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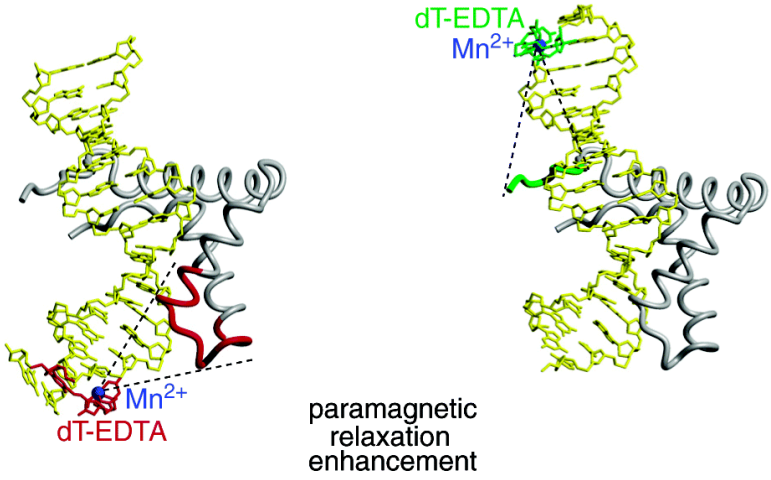

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EDTA-Derivatized Deoxythymidine as a Tool for Rapid Determination of Protein Binding Polarity to DNA by Intermolecular Paramagnetic Relaxation Enhancement

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Despite recent advances in NMR methodology,[1] determining the polarity of protein binding to DNA by NMR still represents a formidable task. Assignment of intermolecular nuclear Overhauser (NOE) interactions is complicated by extensive spectral overlap in the sugar region of the DNA ^{1}H NMR spectrum, a low density of protons on the DNA, lack of sensitivity of isotope-filtered/separated experiments, and potential line broadening due to exchange processes. While relative orientational information (with four-fold degeneracy) can be derived from dipolar coupling measurements,[2] this necessitates isotopic (^{15}N and/or ^{13}C) labeling not only of the protein but also of the DNA. While feasible, the latter is technically challenging and expensive.[3] Moreover, dipolar couplings yield no translational information.[2]

Paramagnetic relaxation enhancement (PRE) of ^{1}H nuclei is a well-known source of long-range distance information but has not gained widespread use for systems that do not possess an intrinsic paramagnetic center.[4] Extrinsic paramagnetic centers can be introduced into proteins by engineering a nitroxide spin-label[5] or metal-binding site.[6] For nucleic acids, nitroxide spin-labeling can be achieved by derivatizing pyrimidines.[7] PRE has been well characterized for paramagnetic metal-binding proteins.[4] If a tight metal-binding site could be introduced into DNA in a simple manner, the polarity of protein binding could be determined by observing intermolecular PRE effects on the protein. Here, we show that DNA containing EDTA-derivatized deoxythymidine (dT-EDTA; shown in Figure 1a) is ideally suited for this purpose.

dT-EDTA was originally developed as a reagent for sequence-specific cleavage of double-stranded (ds) DNA.[8] In the presence of Fe^{2+} and dithiothreitol (DTT), cleavage by chemically active hydroxyl radicals generated by Fe^{2+} chelated to EDTA occurs at sites close to a dT-EDTA nucleotide. This reaction is widely used, and the phosphoramidite derivative of dT-EDTA is commercially available.[9] Thus, solid-phase synthesis of DNA containing dT-EDTA at any desired position is readily feasible and inexpensive. Using HPLC/electrospray mass spectrometry, we found that the cleavage reaction is specific for Fe^{2+} and DTT and does not occur, even after many days, when other metal ions are chelated to dT-EDTA (details provided in Supporting Information). For chelation with Ca^{2+}, Mn^{2+}, and Fe^{3+}, the observed mass of the dT-EDTA containing strand is that of the derivatized oligonucleotide plus the mass of the metal ion minus the mass of displaced protons (two for divalent and three for trivalent cations). Thus, metal ion binding to dT-EDTA is extremely tight, consistent with the known K_D's of $\sim 10^{-8}$, $\sim 10^{-11}$, and $\sim 10^{-21}$ M for the binding of Ca^{2+}, Mn^{2+}, and Fe^{3+}, respectively, to EDTA at neutral pH.[10] DNA−EDTA chelated to Ca^{2+} or Mn^{2+} is therefore highly stable and suitable for the measurement of intermolecular PRE effects.

