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A single base permutation in any loop of a folded intramolecular quadruplex influences its structure and stability

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ABSTRACT

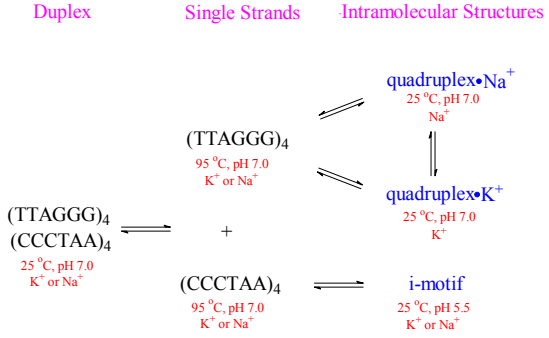
The human telomere sequence $(TTAGGG)_4$ folds into an unusual conformation possessing three G-tetrads linked by TTA loops. The first loop is a propeller loop while the second and third loops are transverse loops. Using Circular Dichroism (CD) spectroscopy, we have investigated the effect of sequence context on the structures and stabilities of intramolecular G-quadruplexes related to the human telomere sequence by considering all permutations of T and A within the loops. The results indicate that changing only one base in any one loop can have a dramatic effect on the conformation of the quadruplex as well as its melting temperature, T_m. Thus, each sequence studied has a unique CD spectrum and T_m. In general, variants with a modified second loop are the most stable while the wild type sequence is the least stable. The observed difference in CD spectra and melting temperature are discussed in terms of base stacking within the loop and stacking of the loop bases with adjacent G-tetrads.

Keywords: Cell DNA Quadruplexes; Sequence Context; Circular Dichroism; Structure; Stability

1. INTRODUCTION

Our research group has been investigating the structure and stability of unusual DNA conformations for many years. Whether we are looking at B - Z junctions [1], DNA hairpins [2,3] or DNA quadruplexes [4], one observation always presents itself—DNA conformation is regulated by sequence and environment. For a DNA oligomer such as $(XXXYYY)_z$, where X is A, C and/or T, Y is G or T and z = 1, 2, 4 or more, the conformation of any secondary structure formed is highly dependent on the identities of X and Y, the number of repeats z and the conditions under which the DNA is prepared (temperature, pH, counterions present and their concentrations, etc.). For example, consider the complex equilibria indicated in **Scheme 1** for $(TTAGGG)_4$ and its Watson-Crick complement $(CCCTAA)_4$. At 95˚C in either Na^+ or K^+, $(TTAGGG)_4$ exists as a single stranded structure. Upon cooling, it can form an intramolecular quadruplex whose conformation depends upon whether the counterion is Na^+ or K^+ [5-8]. Further, the Na^+ form can be converted to the K^+ by titration with KCl and vice versa [7]. Similarly, $(CCCTAA)_4$ at 95˚C, pH 7.0 in the presence of Na^+ or K^+ will form the so called *i*-motif upon cooling to 25˚C and lowering the pH to 5.5 [9-13]. Finally, cooling an equimolar mixture of $(TTAGGG)_4$ and $(CCCTAA)_4$ from 95˚C to 25˚C, in either Na^+ or K^+, will result in the formation of a normal Watson-Crick double helical conformation.

In consideration of the unfolding of the K^+ form of the quadruplex formed from $(TTAGGG)_4$, the human telomere sequence, we have previously demonstrated that the unfolding proceeds via a three state mechanism and is reversible [14]. The proposed conformation of the folded structure is depicted in **Figure 1**. This structure is



Scheme 1. Schematic representation of the equilibria involved with the human telomere sequence and its Watson-Crick complement.

