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Determination of DNA Structure in Solution: Enzymatic Deuteration of the Ribose 2′ Carbon

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Recently, solution NMR spectroscopy has accurately determined the pathway of the helix for DNA helices just over one helical turn by including residual dipolar couplings (RDCs) in refinement.[1,2] Structural analysis of double-helical nucleic acids in the absence of proteins by X-ray crystallography has been limited to less than 1.5 turns and has proven ambiguous.[3] In addition, crystallization of these molecules is problematic due to their surface charge and flexible nature. Structural determination of longer DNA helices will be even more dependent on RDCs, so methods to increase the available set of RDCs are needed. Idek et al. have developed techniques to measure two-bond RDCs in the bases of nucleic acids, which should better define their angles relative to the helical axis.[4] Segmental ^{13}C-labeling already allows for the acquisition of a nearly complete set of C1′−H1′, C3′−H3′, and C4′−H4′ RDCs, but these still do not allow for the accurate determination of the sugar conformation.[2] C2′−H2′ and C2′−H2″ RDCs are necessary to define the ring conformation. The geminal protons on the 2′ carbon produce a doublet of doublets masking the individual couplings. Deuteration at the H2″ position of the ribose allows the one-bond C2′−H′ *J* coupling ($^1J_{C2'H'}$) to be measured since the C2′−D″ bond coupling frequency can be decoupled.[1]

We present here a method that uses ribonucleotide triphosphate reductase (RTPR) from *Lactobacillus leichmannii* for the reduction of the 2′ hydroxyl of uniformly ^{13}C/^{15}N-labeled ribonucleotide triphosphates. When this reaction is performed in D_2O, a deuteron instead of the usual proton is introduced at the H2″ position (see Figure 1, step 1).[5] The conversion of dUTP to dTMP is achieved in two additional enzymatic steps: hydrolysis of the triphosphate to the monophosphate using deoxyuridine 5′-triphosphate nucleotidohydrolase (dUTPase) from *Escherichia coli* (see Figure 1, step 2)[6] and addition of the methyl group to the number 5 carbon of uracil via thymidylate synthase (*Bacillus subtilis*) in the presence of N5,N10-methylene tetrahydrofolate (see Figure 1, step 3).[7] The resulting dTMP is converted to dTTP using standard enzymatic methods.[8]

Previously, deuteration at the H2″ position has been performed via a synthetic scheme starting from ^{13}C-labeled glucose, yielding a protected ribose derivative that was then converted to doubly labeled phosphoramidites following established procedures.[9] Although this synthetic scheme appears useful, doubly labeled phosphoramidites obtained in this manner have only been reported in one DNA structural determination.[1] One drawback is the multistep synthetic route required to produce the doubly labeled phosphoramidites. In addition, a large amount of starting material is needed for automated DNA synthesizers, which were not designed for high yield of product.

