Stability of the Na+ Form of the Human Telomeric G-Quadruplex: Role of Adenines in Stabilizing G-Quadruplex Structure

Brenna A. Tucker,*† Jason S. Hudson,*† Lei Ding,*§ Edwin Lewis,‖ Richard D. Sheardy,§ and David Graves*†,*§

†Department of Chemistry,‡Department of Biochemistry and Molecular Genetics, and§Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294, United States
§Department of Chemistry, Mississippi State University, Mississippi, Mississippi State 39762, United States
‖Department of Chemistry & Biochemistry, Texas Women’s University, Denton, Texas 78204-2, United States

ABSTRACT: G-quadruplexes are higher order DNA structures that play significant roles in gene transcription and telomeric maintenance. The formation and stability of the G-quadruplex structures are under thermodynamic control and may be of biological significance for regulatory function of cellular processes. Here, we report the structural influence and energetic contributions of the adenine bases in the loop sequences that flank G-repeats in human telomeric DNA sequence. Spectroscopic and calorimetric techniques are used to measure the thermal stability and thermodynamic contributions to the stability of human telomeric G-quadruplexes that have been designed with systematic changes of A to T throughout the telomeric sequence. These studies demonstrate that the thermal stability of the G-quadruplex structure is directly related to the number and position of the adenines that are present in the telomeric sequence. The melting temperature (T_m) was reduced from 59 °C for the wild-type sequence to 47 °C for the sequence where all four adenines were replaced with thymines (0123TTT). Furthermore, the enthalpy required for transitioning from the folded to unfolded G-quadruplex structure was reduced by 15 kcal/mol when the adenines were replaced with thymines (37 kcal/mol for the wild-type telomeric sequence reduced to 22 kcal/mol for the sequence where all four adenines were replaced with thymines (0123TTT)). The circular dichroism melting studies for G-quadruplex sequences having a single A to T change showed significantly sloping pretransition baselines and their differential scanning calorimetry (DSC) thermograms revealed biphasic melting profiles. In contrast, the deoxyoligonucleotides having sequences with two or more A to T changes did not exhibit sloping baselines or biphasic DSC thermograms. We attribute the biphasic unfolding profile and reduction in the enthalpy of unfolding to the energetic contributions of adenine hydrogen bonding within the loops as well as the adenine stacking to the G-tetrads of the G-quadruplex structure.

INTRODUCTION

Guanine-rich nucleic acids are capable of forming higher order DNA structures called G-quadruplexes.1–4 Analyses of the human genome have established the presence of a large number of highly conserved guanine-rich sequences that have the potential to form G-quadruplex structures.5–7 These regions were found in certain oncogenic promoter regions and at the telomeric ends of the chromosome, providing evidence for in vivo biological functions of G-quadruplex structures. The potential for G-quadruplex structures to serve regulatory functions have made them attractive targets for the development of therapeutic drugs to modulate genetic transcription and telomeric extension.5–9 At the core of the G-quadruplex structure are the planar G-tetrads composed of four guanines that are held together by Hoogsteen bonding and further stabilized by G-tetrad stacking. In vitro, G-quadruplexes may be constructed via intermolecular or intramolecular interactions of G-rich DNA sequences with significant variations in sequence and molecularity. G-rich oligonucleotides of sufficient length have the innate ability to form unimolecular G-quadruplexes that have demonstrated a high degree of topological variations that are dependent on both the base sequence and cationic environment.10,11

Human telomeric DNA is a noncoding region at the end of the chromosome that consists of repetitive sequences that are approximately 5–15 kb in length with the tandem repeat of 5′- (TTAGGG)_n-3′. The terminal end of this sequence has a 3′-single stranded overhang ranging from 35 to 600 nucleotides in length that has been demonstrated to form stable G-quadruplex structures both in vitro and in vivo.12–15 The formation of the G-quadruplex structure has been demonstrated to inhibit the attachment of telomerase, thus halting the enzymatic extension of the telomere that is often associated with cancerous cells.16–18 The unimolecular human telomeric G-quadruplex

Received: October 26, 2017
Accepted: January 9, 2018
Published: January 24, 2018

DOI: 10.1021/acsomega.7b01649
ACS Omega 2018, 3, 844–855

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structure has three stacked G-tetrads connected by loops comprised of a 5′-TTA-3′ sequence. The focus of the work presented here probes the role of the conserved adenine (in the 5′-TTA-3′ sequence) in each of the loops in stabilizing the G-quadruplex structure. On the basis of X-ray and NMR structural studies of the human telomeric G-quadruplex, the adenines are postulated to provide stability through stacking of the purine base on the G-tetrads of the G-quadruplex structures. To test this hypothesis, systematic replacement of each of the adenines with thymines was followed by an extensive thermodynamic analysis of the energetics of unfolding. These studies reveal the adenines in the 5′-TTA-3′ loop sequences play a significant role in G-quadruplex stability and folding topologies. The nature of the loop connectivity gives rise to a number of alternate conformations that have been characterized both by NMR and X-ray crystallography. The most notable structural differences are between the sodium and potassium cationic forms. Sodium-based buffers result in an all antiparallel strand orientation with glyciscod bond angles in the anticonformation. However, the loop connectivity may differ and be all parallel loops, the “chair” conformation, or have two parallel loops and one diagonal loop, the “basket” conformation. Both of these folding topologies and their loop connectivity are illustrated in Figure 1. Potassium-based buffers result in more complex G-quadruplex structures that are characterized by two lateral loops and one loop that shows a chain reversal that is oriented on the edge of the tetrad core. The result is a mixed strand polarity that has both antiparallel and parallel characteristics with one guanine in a syn-conformation and the remaining three in an anticonformation. Nevertheless, G-quadruplex structures have the amazing ability to form topologically diverse structures that are quite stable under biologically relevant temperatures and conditions. The stabilities of G-quadruplex structures are derived from contributions of the G-tetrad Hoogsteen base pairing and hydrophobic stacking of successive G-tetrads.