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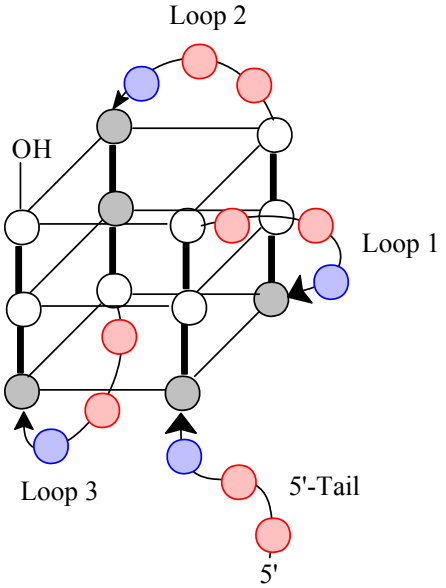

Figure 1. A schematic representation of the folded structure for $(TTAGGG)_4$. The gray circles are anti-guanines, the white circles are syn-guanines, the red circles are thymidine and the blue circles are adenine. Note the designations of Loops 1 through 3 all of which have the sequence of -(TTA)- as well as the 5'-Tail. Loop 1 is described as a propeller loop while Loops 2 and 3 are lateral loops [17].

characterized by having three G-tetrads linked by Loop 1 (a propeller loop) and Loops 2 and 3 (transverse loops). Further, we should note the presence of a 5'-Tail. For the wild type sequence, *i.e.*, the human telomere sequence, all loops and the tail have a sequence of 5'- TTA-3'. Further, the tetrads have a mixture of both syn and anti-guanines. Ultimately, the loops determine the strand orientations**,** which for this conformation, is a mixture of parallel and antiparallel. One possible mechanism for the unfolding is through opening of the quadruplex at Loop 2; *i.e.*, Loop 2 acts as a hinge. This would require breaking six hydrogen bonds on the front face and six hydrogen bonds on the back face of the quadruplex with concomitant release of the sequestered K^+ ions leading to a double hairpin which unfolds in the second step of the transition [4].

It is well established that the folding motif for any DNA intramolecular quadruplex is highly regulated by the types of loops present [15-20]. The types of loops present will determine: 1) the syn or anti conformations of the guanine bases; 2) the strand orientations (*i.e.*, parallel, antiparallel or mixed parallel-antiparallel); and 3) the orientation of the hydrogen bonds within one G-tetrad relative to the G-tetrad above and below [21]. All of these variations will ultimately impact the thermodynamics of the unfolding process.

With our awareness of the relationships between sequence context, conformation and stability, it is quite reasonable to assume that sequence context variations in the loops of intramolecular quadruplexes will influence their confirmations and stabilities. Further, can we use sequence context effects to delineate the unfolding mechanism? We have recently shown that changing the sequence of all loops and the tail systematically influences conformation and stability [4]. Here, we report the effects of changing the sequence context of any one loop, one loop at a time. We demonstrate that changing just one base in any one loop can dramatically influence conformation and stability as determined by circular dichroism (CD) studies.

2. METHODS AND MATERIALS

2.1. Buffer Preparation

Standard potassium phosphate buffer (150 mM K^+, 10 mM PO_4^{3-} and 0.1 mM EDTA) at pH 7 was prepared, filtered through a 0.45 μm Millipore filter and degassed before being stored for use.

2.2. Sample Preparation

HPLC purified oligonucleotides were purchased from Biosynthesis Inc (Lewisville, TX) and used without further purification. All sequences were reconstituted in 1 ml filtered buffer followed by heating to 95˚C. After cooling to room temperature, the sample was stored for 24 hrs at 5˚C.

2.3. Determination of DNA Concentration

DNA concentrations solutions were determined by UV/V are using a Varian Cary 100 Bio model (Varian Associates, Palo Alto, CA) spectrometer. The reconstituted DNA sample was diluted 10 fold in 150 mM K^+ buffer and scanned between 320 nm and 220 nm at 25˚C and 95˚C with subtraction of the buffer baseline. Concentrations were calculated using the molar extinction coefficients provided by the supplier of the DNA oligomers and the observed absorption at 260 nm.