To assess the utility of DNA−EDTA for determining the polarity

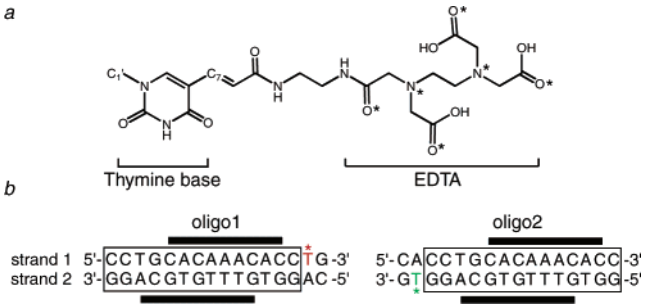

Figure 1. (a) Chemical structure of dT-EDTA.[9] The nitrogen and oxygen atoms involved in metal ion coordination are indicated by asterisks.[11] (b) Sequences of the two 16-bp DNA oligonucleotides. The boxed-in region indicates the DNA used in the structure determination of the SRY/DNA complex, and the underlined regions delineate the SRY binding site.[12] The location of dT-EDTA is indicated by an asterisk. The individual DNA strands were synthesized using cyanoethyl phosphoramidite chemistry.

of a protein bound to DNA, we applied this method to the SRY/DNA complex whose structure has been determined.[12] Two 16 base pair (bp) ds-DNA oligonucleotides (oligo1 and oligo2) encompassing the 14 bp's used previously[12] and two additional base pairs at either end with a single dT-EDTA (Figure 1b) were synthesized. ^{15}N/^{13}C-labeled SRY/DNA−EDTA complexes chelated to either diamagnetic Ca^{2+} or paramagnetic Mn^{2+} were analyzed. Note that it is critical to remove divalent ions at sites other than the attached EDTA, because DNA itself has weak multivalent ion binding sites with K_D's of 10^{-4}−10^{-3} M.[13]

^{1}H−^{15}N HSQC spectra of the diamagnetic Ca^{2+}- and paramagnetic Mn^{2+}-chelated states of the SRY/DNA(oligo1)−EDTA complex are shown in Figure 2a. The ^1H_N/^{15}N chemical shifts of the bound protein are identical for the Ca^{2+}- and Mn^{2+}-chelated states, because pseudocontact shift effects for Mn^{2+} are negligible due to its symmetric $3d^5$ electronic configuration. Cross-peaks for residues 29−34 are visibly broadened in the ^{1}H−^{15}N HSQC spectrum of the Mn^{2+}-chelated oligo1 complex (Figure 2b, right) but not the Ca^{2+}-chelated complex. Values of $^1H_N-T_2$ relaxation enhancement ($^1H_N-\Gamma_2$) for SRY were determined by taking the difference in measured $^1H_N-T_2$ rates in the paramagnetic (Mn^{2+}) and diamagnetic (Ca^{2+}) states.[6d] The residues exhibiting $^1H_N-\Gamma_2$ values ≥ 15 s^{-1} are located in regions closest to the dT-EDTA−Mn^{2+} site: residues 24−37 for the oligo1 complex and residues 74−79 for the oligo2 complex (Figure 2b). These results indicate that metal ion chelated DNA−EDTA represents a powerful tool for rapidly establishing protein binding polarity in a protein−DNA complex, providing the structure of the free protein is already known.

PRE data are not simply qualitative but quantitative (Figure 2c). Simulated annealing refinement[14] against the observed $^1H_N-\Gamma_2$ values[6d] with the positions of the two bp's at either end and the EDTA−Mn^{2+} moieties given their full torsional degrees of freedom

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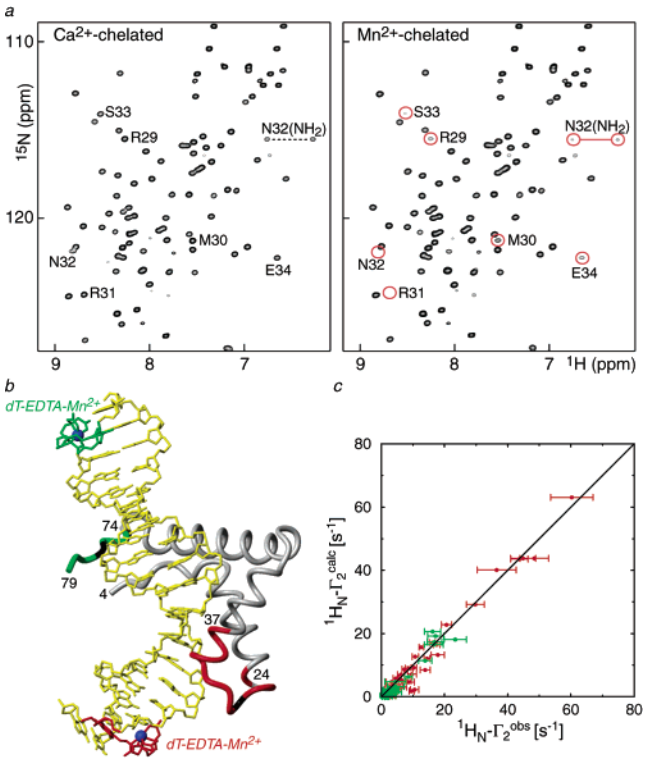

