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³¹P NMR Investigation of Backbone Dynamics in DNA Binding Sites

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Abstract

The backbone conformation of DNA plays an important role in the indirect readout mechanisms for protein-DNA recognition events. Thus, investigating the backbone dynamics of each step in DNA binding sequences provides useful information necessary for the characterization of these interactions. Here we use ³¹P Dynamic NMR to characterize the backbone conformation and dynamics in the Dickerson Dodecamer, a sequence containing the EcoRI binding site, and confirm solid-state ²H-NMR results showing that the C3pG4 and C9pG10 steps experience unique dynamics and that these dynamics are quenched upon cytosine methylation. In addition, we show that cytosine methylation affects the conformation and dynamics of neighboring nucleotide steps but this effect is localized to only near neighbors and base pairing partners. Lastly, we have been able to characterize the %BII in each backbone step and illustrate that the C3pG4 and C9pG10 favor the non-canonical BII conformation, even at low temperatures. Our results demonstrate that ³¹P Dynamic NMR provides a robust and efficient method for characterizing the backbone dynamics in DNA. This allows simple, rapid determination of sequence-dependent dynamical information, providing a useful method for studying trends in protein-DNA recognition events.

Keywords

Dynamic NMR; DNA; methylation; ³¹P NMR; BI/BII; activation barrier

Introduction

DNA-protein interactions are central to biology and play a crucial role in cellular tasks such as DNA replication and transcription. Understanding how proteins recognize particular DNA sequences holds tremendous promise for understanding basic cell cycle events. X-ray diffraction and high resolution NMR experiments have provided detailed structures of

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Supporting Information Available

Two tables containing the %BII as a function of temperature for the C3 and C9 methylated samples and a figure containing the barrier with respect to backbone step number with an extended chemical shift range. This material is available free of charge via the internet at <http://pubs.acs.org>.

numerous DNA sequences. These studies have demonstrated that DNA is not simply a rigid rod of nucleotides for direct readout. Instead, local structural variations exist within DNA strands that most likely play a significant role in recognition of these sequences by proteins.^{1,2} What has also become clear in recent years is that this recognition process is also dependent upon the dynamics of the DNA and proteins involved.³ One part of this indirect readout is the conformational equilibrium between the BI and BII sub-states of the DNA backbone^{4,5} and this sequence-dependent flexibility contributes to an indirect readout by cognate proteins.⁶⁻¹⁰

The local and global dynamics of polynucleotides has been investigated by high-resolution NMR,^{11-16,17} solid-state NMR,^{10,18-20} Fluorescence Polarization Anisotropy and Dynamic Light Scattering,^{21,22} Electron Paramagnetic Resonance,²³ Molecular Dynamics,^{24,25} and X-ray Diffraction.²⁶ However, obtaining accurate information about local dynamics is difficult because the interpretation of X-ray crystallography, molecular dynamics, and high-resolution NMR data requires numerous assumptions about the structure and relaxation mechanisms of the molecule.¹⁸ These assumptions become increasingly complicated in the realm of biological macromolecules.^{21,22}

Solid-state deuterium NMR has been successful in providing accurate dynamical information because it exploits the deuterium quadrupole moment, a single nucleus property. This allows ²H-SSNMR to provide local dynamic information that is free of structural assumptions.¹⁸ Using systematic isotopic labeling schemes, the local dynamics of the base, sugar, and backbone moieties of individual nucleotides within a sequence can be investigated via ²H-SSNMR. Such studies have uncovered local regions of large amplitude dynamics of the sugar and backbone moieties within the EcoRI and HhaI DNA binding sites.^{19,20} In addition, deuterium NMR has been used to demonstrate the quenching of these dynamics due to cytosine methylation.^{10,20}

