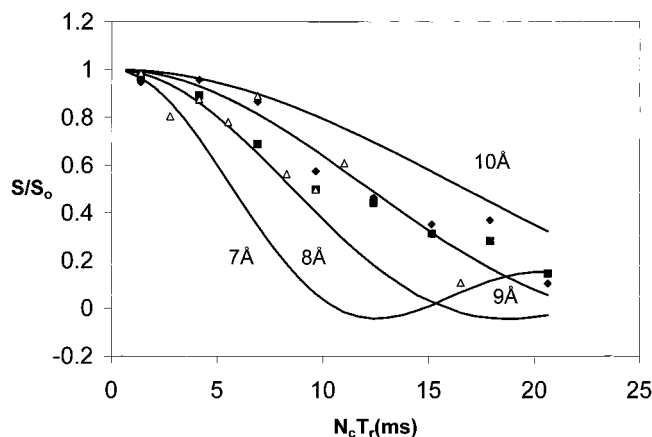


Figure 3. REDOR dephasing curve for $[5'\text{-d}(\text{CGCGAA}-(\text{pS})\text{-T}^{2\text{F}}\text{UCGCG})_2]$. The ^{31}P H_1 field was 50 kHz, and the ^{19}F H_1 field was 66 kHz with active H_1 control. Solid lines are simulations. Open triangles mark data for the DNA at a hydration level of $W = 3$, squares for $W = 10$, and diamonds for $W = 14$.

To test this strategy, modifications were introduced into a structurally well characterized DNA dodecamer.²⁰ We prepared $[5'\text{-d}(\text{CGCGAA}(\text{pS})\text{T}^{2\text{F}}\text{UCGCG})_2]$ by automated chemical synthesis²¹ with a phosphorothioate placed between A6 and T7 and a 2'-deoxy-5-fluorouridine at position eight. Assuming no perturbation by the inclusion of the ^{19}F , the crystallographically determined distance is 7.7 Å (PDB ID# 1BNA). The crystal structure was obtained at a hydration level of 10 water molecules/nucleotide ($W = 10$). Earlier analysis by ^{19}F DRAWS indicates that incorporation of 2'-deoxy-5-fluorouridine at positions T7 and T8 has a minimal effect on the distance between the bases (0.2 Å).²² Figure 3 shows the dephasing curve for the sample as a function of hydration. The distance measured by ^{31}P - ^{19}F REDOR was 8.5 ± 1 angstrom for the sample hydrated to a level of $W = 3$. As the hydration level increased, the measured distance increased from 8.5 ± 1 Å ($W = 10$) to 9 ± 1 angstrom ($W = 14$). The intensity of the distribution of ^{31}P spinning sidebands did not change upon the increase in hydration, indicating that the amplitude of local motions in the backbone of the DNA did not change at this position. As for motion of the base, the transverse relaxation (T_2) of the ^{19}F label at position T8 decreased from 1.5 ms to 1 ms upon hydration to $W = 11$, indicating increased motion at the site. However, ^2H NMR studies of [methyl- ^2H]2'-deoxythymidine at position T8 indicated that the base undergoes only a small amplitude libration ($11 \pm 2^\circ$).²³ A calculation of the averaging of the internuclear distance due to the librational motion indicated that it could account for a change of 0.1 Å at the most. Therefore, the ~ 8.5 -Å distance attained by REDOR is not strongly perturbed by motion at this position in the DNA, and thus, the data agrees with the crystal structure.

(17) Despite the fact that there are relatively strong static homonuclear couplings between the phosphorus nuclei in the backbone (~ 70 Hz), REDOR experiments with only a single phosphorus refocusing pulse, as opposed to 8, gave the same S/S_0 ratio for selected points in the dephasing curve. This result indicates that the XY8 pulse train on the phosphorus channel does not reintroduce phosphorus homonuclear couplings attenuated by magic angle spinning.

(18) Drobny, G. P.; Gladden, J., unpublished work.

(19) Stringer, J. A.; Drobny, G. P. *Rev. Sci. Instrum.* **1998**, *69*, 3384-3391.

(20) Dickerson, R. E.; Drew, H. R. *J. Mol. Biol.* **1981**, *149*, 761-786.

(21) All oligomers were synthesized using standard phosphoramidite chemistry with commercial reagents (Glen Research and ABI).

