B to Z transitions of the short DNA hairpins formed from the oligomer sequences: $d[(CG)_3X_4(CG)_3]$ ($X = A, T, G, C$)

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ABSTRACT

Circular Dichroism (CD) spectra were collected as a function of sodium perchlorate concentration $[NaClO_4]$ for the set of DNA hairpins formed from the oligomer sequences $d[(CG)_3X_4(CG)_3]$ where $X = A, T, G$ or C . Over the range in salt concentration from 0 to 4.0 M $NaClO_4$, the CD spectra invert in a manner characteristic of the B to Z transition. A factor analysis routine is described and employed to determine the least number of basis spectra required to fit the measured spectra of each hairpin over the entire salt range examined. In every case, linear combinations of only two sub-spectra fit the experimental spectra of the hairpins with greater than 98% accuracy, indicating the spectrally monitored structural transitions are two-state. From the relative weights of the individual sub-spectra, B-Z transition curves are constructed. The transitions are analyzed in terms of a simple two-state equilibrium model which yields an evaluation of the transition free-energy, ΔG_{B-Z} , as a function of $NaClO_4$ concentration. At 1.0 M $NaClO_4$ and 21°C, $\Delta G_{B-Z} = 5.4, 4.9, 3.6$ and 2.3 kcal/mole for the G_4, T_4, A_4 and C_4 loop hairpins, respectively.

INTRODUCTION

Cruciform structures in DNA comprised of intramolecular stem-loops or hairpins are widely believed to be crucial to genetic processes. In support of this notion, partially self-complementary DNA sequences that are able to form intramolecular cruciforms have been found near sites involved in transcription and recombination (1–5). Consequently, a number of studies investigating the physical properties of DNA hairpins have been reported (6–12).

Depending on the sequence environment, transitions of local regions of supercoiled DNA to either cruciforms or left-handed Z-DNA, are two means of relieving torsional stress induced by supercoiling (13,14). Since both Z-DNA and hairpins are thought to serve as regulatory structural signals, elucidation of the physical characteristics of both secondary structures is required to understand structural switching in DNA.

Recently, the first crystal structure of a DNA hairpin, formed from the partially self-complementary 16 base DNA oligomer sequence $d[(CG)_3T_4(CG)_3]$, indicated the duplex stem region resides in the left-handed Z conformation (15). Solution NMR, CD and optical melting studies of this 16-mer (6) and several other DNA hairpins formed from similar partially self-complementary sequences (7–12) also demonstrated the duplex stems of these molecules adopt the Z conformation under high-salt conditions. With a single exception (10), most of these studies have focused exclusively on hairpins having single strand loops comprised of four to five thymine residues. The discovery of DNA hairpins with Z-type duplex stems establishes a model system with which to simultaneously investigate potential interactions between both types of DNA secondary structure.

Although effects of loop sequence might be suspected to influence the B-Z transition of a hairpin stem, to our knowledge investigations aimed at verifying this suspicion have not been undertaken. In the study reported in this paper CD spectra as a function of $NaClO_4$ concentration of the DNA hairpins formed from the partially self-complementary oligomers, $d[(CG)_3X_4(CG)_3]$ where $X = A, T, G, C$ were measured. Over the range in salt concentration from 0 to 4.0 M $NaClO_4$, the spectra of the hairpins invert in characteristic spectral regions indicative of the right to left-handed B-Z transition. A factor analysis routine is employed to determine the least number of basis spectra required to fit the measured spectra as a function of salt ($NaClO_4$). Therefore, unlike earlier studies where the changes in ellipticity values from the CD spectrum at a single wavelength (6,9,16) or at most two wavelengths (7,10,17) were used to quantify the B-Z transition, *all* of the collected CD spectral data are utilized to construct transition curves. The analysis reveals the B-Z transitions of the hairpins are two-state and significantly loop sequence dependent.

MATERIALS AND METHODS

DNA Sample Preparation

DNA oligomers for this study are listed in Table I and were synthesized on an Applied Biosystems 380B automated DNA synthesizer (β -cyanoethyl phosphoramidite chemistry) (18).