Solution NMR studies have also been conducted on a number of DNA sequences in an effort to understand conformational changes.^{11,27,28} Much of this work has been focused on the BI→BII transitions of the phosphate backbone. These conformations are defined by the ζ and ϵ torsion angles, C_{4'}-C_{3'}-O_{3'}-P and C_{3'}-O_{3'}-P-O_{5'}, respectively. BI, the most common conformation in B-form DNA, is trans in ζ and gauche- in ϵ (t,g-) while BII is gauche- in ζ and trans in ϵ (g,t). A more general definition of these conformations is determined by the difference in these torsion angles, (ϵ - ζ), the difference being $\sim -90^\circ$ for the BI conformation and >0 for the BII conformation.^{4,12} Phosphorus chemical shifts and proton-phosphorus scalar couplings have been widely used to characterize these backbone conformations. A review of numerous ³¹P NMR studies demonstrates that higher frequency (historically called 'downfield') ³¹P resonances are indicative of nucleotide steps residing in the BII conformation or in conformational exchange where BII conformations are prevalent.¹² In addition, scalar couplings between the phosphorus and H3' of the sugar ring (³J(P-H3')) are larger for BII conformations. A reparameterized Karplus equation for these coupling constants has been used to determine the percentage of BI conformer present in solution.¹³ The need to extract accurate ³J(P-H3') couplings led to development and use of an array of pulse sequences to carefully measure this scalar coupling.²⁹⁻³¹ However, carefully determining scalar couplings is still a difficult task with often unreliable accuracy³² so using chemical shifts is desirable. Recently, a relationship between the ³¹P chemical shift and the %BII has been developed allowing the translation of δP in terms of BI/BII ratios.³² The ability to assign a %BII is extremely useful, but percent population is an equilibrium property that does not provide the detailed dynamical information needed to address the role of DNA dynamics in protein-DNA binding events. In this paper we demonstrate the use of ³¹P dynamic NMR to extract this detailed dynamical information. We have used the relationship derived by the Hartmann group as a starting point to constrain a two-site model of the conformational exchange between BI and BII in the backbones of nucleotide steps using dynamic NMR.³²

We examine the backbone dynamics in the Dickerson Dodecamer, [d(CGCGAATTCGCG)]₂, the first synthetic oligonucleotide crystallized in the biologically relevant B-form helix.^{1,2,26} This structure revealed the presence of sequence-dependent structural variations including; a large twist angle between T8 and C9, a splay in the G4-C9 base pair and an overall bend of 19°. An important feature of the Dickerson Sequence is that it contains the binding site for the EcoRI restriction endonuclease, -G[↓]AATTC-, where the arrow indicates the cutting site. Restriction endonucleases bind and cleave DNA with remarkable selectivity and accuracy making this class of proteins good candidates for preliminary studies of DNA-protein interactions.

Solid-State ²H-NMR has probed the local dynamics of each nucleotide in the Dickerson Dodecamer,^{10,18,19,33,34} providing quality data with which to compare the results obtained from our ³¹P dynamic NMR technique. However, Solid-State ²H-NMR experiments require extensive sample preparation, including the synthesis of isotopically labeled nucleotides and phosphoramidites, and allow the study of only one nucleotide step at a time. Solution ³¹P NMR allows the study of each backbone step simultaneously, using natural abundance DNA samples. This proves to be exceedingly beneficial as it permits the analysis of a large number of DNA sequences rapidly and efficiently and enables the investigation of changes in flanking sequences, methylation states, pH, counterion and numerous other variables that may be important protein-DNA recognition events.

A phosphorus NMR study of the Dickerson Dodecamer has been reported previously.³⁵ It was seen that the ³¹P resonances for the CpG steps (phosphates 3 and 9) were at higher frequency than expected, indicating a change in backbone conformation at these steps. The crystal structure of this dodecamer also showed differences at these steps. The crystal demonstrated a regular B-type structure except for a break at steps 3 and 9 which had unusually small twist angles.¹ Increased temperature resulted in an overall narrowing and a shift to higher frequency of most resonances suggesting an increase in the BII conformer in the conformational equilibrium of the backbone. Phosphates 3 and 9 show the smallest chemical shift change, presumably due to their having a high population of BII even at lower temperatures. This shift and line narrowing with increased temperatures were also observed in the CpG step of the octamer d(CTTCGAAG)₂ and was described as a conformational exchange between the two backbone conformations.³⁶ It is this conformational exchange which we have characterized via dynamic NMR in order to extract rate and thermodynamic parameters.

The wealth of information already known about the Dickerson Sequence makes it the ideal sequence to test the validity of our dynamic NMR approach. Comparison of this sequence and its methylated counterpart with previous deuterium NMR results demonstrates the robustness and efficiency of the method described here. Additional studies demonstrating changes in backbone dynamics with changes in flanking sequences are already underway in our lab.