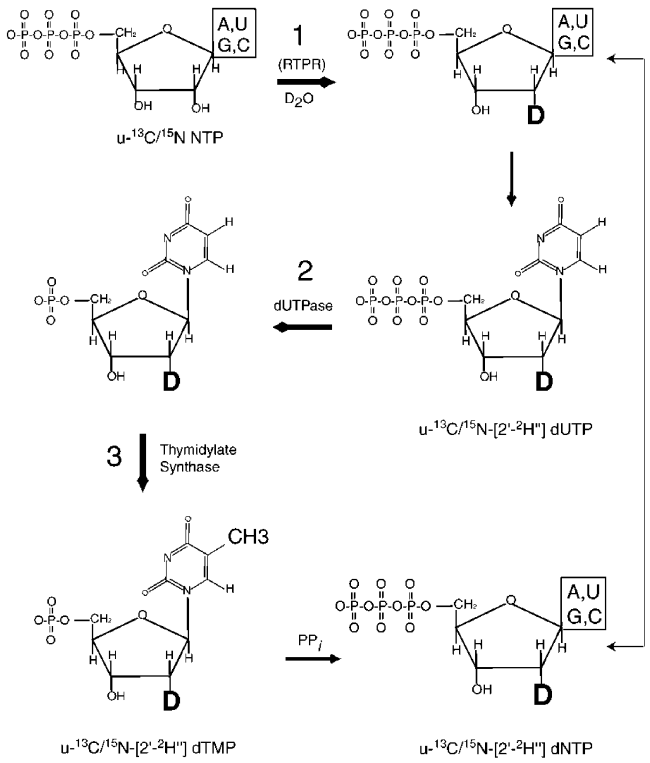

Figure 1. Overall experimental design. Step 1 was carried out in 99% D_2O in the dark to avoid breakdown of the cofactor adenosylcobalamin. The additional steps, 2 and 3, were necessary to convert ^{13}C/^{15}N-[2′-^{2}H″] dUTP to ^{13}C/^{15}N-[2′-^{2}H″] dTMP. The monophosphate (dTMP) was phosphorylated to the triphosphate using standard enzymatic methods.

The deuterated nucleotides obtained as shown in Figure 1 have been used to segmentally label a 20-bp DNA helix that contains the adenovirus major late promoter (AdMLP). Figure 2 shows the C2′−H2′/H2″ region of [^{1}H,^{13}C] ctHSQC spectra recorded on this 20-bp DNA sequence. Nucleotides 6−14, shown in boldface letters, are uniformly labeled with ^{13}C and ^{15}N. The gray box over the sequence indicates the position of the conserved TATA box. Without stereospecific deuteration at the 2H″ position, the recorded $^1J_{CH}$ coupling in the F1 dimension is the sum of both the $^1J_{CH'}$ and $^1J_{CH''}$ couplings (Figure 2A). Deuteration at the H2″ position allows the measurement of the $^1J_{CH'}$ coupling (Figure 2B). Notice that the *J* coupling in spectrum B is approximately half the recorded value of spectrum A. Subtraction of the *J* couplings obtained in spectrum A from spectrum B allows the $^1J_{CH''}$ couplings to be calculated (Figure 2A+B). The slight alignment of these helices will allow the determination of the corresponding RDCs for these bond vectors.[10]

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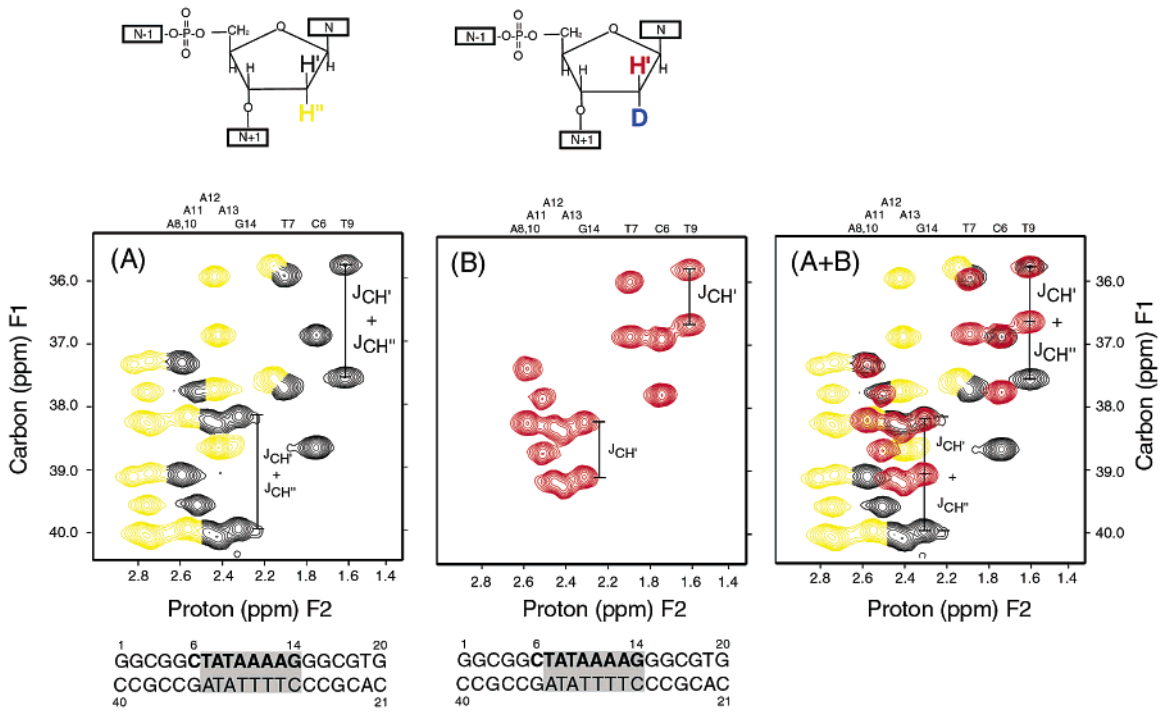