(22) Merritt, M. E.; Stringer, J. S.; Geahigan, K.; Drobny, G. P., manuscript in preparation.

(23) Alam, T. M.; Drobny, G. P. *Chem. Rev.* **1991**, *91*, 1545-1590.

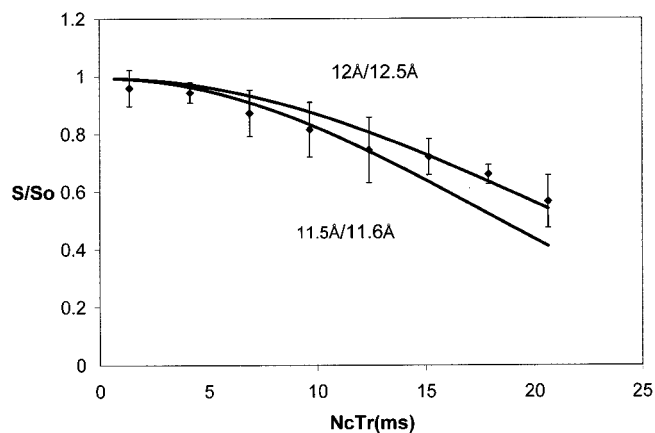


Figure 4. REDOR dephasing curve for the $[5'\text{-d}(\text{CGCGAA}(\text{pS})\text{-T}^{2\text{F}}\text{UCGCG})_2]$ where $^{2\text{F}}\text{U}$ denotes 2'-deoxy-2'-fluorouridine. Solid diamonds mark the data. The lines mark three-spin simulations for two different sets of ^{31}P - ^{19}F internuclear distances (see text for discussion).

Another possible labeling scheme incorporates a fluorine label into the sugar moiety. The second compound studied was $[5'\text{-d}(\text{CGCGAA}(\text{pS})\text{T}^{2\text{F}}\text{UCGCG})_2]$ with a 2'-deoxy-2'-fluorouridine inserted at position 8 and a phosphorothioate (pS) incorporated between A6 and T7. The analysis of the data was complicated by the fact that, according to the crystal structure (PDB ID# 1BNA), each phosphorothioate should be coupled to both fluorines of this self-complementary DNA, i.e., intra- and interstrand interactions are present. Three spin simulations²⁴ using the crystallographically determined distances of 11.6 Å and 11.5 Å (the analogous distances in the opposing strand were 11.1 Å and 11.4 Å) predicted dephasing that was faster than the experimentally determined curve (Figure 4). Our best three-spin fit used distances of 12 Å and 12.5 Å with an uncertainty of ± 1 Å.²⁵ Hence, our data agrees with the crystal structure, which was solved at 2.6 Å resolution.

In conclusion, it has been demonstrated that ^{31}P - ^{19}F REDOR will be useful for generating distance constraints to the backbone of DNA and RNA. The inclusion of 2'-deoxy-5-fluorouridine, 2'-deoxy-2'-fluorouridine, and the phosphorothioates apparently has a negligible effect on the DNA structure. This labeling strategy coupled with REDOR NMR has several advantages. The high gyromagnetic ratios of the two nuclear spins allows distances as large as 16 Å to be measured.⁹ Furthermore, the high sensitivity of the ^{31}P enables small amounts of labeled DNA and RNA to be used for the experiments (on the order of 1 μmol or less at higher fields). Finally, backbone distances to both the sugar ring and the base of nucleic acids are measurable.

The strategy described in this paper will be useful for several different applications. First, it could be applied to the study of the interaction of drugs or proteins with the backbone of nucleic acids. Second, distances to the backbone could be used to refine current DNA or RNA structures which are under-constrained in this area. Finally, this methodology has great potential in the study of tertiary and quaternary structures of large nucleic acids that defy analysis by current analytical techniques.

Acknowledgment. The authors are grateful to Dr. S. A. Weerawarna for help in sample preparation. This work was supported by NIH grant R01GM47802-04. JA9841730

(24) Goetz, J. M.; Schaefer, J. J. *Magn. Reson.* **1997**, *127*, 147-154.

(25) The simulations were carried out according to Goetz and Schaefer²⁴ with an angle of 67° between the two fluorine labels, as defined by the ^{31}P - ^{19}F dipolar vectors. Further simulations indicate that the angle between the vectors has a minimal effect on the simulated distance unless the three spins are arranged in a linear fashion, with the ^{31}P between the ^{19}F 's.