Experimental Methods

Theory

The BI/BII conformational exchange in DNA oligonucleotides falls into the fast exchange regime, where the exchange rate is larger than the difference in chemical shifts between the two sites.³⁷⁻³⁹ In this limit, a single resonance line is observed experimentally for each XpY step of the flanking sequence. We employ a two-state model to describe this exchange using the potential energy well shown in Figure 1. In this model, BI is taken as the zero-energy state, ΔG is the free energy difference between the BI and BII conformers, and $\Delta G^\ddagger$ is the free energy of activation, again defined relative to the BI state. The system is in dynamic equilibrium, with

$$B_I \xrightleftharpoons[k_r]{k_f} B_{II} \quad (1)$$

and the observed magnetization in the NMR experiment can be written as the sum of unitary magnetization precession, relaxation, and exchange.⁴⁰⁻⁴⁴

$$\frac{d}{dt} \begin{pmatrix} M_{BI} \\ M_{BII} \end{pmatrix} = -(iL + R + K) \begin{pmatrix} M_{BI} \\ M_{BII} \end{pmatrix} \quad (2)$$

given respectively by the Liouvillian matrix,

$$L = - \begin{pmatrix} \omega_{BI} & 0 \\ 0 & \omega_{BII} \end{pmatrix}, \quad (3)$$

the relaxation matrix,

$$R = \begin{pmatrix} \frac{1}{T_2} & 0 \\ 0 & \frac{1}{T_2} \end{pmatrix} \quad (4)$$

and the exchange matrix,

$$K = \begin{pmatrix} k_f & -k_r \\ -k_f & k_r \end{pmatrix} \quad (5)$$

T_2 is the spin-spin relaxation time, which we assume to be equal for both conformers (this assumption can be easily relaxed), ω is the chemical shift for each of the exchanging conformer sites (in rad/s), and the k 's are the exchange rates defined by equation 1. This set of linear coupled differential equations can be solved by the eigenvector method, in which the eigenvalues and eigenvectors of the combined matrix,

$$-(iL + R + K) = \begin{pmatrix} i\omega_{BI} - k_f - \frac{1}{T_2} & k_r \\ k_f & i\omega_{BII} - k_r - \frac{1}{T_2} \end{pmatrix} \quad (6)$$

define the resulting lineshapes in the time and frequency domains.^{40,42,43} The eigenvalues of this matrix are

$$\Lambda_{+/-} = -\frac{1}{T_2} - \frac{1}{2} \left(k_f + \frac{k_f}{K} - i(\omega_{BI} + \omega_{BII}) \pm \sqrt{\left(k_f + \frac{k_f}{K} \right)^2 + 2i \left(k_f - \frac{k_f}{K} \right) (\omega_{BII} - \omega_{BI}) - (\omega_{BII} - \omega_{BI})^2} \right) \quad (7)$$

where the equilibrium constant $K = k_f / k_r$. As is well-known in chemical exchange, the positive root leads to a quickly decaying time-domain signal with vanishing intensity in fast-exchange, while the negative root leads to a line that continues to narrow.³⁷ Taking this fast exchange limit with $k_f, k_r \gg (\omega_{BII} - \omega_{BI})$, the real part of equation (7) gives an effective line width,

$$r = \frac{1}{\pi T_2} + \frac{K^2(\omega_{BII} - \omega_{BI})^2}{\pi(1+K)^3 k_f}, \quad (8)$$

while the imaginary part gives the resonance offset leading to the observed averaged chemical shift

$$\langle \omega \rangle = \frac{\omega_{BI}}{(1+K)} + \frac{K \omega_{BII}}{(1+K)}. \quad (9)$$

The connection with the free energy of activation is provided by Eyring's equation,^{37-39,42,43,45-47} with

$$k_f = \frac{K_b T}{h} \exp(-\Delta G^\ddagger / RT) \quad (10)$$

Here K_b is Boltzmann's constant, h is Planck's constant, and R is the gas constant, which allows the effective line width to be written as

$$r = \frac{1}{\pi T_2} + \frac{K^2(\omega_{BII} - \omega_{BI})^2}{(1+K)^3} \frac{h}{\pi K_b T} e^{\Delta G^\ddagger / RT} \quad (11)$$

While equation (11) is valid in the fast-exchange limit, its application can be troublesome if independent measures of K and the slow-exchange chemical shifts are not available.^{12,32} Assuming that the pure BI and BII conformations each have a single known ³¹P chemical shift, ω_{BI} and ω_{BII} , respectively, that is independent of sequence, one can use equation (9) to write a linear relationship between the observed fast-exchange chemical shift and the %BII conformation:

$$\%BII/100 = \frac{1}{\omega_{BII} - \omega_{BI}} \langle \omega \rangle - \frac{\omega_{BI}}{\omega_{BII} - \omega_{BI}} \quad (12)$$

as well as the equilibrium constant

$$K = \frac{\langle \omega \rangle - \omega_{BI}}{\omega_{BII} - \langle \omega \rangle}. \quad (13)$$