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Following detritylation and deprotection, DNA samples were dried under vacuum and resuspended in ddH₂O. DNAs were purified electrophoretically from 20% polyacrylamide gels by brief shadowing with short wavelength uv light ($\lambda = 254$ nm) and slicing the major synthetic product out of the gel. Excised gel slices were crushed and soaked in 10 volumes of elution buffer (100 mM NaCl, 10 mM sodium phosphate, pH = 7.0) and incubated at 55°C for at least 18 hours. Gel particles were removed by filtration through 0.4 μ m filters and a column of hydroxylapatite. Purified samples were exhaustively dialyzed, evaporated to dryness and stored at -20°C. Post-purification electrophoresis verified fidelity of DNA samples.

Prior to CD measurements, an aliquot of each DNA oligomer was dissolved in ~5 ml of buffer solution (10 mM sodium phosphate, pH=6.2). DNA concentrations were determined from the extinction coefficients evaluated for each oligomer. Values of the extinction coefficients used for the hairpins in this study were 8016, 8606, 9041 and 8320 M⁻¹·cm⁻¹ for the C₄, G₄, A₄ and T₄ loop hairpins, respectively. The method employed for determining the hairpin extinction coefficients was a modification of that recently developed (19). The salt concentration of DNA solutions was adjusted by adding the appropriate amount of solid NaClO₄ (twice re-crystallized, Sigma). At each salt concentration, corrections of the DNA concentration were made to account for any volume changes due to added salt. DNA concentrations were between 30 and 50 μ M strands (~4 OD/ml). Aliquots taken from these concentrated DNA solutions, in either high or low salt, electrophoresed on 20% polyacrylamide gels revealed sharp, single bands at approximately the same migration distance as the previously characterized T₄ loop hairpin (6). Likewise, the absorbance spectra above 300 nm of these DNAs as a function of salt did not indicate appreciable aggregation that might be expected to arise from intermolecular pairing, particularly for the G₄ loop hairpins.

Circular Dichroism (CD) Measurements

CD spectra of DNA solutions were recorded on a J-600 spectropolarimeter at ambient temperature of the sample holder (~21°C). Spectra were measured at 0, 0.1 M, 0.5 M and in increments of 0.5 M thereafter up to 4.0 M NaClO₄, in a 1 cm path length quartz cell. DNA solutions were allowed to equilibrate for at least 4 hours at each titration point at 21°C prior to recording of CD spectra. Three scans at 50 nm/min were made over the wavelength range from 185 to 330 nm. The average spectrum of these three scans was used in the analysis. Measurements were made at 10 salt concentrations. CD spectra collected at 0.1 M and 4.0 M NaClO₄ on a second set of independently prepared samples were in exact agreement with those data measured on the actual sample set. From this observation the spectral data are considered to be highly reproducible.

FACTOR ANALYSIS

Theory

To probe characteristics of the right to left-handed B-Z transition in DNA hairpins the collected CD data of the DNAs in Figure 1 were subjected to factor analysis. Numerous versions and variations of this classical technique, applied to analyze CD spectral data from DNA and proteins, have been reported (20–25). Here we provide only a brief summary of the essential features underlying our quantum mechanical approach.

Figure 1: Circular Dichroism spectra of the DNA hairpin formed from d[(CG)₃T₄(CG)₃] as a function of NaClO₄ concentration from 0 to 4.0 M in increments of 0.5 M at 21°C. Units of $\Delta\epsilon$ are M⁻¹·cm⁻¹. Analogous plots were obtained for the other hairpins as a function of salt concentration. Values of $\Delta\epsilon$ every 0.2 nm from 185 to 330 nm were subjected to factor analysis as described in the text.