2.4. Circular Dichroism Spectrophotometry

Circular dichroism spectra were collected with DESA Rapid Scanning Monochromator Spectrophotometer (Model Olis RSM 1000) attached with a temperature controller (Julabo CF31) and a nitrogen purging unit. CD spectra were collected from 320 nm to 220 nm at 1 nm intervals with an integration time of 3 seconds at 25˚C or 95˚C. For optical melting profiles, spectra were collected from 25˚C to 95˚C at an interval of 5˚C with an integration time of 3 seconds. After equilibration for 5 minutes at each temperature, the spectrum from 320 to 220 nm was collected. A baseline of the 150 mM K^+ buffer was obtained from 320 - 220 nm using an integration time of

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3 seconds and was subtracted from each spectrum. Samples were run in a 1 mm circular quartz cuvette. Data were analyzed using Olis Global Works and SigmaPlot version 11.

3. RESULTS AND DISCUSSION

Keeping in mind that the wild type sequence is $(TTAGGG)_4$, we examined the conformation and stability of all permutations of the A and T bases in each loop via CD optical spectroscopy. The CD spectrum of each oligomer is unique. **Figures 2** and **3** compare spectra, arranged by loop number or loop sequence context, respectively, for the quadruplex forming DNA sequences possessing a single base permutation of the TTA loop sequence found in each loop of the wild type structure. As can be seen, changing just one base in any one loop gives rise to a CD spectrum different from the wild type and all other sequences. The differences appear in peak or trough wavelength, peak or trough intensity and the presence or an absence of a shoulder in the 260 - 280 nm range. Similar trends are observed for the double and triple variants (data not shown).

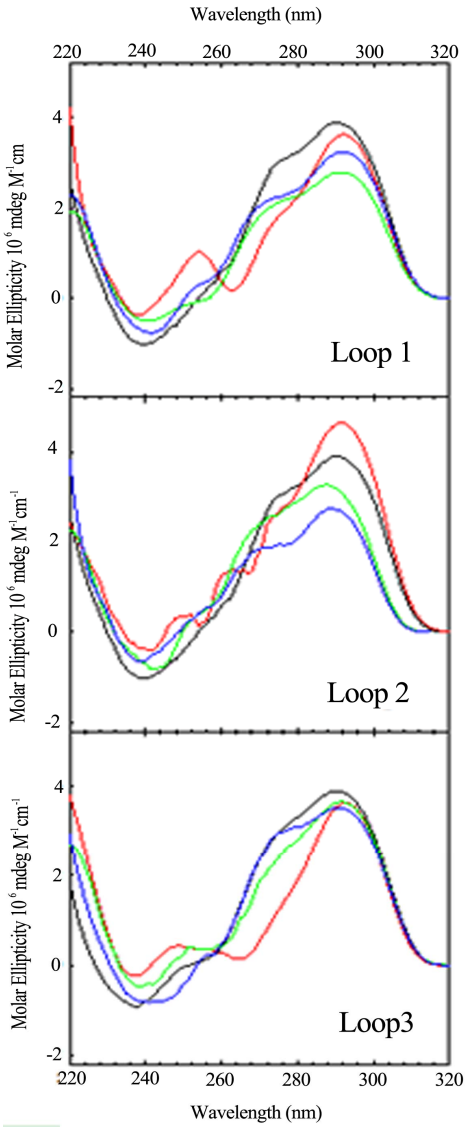

Figure 2. CD spectra of the loop variants with just one base different from the wild type, $(TTAGGG)_4$, as a function of sequence: TTA (black); TTT (red); TAA (blue); ATA (green). CD spectra were collected at 25˚C in 10 mM potassium phosphate buffer, pH 7.0, with a total of 150 mM K^+.