Such a linear relationship for %BII was recently introduced by Heddi et al.³² as an empirical equation, but as shown above it is also the result of a two-site exchange model. Estimates of the values for ω_{BI} and ω_{BII} will be obtained experimentally from the extrema of a large sampling of observed chemical shifts (*vide infra*). Along with equation 12, they provide the needed independent measures of K and $(\omega_{BII} - \omega_{BI})$ that allow us to fit the ³¹P line widths in the fast exchange limit and extract T_2 and $\Delta G^\ddagger$ for each step in the sequence.

Experiments

NMR experiments were carried out on a Bruker Avance spectrometer (¹H frequency 600 MHz) with a 5 mm gradient probe. Spectra were recorded over a range of temperatures from 12 to

44° C using an FTS System AIRJET temperature preconditioner (FTS, Stone Ridge, NY). Data were collected every 2 degrees. The probe thermocouple was calibrated and then used to provide all temperatures. DNA samples were obtained from Alpha DNA (Montreal, Canada). These were salted with 100 mM phosphate buffer at pH = 7.4 then lyophilized. The samples were re-hydrated in 98% D₂O, re-lyophilized and finally dissolved in 99.9% D₂O, immediately before use.

1D ¹H and ³¹P experiments were acquired to set the spectral window at each temperature. 2D ¹H nuclear Overhauser effect (NOESY) experiments were performed with mixing times of 500 ms and a gradient pulse applied during the mixing time. 4096 data points were collected in the t₂ dimension and 512 increments in t₁. The matrix was zero-filled to 4096 × 1024 points before double Fourier transformation. Phase sensitive ³¹P-¹H HSQC were recorded using Echo/Antiecho-TPPI gradient selection {Kay, 1992 #105; Palmer, 1991 #104; Schleucher, 1994 #106} and decoupling during acquisition. All HSQC were performed with 2048 points in the t₂ dimension and 256 t₁ increments using a relaxation delay of 4 s. The chemical shift of H₂O and the center of the DNA phosphorus resonances were used to set the center of HSQC and NOESY spectra. A spectral width of 9 ppm was used for all 1D experiments and in both dimensions of the 2D experiments.

Since H2' and H2'' are strongly coupled with H1', H3', H5' (sugar protons) and to the base protons, we are able to assign protons on the NOESY using a sequential walk⁵⁰. The strong correlations between ³¹P and H3' and H5'/H4' then enabled us to assign the HSQC using the proton assignments determined from the NOESY. The difficulty in line width analysis caused by overlapping resonances in the HSQC was mitigated somewhat by the fact that each phosphorus is coupled to both H3' and H4', which provides two HSQC cross peaks.

1D ³¹P traces were extracted from the indirect dimension of the HSQC spectra for each XpY step of the DNA sequences and fit to a single Lorentzian plus a simple base line offset in *Mathematica*TM (Wolfram Research, Champaign, IL). The Lorentzian half-widths from this fitting procedure were then fit as a function of temperature to equation 11 to extract T₂ and the free energy of activation. Due to the accuracy of our temperature and chemical shift data the %BII is reported with three significant figures and the error bars for the transition state parameters were determined through a numerical calculation of the covariance matrix and are reported as ± one standard error.

In this procedure, the equilibrium constant was calculated at each temperature using equation 13. ³¹P chemical shifts were referenced to 85% phosphoric acid using a coaxial tube insert at each temperature. No attempt was made to separate out entropy and enthalpy terms in the free energy, which was assumed to be negligibly temperature dependent over this rather restricted temperature range.