The starting point is assuming that each collected experimental CD spectrum within a given data set, $\theta_i(\lambda)$, can be expressed as a linear combination of p basis spectra or sub-spectra, $S_j(\lambda)$, i.e.

$$\theta_i(\lambda) = \sum_{j=1}^p \alpha_{ij} S_j(\lambda) \quad (1)$$

α_{ij} are weighting coefficients of each sub-spectra (unrestricted in sign). Because the actual input data is comprised of a number of CD spectra of the same molecule measured at different salt concentrations, equation (1) represents the columns of the input matrix, $[\theta(\lambda)]$,

$$[\theta(\lambda)] = [S(\lambda)][\alpha] \quad (2)$$

$[S(\lambda)]$ is the matrix of sub-spectra with columns $S_j(\lambda)$. $[\alpha]$ is the coefficient matrix with elements, α_{ij} , the coefficient of the j^{th} sub-spectrum in the i^{th} spectrum.

The set of n experimental spectra, $\theta_i(\lambda)$ ($i=1, n$), are considered to be continuous real functions of the wavelength, λ , and thus define a finite p -dimensional Hilbert space, H_p ($p \leq n$). Equation (1) represents the expression of any experimental spectrum as a vector $\theta_i(\lambda)$ in the basis of H_p , which can be decomposed into the orthogonal subspaces generated by the basis vectors $S_j(\lambda)$. To find p and $S_j(\lambda)$, a projection operator $\hat{P}$ that projects $[\theta(\lambda)]$ onto the orthogonal subspaces of H_p must be found.

Such an operator can be found by considering the square matrix of the input spectra defined as,

$$[C] = [\theta]^T[\theta] \quad (3)$$

The elements of $[C]$ are given by the overlap integrals

$$C_{ij} = \int \theta_i(\lambda) \theta_j(\lambda) d\lambda \quad (4)$$

In the space H_p , $\hat{C}$ is an operator with eigenfunctions $S_j(\lambda)$. The projection operator $\hat{P}$ that projects an arbitrary vector from H_p onto the basis formed by the eigenfunctions of $\hat{C}$, obeys the operator eigenvalue equation,

$$\hat{C} \hat{P} = \hat{P}[\Lambda] \quad (5)$$

$[\Lambda]$ is the diagonal eigenvalue matrix of eigenvalues, Λ_i . Expressing the respective operators in matrix notation gives the matrix eigenvalue equation,

$$[P][C] = [\Lambda][P] \quad (6)$$

$[P]$ is obtained by constructing $[C]$ from the input data and solving the resulting eigenvalue problem in equation (6). The sub-spectra are then given by

$$[S(\lambda)] = [\theta][P] \quad (7)$$

Substitution of equation (7) in equation (2) yields the coefficients,

$$[\alpha] = [P]^T \quad (8)$$

The dimension of space H_p is equal to the number of non-zero eigenvalues in $[\Lambda]$.

Analysis of the CD Spectra

CD spectra comprised of 725 points, $\Delta \epsilon$ values at every 0.2 nm from 185 to 330 nm, served as the input data. The factor analysis software employed was a slightly modified version of that previously described (20). The integrals of equation (4) were evaluated numerically using Simpson's rule. The matrix diagonalization routine used to solve the eigenvalue problem of equation (6) was previously described (20).

CD spectra were analyzed in individual sets. The factor analysis routine returns the eigenvalues in ranked order indicating the relative contribution of the corresponding sub-spectra to the actual experimental spectrum. The minimum number of these most significant sub-spectra required to reproduce the experimental spectra within the known experimental error ($< 2\%$) is $p_{\min}$. The corresponding weight of the j^{th} sub-spectrum in the i^{th} experimental CD spectrum, W_{ij} , is found from the coefficients, α_{ij} , according to,

$$W_{ij} = \frac{\alpha_{ij}}{\sum_{j=1}^{p_{\min}} |\alpha_{ij}|} \cdot 100 \quad (9)$$

The sub-spectra at each salt concentration are taken to be indicative of structural features of the DNA molecules within a particular set. Plots of the weights of the sub-spectra versus salt concentration, constructed using equation (9), therefore mirror salt induced structural transitions.