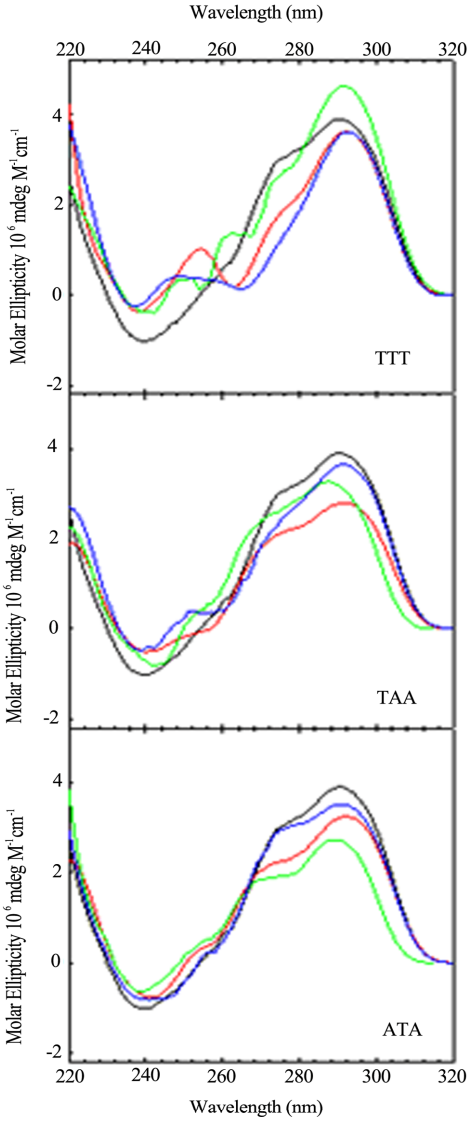

Figure 3. CD spectra of sequence variants with just one base different from the wild type, $(TTAGGG)_4$, as a function of loop location: Wild Type (black); Loop 1 (red); Loop 2 (blue); Loop 3 (green). CD spectra were collected at 25˚C in 10 mM potassium phosphate buffer, pH 7.0, with a total of 150 mM K^+.

These spectral differences are partly due to the chan-

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ging of the chromophore from T to A or A to T, each of which contribute differently to the total CD spectrum [22, 23]. The other contribution to spectral changes is due to alteration of stacking of the loop bases within the loops. Hence, changing a single base in a loop changes its conformation due to differences in stacking. Sequence context plays a role in the structure and stability of DNA duplexes due to differences in base stacking for DNAs of identical GC content [24-28] and also plays a role in stacking of bases in single strand loops in duplex DNA [29,30]. The folding motifs of quadruplexes are dependent upon loop formation for the non G-rich segments and theses short loops are conformational restricted. Changing the conformation of one loop can lead to changing the conformations of the stacked tetrads and the other loops. So it should not be surprising that a single base change can influence loop conformation and overall structure.

Since base stacking influences conformation, it also influences stability. We carried out CD optical melting studies on all DNA sequences. Experimentally, we recorded the CD spectrum from 320 to 220 nm every 5˚C from 25˚C to 95˚C. To obtain T_m values **(**the melting temperature determined at the midpoint of the quadruplex to single strand transition), we plotted the fractional change of the molar ellipticity at 292 nm as a function of temperature. **Figure 4** displays these optical melting curves grouped by loop variant. T_m values were obtained at the midpoint of the transition (*i.e.* fractional change = 0.5). The ΔT_m values listed in **Table 1** were determined by subtracting the T_m of the wild type sequence (71.4˚C) from that of the particular oligomer. As can be seen from **Figure 4**, not all quadruplexes are fully denatured at 95˚C so the ΔT_m values in **Table 1** are estimated for five of the quadruplexes as indicated by a >in front of the temperature.

It is quite remarkable that a single base change can influence the melting temperature in sometimes a dramatic fashion. For example, changing TTA to TTT increases the T_m by 8˚C to over 11˚C, depending upon the loop location of the permutation. It should be noted that we are using T_m as a basis for comparison of relative quadruplex stability. Our previous work demonstrated that the wild type sequence $(TTAGGG)_4$ unfolds via a three state mechanism. None the less, we can still use T_m values to compare relative stabilities. Data for the double base variants and triple base variants are also included in **Table 1**. In general, these quadruplexes are more stable than the wild type as well.