Results

Figure 2 shows a plot of ³¹P chemical shifts vs. temperature (referenced at each temperature relative to 85% phosphoric acid) for the following 7 sequences: [d(CGCGAATTCGCG)]₂ in native, C3 methylated and C9 methylated forms; [d(TTCGAATTCGAA)]₂, both native and C9 methylated; and [d(AAAGAATTCCTT)]₂, native and C9 methylated. These shifts all lie between the two flanking lines:

$$\delta P_{BI} = 0.002031T (K) - 1.345 \quad (14)$$

and

$$\delta P_{BII} = -0.003079T (K) + 1.023, \quad (15)$$

which we take as the temperature-dependent limiting shifts for the pure BI and BII conformations. This gives for equation 12

$$\%B_{II} = \frac{100}{2.368 - 0.005110T} \delta P (ppm) + \frac{134.5 - 0.2031T}{2.368 - 0.005110T} \quad (16)$$

which compares fairly well with the original equation of Heddi et al near room temperature,

$$\%B_{II} = 143 \delta P (ppm) + 85, \quad (17)$$

although we find that Heddi's equation leads to %BII values outside the range of 0 to 100%.

A representative HSQC spectrum of the native Dickerson Sequence at 30 °C is shown in Figure 3. From these, the phosphorus 1D spectra were extracted for line shape analysis. For example, figure 4 shows the C9pG10 phosphorus resonance at 20, 30 and 40 °C and the Lorentzian fit for each, providing the chemical shift and line width for the subsequent analysis.

The %BII (calculated from equation 16) as a function of temperature for each resonance in the native Dickerson sequence is shown in Figure 5. Similar plots for the C3 and C9 methylated sequences can be found in Figure 6 and 7, respectively. From Figure 5 it can be seen that as the temperature is increased, the majority of the backbone steps are shifting to higher frequency, toward a greater percentage of BII. However C3pG4 and C11pG12, the resonances already experiencing a great extent of %BII at 12 °C (60.5% and 65.7%, respectively), actually shift to lower frequency, demonstrating an increased sampling of the BI conformation. The phosphate step at the C9pG10 position also shows a large %BII at low temperatures, but its chemical shift remains relatively constant across the temperature range studied (−0.137 ppm). The phosphate steps at G2pC3 and G10pC11, the base pair partners of C11pG12 and C3pG4, also shift toward lower frequency with increasing temperature, although, like C11pG12, they experience a much smaller change in chemical shift than C3pG4. As expected, the %BII is lowest in the center of the sequence and increases as we move out toward the ends of the sequence, and toward decreased base stacking. For example, note the lines for A6pT7 and T7pT8 and for A5pA6 and T8pC9. These center resonances, presumed to be the most stable by virtue of their position and the increased base stacking conferred upon them, have chemical shift profiles that have the lowest %BII and lie nearly on top of each other. G4pA5 has the next highest %BII, but C9pG10 is significantly higher. The variation is also large for the C3/G10 base pair. C3 methylation (Figure 6) causes an increase in %BII for the C3pG4 and C1pG2 steps and a decrease in the G2pC3 step. C9 Methylation (Figure 7) causes an increase in %BII for the C9pG10, G10pC11, T7pT8 and G4pA5 steps. Table 1 provides the %BII as a function of temperature for all eleven resonances. Similar tables for the C3 and C9 methylated sequences can be found in the supplemental materials. These trends in %BII can be better observed in Figure 8.

Figure 8 shows the effect on the percentage of BII at 25 °C as a function of sequence position. The plot is shown for the native and two methylated sequences. The plot of the native sequence shows the dramatic increase in %BII at positions C3pG4 and C9pG10. Methylation of C3 results in an increase in %BII at C3pG4, but also a significant decrease in %BII of the C1pG2 and G2pC3 steps. However, methylation of C3 does not affect the backbone conformation of steps on the other end of the sequence. Methylation of C9 causes an increase in the %BII of

the C9pG10 and G10pC11 steps. Again, this effect is localized to the nucleotides near the site of methylation.