RESULTS AND DISCUSSION

The DNA Oligomers Preferentially form Uni-molecular Hairpins

The partially self-complementary sequence of these oligomers indicates they can form uni-molecular hairpins. To verify they preferentially exist in the uni-molecular state, optical melting curves of the DNAs listed in Table I were measured in 0.1 M and 4.0 M NaClO₄ as a function of DNA strand concentration. For the A₄, G₄ and C₄ loop molecules, melting curves were measured at molar strand concentrations between 1 and 10 μ M. For the T₄ loop oligomer, melting curves were measured over a 10-fold greater concentration range (1 to 100 μ M). All collected melting curves displayed very similar characteristics and were mono-phasic over the entire temperature range sampled (10 to 95°C, data not shown). In addition, neither the melting transition temperature, t_m , or curve shapes were sensitive to changes in the DNA concentration over the range examined. In 0.1 M NaClO₄, the DNAs melt with t_m 's of 84.4, 86.9, 89.5 and 85.8°C, for the A₄, C₄, G₄ and T₄ loop molecules, respectively. In high salt, the transition temperature of each oligomer was $\sim 20^\circ\text{C}$ lower. The average experimental error in t_m determinations was ($\pm 0.4^\circ\text{C}$). In either high or low salt the t_m determined at every DNA concentration examined never deviated outside the experimental uncertainty. In contrast to what was observed, self-complementary DNA oligomers which form bi-molecular bulged duplexes clearly display bi-phasic melting curves and concentration dependent t_m values over a similar range in DNA concentration (10,26,27). From these findings combined with results of gel electrophoresis of the concentrated DNA solutions in high and low salt, (see MATERIALS AND METHODS), we confidently assume for the remainder of the analysis that *all* of the oligomers of this study preferentially exist as uni-molecular hairpins.

Input Spectra for Factor Analysis

10 CD spectra at salt concentrations from 0 to 4 M NaClO₄ were recorded for the DNA oligomers listed in Table I. Each set of 10 CD spectra collected for the hairpins formed from $d[(CG)_3X_4(CG)_3]$, $X = A, T, G, C$ were subjected to factor analysis. Figure 1 shows the 10 CD spectra of $d[(CG)_3T_4(CG)_3]$ measured from 185 to 330 nm as a function of $[NaClO_4]$. The data in Figure 1 and analogous data for the A₄, G₄ and C₄ loop hairpins (not shown) comprised the input data for the factor analysis routine. The input matrix of each hairpin was comprised of 10 columns and 725 rows.

The B-Z Transitions of the Hairpins are Two-State

For each of the A₄, T₄, G₄ and C₄ loop hairpins factor analysis returned only two sub-spectra. The first sub-spectra of the hairpins (shown in Figure 2a) are clearly B-like (28). Identifiable characteristic features of B-DNA displayed in the first sub-spectrum are the positive bands around 275 and 190 nm and the negative bands around 250 and 205 nm. The second sub-spectra are shown in Figure 2b. From a comparison of the sub-spectra in Figures 2a and 2b, five notable differences provide evidence of a structural transition. Most notable is the inversion of the second sub-spectra and emergence of a negative band at 290 nm not present on the first sub-spectra. The position of the positive band on the high wavelength end of the first sub-spectra is blue-shifted approximately 10 nm in the second sub-spectra. The negative rotational strength at 250 and 205 increases on the second sub-spectra. The positive band at 190 nm is also greatly decreased

Figure 2a: First sub-spectra of the C₄ (I), G₄ (II), A₄ (III) and T₄ (IV) loop hairpins returned by factor analysis of ten spectra of each of the hairpins as a function of NaClO₄ concentration. Units of $\Delta\epsilon$ are $M^{-1} cm^{-1}$. **Figure 2b:** Second sub-spectra of the four hairpin loops returned by factor analysis of ten CD spectra as a function of NaClO₄. Symbols on the spectra and units are the same as in Figure 2a.

on the second sub-spectra. In addition, the rotational strength of the spectral region between 245 and 200 nm increases slightly in the second sub-spectra. These observations for the second sub-spectra are consistent with a left-handed Z-like DNA structure (28). These observations are also consistent with results of our recently published NMR, CD and optical melting study of the T₄ loop hairpin which verified the stem sequence is in the right-handed B-form in low salt and converts to the left-handed Z-form in 4 M NaClO₄ (6). Differences between the individual sub-spectra in both Figures 2a and 2b are observed, particularly for the A₄ loop. These may be attributed to salt dependent structures that form within the loop as well perturbations of duplex stacking in the stem induced by the different loop sequences.