Figure 2. PRE for the SRY/DNA−EDTA−Mn^{2+} complex. (a) 500 MHz $^1H-^{15}N$ HSQC spectra (35 °C) with DNA(oligo1)−EDTA chelated to Ca^{2+} (left) and Mn^{2+} (right). Severely broadened cross-peaks ($^1H_N-\Gamma_2 > 30\ s^{-1}$) in the presence of Mn^{2+} are shown by red circles. (b) Residues of SRY with $^1H_N-\Gamma_2 \geq 15\ s^{-1}$ are displayed in red (oligo 1) and green (oligo 2) on the 3D structure of the SRY/DNA−EDTA−Mn^{2+} complex (with intervening proline residues in black). Two additional base pairs, at either end, including dT-EDTA−Mn^{2+} (in red for oligo1 and green for oligo 2 with Mn^{2+} shown as a blue sphere) were added to the original SRY/DNA structure,[12] optimized by simulated annealing (keeping the protein and DNA fixed), followed by restrained minimization of all coordinates against all experimental restraints. The resulting comparison between the 130 observed and calculated $^1H_N-\Gamma_2$ values is shown in (c). Complexes at low concentration (~30 μM) were extensively washed with a high ionic strength buffer (20 mM Tris·HCl, pH 6.8 and 0.5 M NaCl) to remove any trace metal ion contaminants. The final NMR samples contained 0.3 mM SRY/DNA−EDTA chelated to Ca^{2+} or Mn^{2+} in a buffer of 20 mM Tris·HCl, pH 6.8, 20 mM NaCl, and 93% H_2O/7% D_2O.

(in the context of an 18 bp oligonucleotide, thereby combining the data for oligo1 and oligo2) with the coordinates of the protein and central 14 bp of DNA held fixed, followed by conjugate gradient minimization of all coordinates against all of the experimental restraints[12] (i.e., interproton distances, torsion angles, $^3J_{HN\alpha}$ couplings, $^{13}C\alpha/\beta$ shifts, dipolar couplings, and $^1H_N-\Gamma_2$) and nonbonded terms (van der Waals repulsion and conformational database torsion angle and base−base potentials[12]), yields structures with excellent agreement between observed and calculated $^1H_N-\Gamma_2$ values while still agreeing with all other experimental restraints within their errors (see Supporting Information). For the restrained regularized mean structure, the rms difference between observed and calculated $^1H_N-\Gamma_2$ values is 2.0 s^{-1}, the correlation coefficient is 0.984, the slope is 0.998, and the *Q*-factor, $100[\sum(\Gamma_2^{obs} - \Gamma_2^{calc})^2/\sum(\Gamma_2^{obs})^2]^{1/2}$, is 17% (Figure 2c). The atomic rms shift for the protein and central 14 bp of DNA relative to the previously solved structure[12] is only 0.25 Å for all heavy atoms, which is well within the errors of the coordinates. Note that the position of the Mn^{2+} ion is well defined and the seventh coordination ligand could readily be provided by a water molecule hydrogen bonded to the N7 or N6 of the adenine that is Watson−Crick base paired to the dT-EDTA.

The approach presented in this paper can be readily applied to any protein−DNA complex providing no tight metal-binding site other than the DNA-attached EDTA is present. The paramagnetic center is located in the major groove since EDTA is linked to the C5 position of the T base. Hence, it is essential to place the dT-EDTA nucleotide outside of the protein binding site. The range of Γ_2 can be tuned by judicious choice of paramagnetic metal ion, thereby offering a unique advantage over techniques employing nitroxide spin-labels.[7] At 500 MHz and a rotational correlation time of 10 ns, Γ_2 ranges from 70 to 1 s^{-1} for distances from ~17 to ~35 Å for Mn^{2+} (electron relaxation time $\tau_e \approx 5$ ns) but from ~11 to ~22 Å for Cu^{2+} ($\tau_e \approx 3$ ns).[4,6d] Quantification of the PRE is strightforward and provides a ready source of highly valuable long-range distance information for structure refinement as evidenced by the excellent agreement between observed and calculated values of $^1H_N-\Gamma_2$ for the SRY−DNA complex (Figure 2c). Because the incorporation of dT-EDTA into synthetic DNA is so simple, we expect that the present method will gain widespread utility in structural and chemical studies of protein−DNA complexes.

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Supporting Information Available: Details of HPLC-MS analysis; pulse sequences used to measure $^1H_N-\Gamma_2$ and $^1H_N-\Gamma_1$; structure refinement and structural statistics (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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