The dynamic behavior of the phosphate steps can be quantified using the two-site exchange model described in the methods section. Representative plots of the line width as a function of temperature for the native and C9 methylated Dickerson Sequence are shown in Figure 9. These fits allow us to determine both $\Delta G^\ddagger$ and T_2 for this phosphate step and table 2 summarizes the same fits for each step in the native, C3 and C9 methylated samples. The center nucleotides have the highest $\Delta G^\ddagger$ and are the least dynamic, as expected for phosphate steps experiencing the greatest base stacking. The $\Delta G^\ddagger$ values of C3pG4 and C9pG10 steps in the native Dickerson Sequence are similar to those of the ends, and in the case of the C3pG4 lower, explaining the increased dynamics observed for these nucleotides in ^2H -SSNMR studies.¹⁹ Methylation causes an increase in the barrier for the C3pG4 and C9pG10 steps. However, these changes in $\Delta G^\ddagger$ shown in Table 2 are deceptively low. This is because the activation barrier was defined in relation to the BI conformation. The C3pG4 and C9pG10 phosphate steps have a more stable BII state (as evidenced by their negative ΔG values). Thus, a better measure of the conformational locking that occurs upon methylation is an activation barrier defined as the difference between the lowest energy state and the transition state. This definition clearly shows significant changes in the activation barriers for the C3pG4 and C9pG10 steps upon methylation. This is evident in Figure 10, which illustrates the change in the barrier between the most stable state and the transition state for each nucleotide step. The increase in the barrier for conformational exchange upon methylation at C9pG10 is in agreement with the methylation induced dynamics quenching of the C9 step observed via ^2H -SSNMR.¹⁰ However, since ^2H -SSNMR can only observe one nucleus at a time, information about the increased conformational barrier of other steps local to the site of methylation has not been previously observed.

Another insight unobtainable from ^2H -SSNMR is the differences and changes in the ΔG values. Our technique not only provides information about the dynamics of the backbone but also about the conformation at each phosphate step. Most significantly, the negative ΔG values for the native C3pG4 and C9pG10 steps indicate that at these steps, BII is the most stable conformation, even before methylation. The C11pG12 step also has a negative ΔG values, however it is unlikely that this is sequence specific but is instead a result of their position in the sequence; the ends of DNA sequences fray, resulting in a loss of base stacking and thus an increase in conformational freedom. Methylation of C9 results in significant decreases in the ΔG of C9pG10 and G10pC11, its 5' neighbor, and G4pA5, its base pairing partner, indicating an increase in the stability of the BII conformation for these steps. However, methylation of C9 does not cause any significant changes in the ΔG of phosphate steps on the other (3') end of the sequence suggesting that cytosine methylation causes only local structural perturbation of the sequence. Methylation of C3 also results in a drop in the ΔG of the C3pG4 step and increases the ΔG of its 3' neighbor G2pC3. However, there is no significant change in the ΔG of the phosphate steps at the other (5') end of the sequence.

Discussion

Our results clearly indicate that the C3pG4 and C9pG10 steps of the Dickerson Dodecamer are conformationally unique with respect to the other nucleotides in the sequence. They favor the BII conformation and have activation energies that are lower than other internal phosphate steps. The increased dynamics at these sites is expected in light of ^2H -SSNMR studies of this same sequence which found that C3 and C9 experience unique, large amplitude (± 38 degree half-angle of libration) furanose dynamics and that C9 had large amplitude backbone dynamics compared to other backbone steps previously studied (the backbone dynamics of C3 were not studied).¹⁰ In addition to obtaining results consistent with ^2H -SSNMR studies of the native

dodecamer, our methylation results are also consistent with a ^2H -SSNMR study of C9 methylation effects. This study showed increased $T_{1\rho}$ relaxation times for the C9pG10 backbone step upon C9 methylation (0.03 and 0.055 ms, native and methylated, respectively) and a significant increase in the quadrupole coupling constant, QCC, indicative of a loss of large amplitude dynamics.¹⁰ This ^2H -SSNMR study only investigated the effects of C9 methylation (not C3 methylation) and, due to the quadrupole being a single nucleus property, could not address the effects of this methylation on other steps in the sequence.

In addition to confirming that ^{31}P dynamic NMR detects increased dynamics of the phosphates at these nucleotide steps, our data uncover increased stability of the BII conformation for these steps. Our results also confirm that while these two nucleotide steps are uniquely dynamic, they are not the same. This difference between these steps is further confirmed by analysis of the temperature-induced conformational changes. The C3pG4 step shows a dramatic decrease (60.5–53.6%) in the %BII as a function of temperature, while the C9pG10 step increases (61.6–67.5%). Interestingly, this response to temperature change is reversed in the base pairing partners of these steps. The G4pA5 step, the base pairing partner of C9, shows a dramatic increase in %BII (28.6–41.0%) while the G10pC11 step, the base pairing partner of C3, shows little change in %BII (47.6–46.4%). The low %BII of the G4pA5 step coincides with the stiffness of this nucleotide step observed via ^2H -SSNMR. It must be noted however that the G10 step was not studied via ^2H -SSNMR due to its position so near to the end of the sequence. Its location at the end of the sequence may explain its relatively high %BII even at low temperatures and thus its small changes in %BII with increasing temperature. It may be very significant that at physiological temperature (37 °C), C3 is strongly BII but G4 is strongly BI (54.7 and 39.5%, respectively). This creates a step with a sharp switch in backbone conformation at this location, the step that is recognized by the protein, which cuts between G4 and A5. The only other step with such a significant switch in backbone conformation is the T8pC9 step (24.2 and 66.5%, respectively), across from the G4 cutting site. Enzymologists, recognizing that restriction endonucleases transiently bind to non-selective sites but do not cleave, proposed that enzymes roll along DNA strands dropping in and out of non-selective sites on the DNA. However, the enzyme binds more strongly when it comes to a high affinity site, postulated to be a region of increased dynamics where the protein can distort the DNA to enhance necessary contacts for binding and cleavage.⁵¹ This site is necessarily the result of local structure and dynamics in the DNA. Since the EcoRI binds to its palindromic DNA sequence as a dimer, it is not surprising to have discovered regions of enhanced dynamics at either end on both strands of this sequence.