Figure 5 graphically displays the T_m values for all permutations studied. Some generalizations can be made from these data. First, any loop triplet with a T in the first and third position gives rise to the most stable variant. The variant with all A bases in Loop 3 is the least

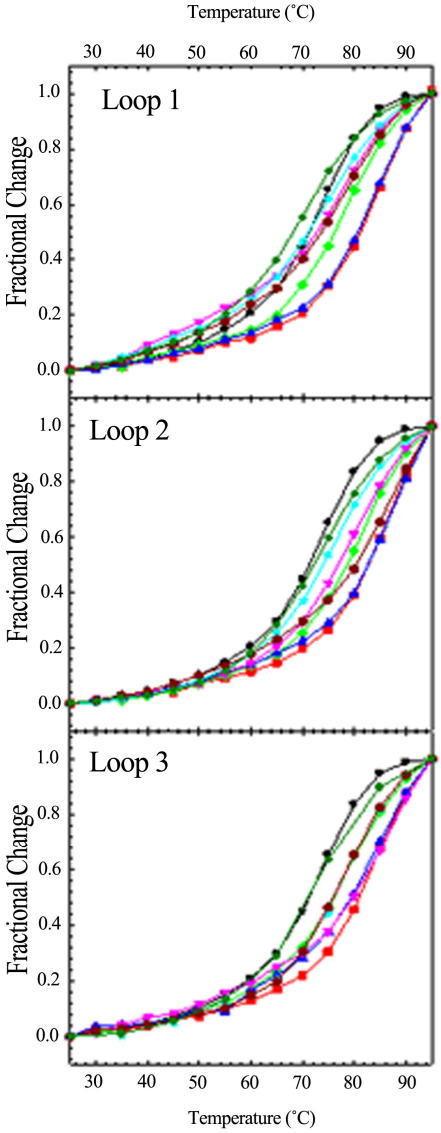

Figure 4. CD optical melting profiles of all loop variants: TTA (black circle); TAT (red square); ATT (green diamond); TTT (blue up triangle); AAT (pink down traingle); ATA (cyan star); TAA (dark red hexagon); AAA (dark green cross). CD spectra were collected in 10 mM potassium phosphate buffer, pH 7.0, with a total of 150 mM K^+, every 5˚C from 25 to 95˚C with a five minute equilibration time at each temperature.

stable of all sequences. In general, the most stable variants, with respect to loop, are the Loop 2 variants.

Finally, with the exception of Loop 3 AAA, all other sequences are as stable as or more stable than the wild type sequence of TTA. In other words, the wild type se quence forms one of the least stable quadruplexes.

Table 1 also includes ΔT_m values for DNA sequences previously studied in which all loops and the tail possess all permutations of A and T. As previously reported, these

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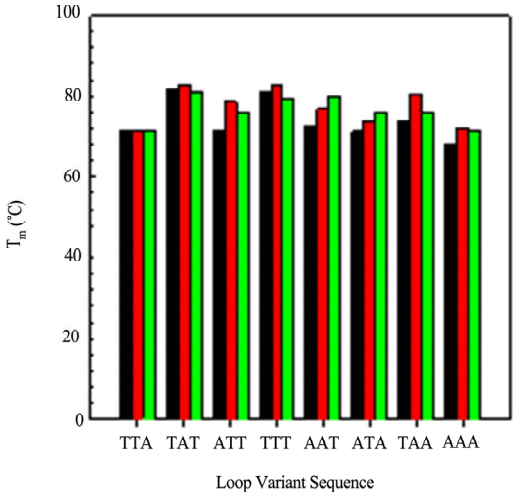

Figure 5. A comparison of the T_m values obtained from the CD optical melting profiles: Loop 1 variants (black); Loop 2 variants (red) and Loop 3 variants (green).