Since combinations of only two sub-spectra are apparently able to be combined to yield the actual input spectra of each hairpin over the entire transition range, we are justified in employing a simple two-state transition model to extract thermodynamic parameters of the B-Z transition of each hairpin. This analysis has been widely employed in the analysis of B-Z transitions of DNA polymers and oligomers (16,17,29–31). From such an analysis the influence of loop sequence on the transition free-energy of the hexamer hairpin stem can be determined.

To facilitate the two-state analysis, transition curves were constructed for each DNA by normalizing to 1.0 the absolute difference between the lowest and highest relative weights,

determined from factor analysis and equation (9), of each of the sub-spectra versus [NaClO₄]. Curves of the normalized weights for both the disappearance of the first sub-spectrum (W_B versus NaClO₄) and appearance of the second sub-spectrum (W_Z versus NaClO₄) were constructed (data not shown). f_Z , the fraction of Z-form was determined from the average of the normalized weights, $1 - W_B$ and W_Z , as a function of [NaClO₄]. These resulting average normalized transition curves for each hairpin are plotted as f_Z versus [NaClO₄] (open circles) in Figure 3. The displayed error bars are not the actual experimental error, rather they indicate the deviation between the transition curves constructed from the normalized weights of the first and second sub-spectra.

From the transition curves in Figure 3, the energetics of the B-Z structural transition of the hairpins can be evaluated. According to the two-state model, the equilibrium transition from the B to the Z form can be written as (29–31),

B and Z represent molecules in the B and Z forms. n includes all the differences in salt dependent polyelectrolyte behavior for a molecule in the B versus Z conformation and is assumed to be constant for a particular molecule. In this context n is the coefficient of salt concentration required to induce right to left handed structural conversion. In this vein, NaClO₄ is put in

Figure 3: Transition curves of the C₄ (I), G₄ (II), A₄ (III) and T₄ (IV) loop hairpins. Open circles: f_Z , the fraction of Z-form determined from the average of the normalized weights of the disappearance of the first sub-spectrum (Figure 2a) and the appearance of the second sub-spectrum (Figure 2b) for each hairpin at each NaClO₄ concentration. Solid lines: transition curves calculated using a two-state model and the empirically evaluated values of n and $\ln[K_{eq}]$ given in Table I as described in the text.

brackets in equation (10) to indicate it is not merely a site-binding reactant but the concentration of NaClO₄ required to induce the transition. n in equation (10) should *not* be confused with Δn that would be used to represent merely the counterion uptake in the B-Z transition if the reaction were governed entirely by the processes modeled in Manning's counterion condensation theory (32–34). In fact, Soumpasis (35) has recently shown by theoretical arguments that counterion condensation theory does not account for the experimentally observed linear dependence on salt concentration (above ~ 0.9 M) of the free-energy of B-Z conversion, apparently suggesting counterion condensation theory may be inappropriate in the thermodynamic analysis of the B-Z transition. K_{eq} in equation (10) is the true equilibrium constant and $[\text{NaClO}_4]$ is the molar concentration of sodium perchlorate. The observed equilibrium constant for the reaction, K_{obs} , is given by

$$K_{obs} = [Z]/[B] = f_Z/(1-f_Z) = K_{eq}[\text{NaClO}_4]^n \quad (11)$$

where $[Z]$ and $[B]$ are the molar concentrations of Z and B type structures and f_Z is the fraction of Z-form. From the expression in equation (11) the observed free-energy of the transition, ΔG_{obs} , is obtained,