It is important at this point to add two caveats. The first is that the accuracy of the kinetic and thermodynamic parameters we extract depends on the validity of equation (16). We have tested the sensitivity of our analysis to the exact form of this empirical relationship by examining alternate parameterizations of %BII vs. chemical shift. In particular, the observed values of extrema for the chemical shifts do serve as limits of these values, but it is quite probable that the actual range is larger. By increasing the width of this range by 0.10 ppm on each edge, we find that the general trends remain intact, while the exact values vary by about 0.4 kcal/mol (figure provided in the supplementary materials).

The second caveat is to emphasize that we used a two-site jump model, exchange between BI and BII, to model the dynamics of the phosphate backbones in this sequence and extract an activation energy. This is no doubt a very simplistic model for such a complex system and clearly has several shortcomings. That said, a two-site jump model, including the estimate of %BII, provides a robust and convenient model for studying dynamics in this complicated biological system and provides a quantitative comparison of activation energies and dynamics. For example, the G2pC3 step in C3-methylated sequence shows an increase of ~1.2 kcal/mol for the activation energy, corresponding to rates of exchange that are only 15% of those in the

native sequence. While we caution against the over interpretation of these numbers, our experiment and analysis do provide a consistent and efficient way to compare the dynamics of each backbone step in an oligonucleotide, affording important sequence-dependent information. This screening method is complementary to previously reported methods and once backbone steps with increased dynamics are identified, ^2H -SSNMR, for example, can be used if more complete modeling of the dynamics is desired.

Conclusions

We have presented a robust and efficient method of studying the local dynamics in DNA backbones. We have confirmed the presence of unique dynamics at the C3pG4 and C9pG10 steps of the Dickerson Sequence. In addition, we have shown that these steps exist in the less common BII conformation even at low temperatures and cytosine methylation at C3 and C9 increases the %BII at these and neighboring steps. Our goal is to provide sequence-dependent dynamical information about DNA sequences in an effort to uncover any trends in protein-DNA recognition. The method described herein is an efficient means of collecting this data, allowing us to look at a myriad of sequences under a variety of conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The potential energy diagram describing the two-state conformational exchange between BI and BII.

Figure 2.

^{31}P chemical shifts vs. temperature (referenced at each temperature relative to 85% phosphoric acid) for 7 sequences: $[\text{d}(\text{CGCGAATTCGCG})]_2$ native, C3 methylated and C9 methylated; $[\text{d}(\text{TTCGAATTCGAA})]_2$ native and C9 methylated; $[\text{d}(\text{AAAGAATTCTTT})]_2$ native and C9 methylated.

Figure 3.

A representative HSQC spectrum of the native Dickerson Sequence at 30 °C showing the DNA backbone ^{31}P resonances vs. the H3'/H4' region.

Figure 4.
The C9pG10 phosphorus resonances at 20, 30 and 40 °C with their Lorentzian fits and residuals.

Figure 5.
The calculated %BII for each nucleotide step in the native Dickerson Sequence as a function of temperature.

Figure 6.
The calculated %BII for each nucleotide step in the C3 methylated Dickerson Sequence as a function of temperature.

Figure 7.
The calculated %BII for each nucleotide step in the C9 methylated Dickerson Sequence as a function of temperature.

Figure 8.
The %BII at 25 °C as a function of position within the sequence.

Figure 9. Line widths as a function of temperature for the C9pG10 step in the native (bottom) and C9 methylated (top) Dickerson Sequences.