Table 1. Tabulate ΔT_m values (˚C) for the various DNA oligomers.[1]

Single Base Variants			
Loop	TTA → TTT	TTA → TAA	TTA → ATA
1	>9.4	2.3	−0.1
2	>11.2	>9.0	2.5
3	>8.0	4.1	4.7
All	−8.0	1.2	0.5
Double Base Variants			
Loop	TTA → AAA	TTA → ATT	TTA → TAT
1	0.0	2.0	>9.6
2	0.8	7.2	>11.2
3	−3.3	−0.1	>9.9
All	−29.7	−7.0	2.3
Triple Base Variants			
Loop	TTA→ AAT		
1	1.3		
2	5.4		
3	>8.5		
All	0.7		

[1]All ΔT_m values are ±0.2˚C. The T_m values were obtained from the midpoints of the CD optical melting profiles and that of the wild type sequence subtracted to obtain ΔT_m. Values for variants in which all loops were modified were obtained from [4]. In many cases, the melts of the single loop variants were incomplete even at 95˚C, the upper limit of our temperature range for the CD, and those ΔT_m values are estimated as indicated by the >symbol.

permutations can have dramatic effects on the overall stability and structure of the folded quadruplex [4]. However, the effect of changing one base in the loop is not additive when all loops are changed. For example, permuting TTA to TTT stabilizes the quadruplex if the permutation is only in one loop. However, changing all the loops and the tail leads to dramatic destabilization. Thus, the sequence context and length of the tail may have an effect as well on the overall stability. We are currently investigating that question.

Just as base-pair stacking in duplex DNA contributes to the total free energy of duplex formation in a sequence specific fashion [24-28], the stacking of the tetrads in an intramolecular quadruplex will also contribute to the total free energy of its folding. Additional contributions to the total free energy of quadruplex folding also comes from the base-stacking within the tail, within the loops and the stacking of third base of the tail with the first G-tetrad and the stacking of the first and third bases of the loops with the G-tetrads to which they are connected. There may also be stacking of the second base of the loops with the G-tetrads.

The stacking of unpaired bases to DNA or RNA duplexes has been thoroughly studied [31-36]. For duplex RNA, the presence of a dangling end (an overhang of unpaired bases) stabilizes the duplex relative to the blunt ended duplex with a 3'overhang more stabilizing than a 5'overhang [31,32]. For duplex DNA, the 5'overhang stabilizes more than the 3'overhang and in a sequence specific fashion of purine > T > C [33-36].

All quadruplexes studied here have a 5'-TTA tail giving rise to the stacking of the A base with the first G-tetrad. The contribution of that stack plus the stacking within the tail should be the same for all sequences studied. We have also investigated the effects of dangling ends on the human telomere quadruplex using $(GGGTTA)_3GGG$ with no overhangs, $(TTAGGG)_4$ with a 5'-TTA overhang, $(GGGTTA)_4$ with a 3'-TTA overhang and (TTAGGG)TTA with both a 5' and 3' TTA overhang via CD optical melting studies. Surprisingly, the stability order based on T_m is: no overhang > 3'-overhang > 5'- overhang > 5'- and 3'-overhang in both sodium and potassium solutions [37]. This observation is consistent to that recently reported for a single quadruplex with a 5'-TTA and a 3'-TT overhang having a lower T_m than the quadruplex with just the 5'-TTA overhang [38]. The tetrads will stack with either a 5'-T or 5'-A going into the loop and a 3'-T or 3'-A coming out of the loop. This stacking may also depend upon the type of loop (propeller vs transverse). Hence, the difference in observed T_m values also are due to changes in stacking of the tetrads with the bases in the loops and may also be due to difference in stacking of the tetrads induced by the conformational differences of the loops. A calorimetric study is currently underway to address the issues of stacking.

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4. CONCLUSION

These studies have demonstrated the sensitivity of DNA G-quadruplexes to subtle sequence context changes in the loops that link the tetrads together. This sensitivity arises from the stacking interactions of the loop bases with themselves and/or with the G-tetrads. Since base-base stacking is dependent upon the nature of the bases, the preferred stacking would give rise to the lowest energy conformation of the loop. A conformational change in the loop will be compensated by an overall conformational change in the quadruplex. In general, the T_m values are both sequence specific as well as loop specific. Further, most variants have higher T_ms than that of the wild type. Since unfolding of the quadruplexes located at the 3 termini of human chromosomes is the first step in DNA replication, it should not be surprising that the least stable structure evolved.

5. ACKNOWLEDGEMENTS

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