Figure 2. Acquisition of *J* coupling. (A) C2′−H2′/H2″ region of a [^{1}H,^{13}C] ctHSQC spectrum of a 20-bp DNA helix containing the AdMLP with nucleotides 6−14, boldface letters, isotopically labeled ^{13}C/^{15}N. The measured *J* coupling in the F1 dimension is the sum of both the $^{1}J_{CH'}$ and $^{1}J_{CH''}$ values. The individual coupling values cannot be determined. The gray box indicates the conserved TATA box. (B) Same region but with the proton at the H2″ position of nucleotides 6−14 replaced with a deuteron. The observed coupling is equal to the $^{1}J_{CH'}$ value. (A+B) Overlay of spectra A and B. The slight alignment of these two helices will enable the accurate determination of one-bond $D_{CH'}$ and $D_{CH''}$ couplings.

The overall yield for this enzymatic method is >95% for the synthesis of ^{13}C/^{15}N-[2′-^{2}H″] dATP, dGTP, and dCTP. The synthesis of ^{13}C/^{15}N-[2′-^{2}H″] dTTP requires an additional three steps, with an overall yield >80%. This method allows for the easy and high-yield conversion of ^{13}C/^{15}N NTPs that are already isolated when producing ^{13}C/^{15}N dNTPs. Furthermore, these NTPs are present in the cell at ∼4.5× greater amounts compared to dNTPs. This enzymatic deuteration at the 2H″ position along with segmental labeling allows the recording of the C2′−H2′ and C2′−H2″ *J* couplings and residual dipolar couplings. These additional data allow the accurate determination of sugar pucker and add to the list of easily obtainable RDCs. Interesting DNA double helices of 2−3 turns are now within the window measurable by solution NMR spectroscopy.

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Supporting Information Available: Detailed methods describing the isolation and purification of all enzymes, reaction conditions for all steps (PDF), and ref 12 as a Word2000 document. This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) The plasmid pSQUIRE that contains the gene for RTPR from *Lactobacillus leichmannii* was a kind gift from JoAnne Stubbe (MIT). The RTPR was expressed and purified according to Booker and Stubbe: Booker, S.; Stubbe, J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8352−8356.
(6) The gene corresponding to dUTPase from *E. coli* was cloned directly from the E. coli genome with PCR primers based on the sequence in genbank for *E. coli* K12 gene=dut. The amplified gene was ligated in phase at the 3′ of the sequence corresponding to the maltose binding protein (MBP). The MBP−dUTPase fusion protein was expressed and purified using standard techniques.
(7) Thymidylate synthase was a generous gift from Frank Maley (Department of Health, New York) and used with a folate cocktail (personal communication with F. Maley). The cofactor (6*R*,*S*)-5,10-methylene-5,6,7,8-tetrahydrofolic acid, magnesium salt, was purchased from Schiracks Labs (Switzerland).
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