Figure 10.

The energy difference between the transition state and most stable conformation for each nucleotide step. The squares represent the native Dickerson Sequence, the circles the C9 methylated sequence and the triangles the C3 methylated sequence.

Table 1

The %BI for each nucleotide step in the native sample.

TempK	C1pG2	G2pC3	C3pG4	G4pA5	A5pA6	A6pT7	T7pT8	T8pC9	C9pG10	G10pC11	C11pG12
285.2	44.0	34.5	60.5	28.6	18.7	5.9	9.7	20.4	61.6	47.6	65.7
287.2	44.6	35.1	60.1	30.0	18.3	6.2	8.3	20.4	62.1	47.5	65.6
289.2	45.4	35.2	59.7	31.6	19.3	8.2	11.3	22.1	63.4	48.1	65.9
291.2	45.4	35.0	58.9	32.1	19.6	7.7	10.0	21.7	63.5	47.8	65.4
293.2	45.8	34.8	58.3	33.4	19.8	7.8	8.3	21.9	63.9	48.0	65.4
295.2	46.2	34.7	58.0	35.0	20.7	5.9	8.9	22.4	64.4	47.5	65.4
297.2	46.7	34.3	56.9	35.6	21.1	6.6	7.9	22.3	64.8	47.9	65.0
299.2	47.0	34.0	56.8	36.7	21.6	6.5	8.2	22.7	65.2	48.0	65.1
301.2	47.5	34.0	56.4	37.3	22.1	6.6	7.6	23.2	65.5	48.0	65.1
303.2	47.7	33.6	55.9	38.0	22.9	6.9	6.8	23.7	65.8	48.1	64.7
305.2	48.1	33.4	55.5	38.6	23.0	6.2	6.7	23.8	66.2	48.0	64.5
307.2	48.5	32.9	55.2	39.1	23.0	6.7	6.5	24.0	66.4	47.9	64.6
309.2	48.9	32.5	54.7	39.5	23.3	6.1	5.7	24.2	66.5	47.8	64.5
311.2	49.3	32.3	54.3	39.9	23.7	6.1	5.7	24.5	66.9	47.1	64.2
313.2	49.7	31.7	54.1	40.2	24.0	5.5	5.3	24.7	67.2	46.8	64.1
315.2	50.2	31.3	53.8	41.0	24.2	5.6	4.8	24.7	67.4	46.7	64.0
317.2	50.7	31.1	53.6	41.0	24.7	5.4	4.9	24.6	67.5	46.4	63.9

Table 2
Calculated thermodynamic parameters for each nucleotide step.

	Native Dickerson			C9Me Dickerson			C3Me Dickerson		
	$\Delta G^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	T_2 (sec)	$\Delta G^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	T_2 (sec)	$\Delta G^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	T_2 (sec)
ClpG2	12.17±0.05	0.0783	0.0371	12.30±0.05	0.0801	0.0365	12.61±0.04	0.0478	0.0262
G2pC3	12.57±0.10	0.383	0.0319	12.69±0.07	0.431	0.0324	13.67±0.06	1.14	0.0310
C3pG4	12.08±0.10	-0.165	0.0312	12.23±0.03	-0.166	0.0318	12.19±0.06	-0.368	0.0321
G4pA5	12.93±0.08	0.349	0.0376	12.75±0.10	0.219	0.0341	13.01±0.06	0.396	0.0390
A5pA6	13.28±0.08	0.779	0.0365	13.44±0.07	0.792	0.0339	13.51±0.08	0.930	0.0324
A6pT7	14.32±0.16	1.57	0.0306	15.31±0.11	1.98	0.0290	14.89±0.17	1.69	0.0265
T7pT8	13.98±0.14	1.45	0.0262	13.77±0.06	0.989	0.0304	14.44±0.21	1.56	0.0260
T8pC9	13.54±0.06	0.737	0.0362	13.69±0.05	0.667	0.0308	13.56±0.04	0.854	0.0331
C9pG10	12.21±0.08	-0.360	0.0330	12.44±0.04	-0.534	0.0365	12.22±0.07	-0.347	0.0319
G10pC11	12.06±0.09	0.0502	0.0355	12.26±0.08	-0.0925	0.0313	12.37±0.06	0.0534	0.0322
Cl1pG12	11.93±0.07	-0.366	0.0406	12.18±0.08	-0.309	0.0366	11.99±0.10	-0.266	0.0285