$$\Delta G_{obs} = -RT(\ln[K_{eq}] + n \ln[\text{NaClO}_4]) \quad (12)$$

$\ln[K_{eq}]$ and n graphically determined from the y-intercept and

TABLE I: Parameters of the DNA Hairpin Oligomers

Oligomer Sequence	n	$\ln(K_{eq})$	ΔG_{B-Z}^a	ΔG_{B-Z}^b
(I) d[(CG) ₃ C ₄ (CG) ₃	5.0	-3.89	2.3	-0.39
(II) d[(CG) ₃ G ₄ (CG) ₃	9.8	-9.24	5.4	0.14
(III) d[(CG) ₃ A ₄ (CG) ₃	7.3	-6.05	3.6	-0.37
(IV) d[(CG) ₃ T ₄ (CG) ₃	8.6	-8.22	4.9	0.21

^a In kcal/mol, evaluated at 21°C from n and $\ln(K_{eq})$ at $[\text{NaClO}_4] = 1.0$ M using equation (12).

^b $[\text{NaClO}_4] = 2.5$ M, 21°C.

Figure 4: The free-energy of the B-Z transition, ΔG_{B-Z} , at 21°C as a function of $\ln[\text{NaClO}_4]$ for the C₄ (I), G₄ (II), A₄ (III) and T₄ (IV) hairpins. ΔG_{B-Z} was determined from the plots in Figure 3 employing a two-state equilibrium analysis of the B-Z transitions in the hairpins.

slope of $\ln[K_{obs}]$ versus $\ln[\text{NaClO}_4]$ plots in the transition region (not shown) are given in Table I for each hairpin. The f_Z versus $[\text{NaClO}_4]$ transition curves calculated from the two-state model using equation (11) and the empirically evaluated n and $\ln[K_{eq}]$ in Table I are plotted in Figure 3 (solid line) for comparison with experimentally derived f_Z versus $[\text{NaClO}_4]$ plots (open circles). Clearly, the two-state model calculations quite accurately represent the experimental observations within the uncertainty indicated by the error bars. The resulting graphically evaluated free-energies calculated from equation (12) are plotted for each of the hairpins in Figure 4 as a function of $\ln[\text{NaClO}_4]$.

Effects of Loop Sequence on the B-Z Transition of d(CG)₃

Plots of the B-Z transition free-energy, $\Delta G_{B-Z} = \Delta G_{obs}$, as a function of $\ln[\text{NaClO}_4]$ concentration shown in Figure 4 readily reveal that the sequence of the four base loop of each hairpin has a substantially different effect on the B-Z transition of the d(CG)₃ duplex stem. According to Figure 4, the B-Z transition is most easily accomplished in the hairpin with a C₄ loop, while the transition is most difficult for the hairpin with a G₄ loop. The base sequence of the four base loop also affects the transition midpoint, defined as the NaClO₄ concentration where $\Delta G_{B-Z} = 0$. The transition midpoints of the G₄ and T₄ loops are approximately equal at 2.6 M NaClO₄ while the C₄ and A₄ loops have midpoints of 2.2 and 2.3 M NaClO₄, respectively. Values of ΔG_{B-Z} for each hairpin evaluated at 1.0 M and 2.5 M NaClO₄ and 21°C are given in Table I and range from 5.4 kcal/mole for the G₄ loop to 2.3 kcal/mole for the C₄ loop. The error of the values given in Table I was taken as the variance of ΔG_{B-Z} , individually calculated from the normalized weights

of the first and second sub-spectra, which is ± 250 cal/mole.

Our results confirm the thermodynamics of the B-Z transition of a d(CG)₃ duplex stem significantly depend on the sequence of the adjoining hairpin loop and also depend on salt dependent structures that potentially form within the loop. Differences observed between the B and Z sub-spectra of the individual hairpins shown in Figure 2a and 2b provide an indication that differences in transition thermodynamics probably arise from both loop sequence dependent perturbations of the stem and sequence dependent structures of the loops. Quantitative verification of these propositions however must await further structural studies of B-Z transitions in these and similar short DNA hairpins.

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