The energetics of G-quadruplex unfolding and the enthalpic (ΔH<sub>unf</sub>) and entropic (ΔS<sub>unf</sub>) contributions to the thermal stability have been reported by numerous research groups using a variety of biophysical methods. There are variations in the unfolding energies reported for G-quadruplex forming sequences. These variations arise from subtle differences in base sequences as well as buffer conditions. Herein, we present an analysis of the thermal stabilities and the energies of unfolding of the wild-type human telomeric sequence 5′-(AGGGTTAGGGTTAGGGTTAGGG)-3′ using circular dichroism (CD) spectropolarimetry and differential scanning calorimetry (DSC). Starting with the wild-type sequence, a series of deoxyligonucleotides were designed to probe contributions that the adenines within the sequences had on the structural and energetic contributions (see Table 1 for sequences). The buffer conditions (100 mM NaCl supplemented buffer) and loop length (three nucleotides) were consistent for CD and DSC measurements on each of the DNA sequences. In an effort to discern the number of structurally relevant and or intermediate components present or arising during the unfolding event, three-dimensional CD melting curves were generated and analyzed by singular value decomposition (SVD).

RESULTS AND DISCUSSION

CD Studies on Conformational and Thermal Denaturation. CD spectroscopy was used to probe the conformational changes that result from the A to T conversion(s) in the TTA loops of the human telomeric G-quadruplex sequences. The CD spectra provided in Figure 2 are assembled with respect to the number of loops modified (i.e., either single loop change or multiple loop changes). The CD spectrum for the wild-type sequence (black) is included in both panels for comparison. Structural analyses by CD of the wild-type human telomeric DNA sequence and concomitant sequences containing A to T loop modifications reveal that under Na⁺ conditions, all of the selected sequences used in this study formed antiparallel G-quadruplex structures, as indicated by their positive maximum at 295 nm and a negative minimum at 265 nm. The CD spectra for oligonucleotides with base changes in the TTA loops (A to T conversions) are all consistent with antiparallel topology; however, subtle differences are observed in the magnitudes and the wavelength of the maxima and minima. This is in the case of single A to T loop conversion, differences in magnitudes of the maxima at 295 and 245 nm and minimum at 265 nm, relative to the wild-type, are observed. In the modified sequences with two or more A to T loop conversions, differences in the magnitudes for all maxima and minima are observed. Additionally, slightly higher shifts in wavelengths for the maximum and minimum are observed.

The CD spectra are clearly influenced by sequence effects, most likely because of the geometries associated with differences between adenine base stacking with G-tetrads that are adjacent to the loops in the folded structures in the case of the wild-type sequence or the lack thereof when the adenine is replaced with thymine. CD analyses of G-quadruplexes are most suited for classifying the strand polarities (parallel and antiparallel orientations) and observing changes in the G-quadruplex structure and cannot distinguish between conformations or structural isoforms of the same strand polarity. However, when analyzed in conjunction with the DSC data, we speculate that the changes in the CD spectra are likely due to the disruption of the stacking interactions resulting from the removal of the adenine bases from the loop sequences. It has been previously reported that changing the thymine(s) in the loop sequences to cytosines results in structural changes to the G-quadruplex formed when in the presence of potassium. The loss of adenine stacking interactions may result in structural distortion of the G-tetrads that result in subtle structural changes relative to the wild-type sequence that would give rise to the observed variations in the CD spectra.
Chaires and co-workers have provided considerable insights into gleaning G-quadruplex unfolding mechanisms by CD spectropolarimetry. In this study, CD spectra are used to qualitatively determine the nature of strand polarity for the folded structures. For these Na+-induced G-quadruplex sequences, all are indicative of antiparallel conformations. Melting studies derived by monitoring the change in CD signal at 295 nm were conducted to probe the effects of A to T loop conversions on the thermal stabilities for each of the G-quadruplex sequences. Overlays for the single loop and multiple loop modifications are provided in Figure 3. The curves appear to be monophasic and the $T_m$ was calculated by taking the first derivative of the sigmoidal melting curve. The wild type G-quadruplex resulted in a melting temperature of 58 °C. The melting temperatures of the majority G-quadruplexes harboring one or more A to T loop changes were within ±4 °C from the wild type. These results are summarized in Table 2. In the case of the 0123TTT oligonucleotide, where all four adenine residues are replaced with thymines, a significant reduction in the thermal stability of the G-quadruplex structure is observed with a $T_m$ of 44 °C, a decrease in thermal stability of 14 °C. In contrast to this trend, if the sequence of loop 2 was changed from TTA to TTT, a slight enhancement in the

Table 1. Sequences Showing the A to T Base Modifications in the Loops of the Human Telomeric G-Quadruplex Sequences Used in This Study

| Sequence | Loop Modification | Nomenclature |
|----------|-------------------|--------------|
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | - | wild-type |
| 5'-TGGTTAGGGTTAGGGTTAGGG-3' | 0 | 0T |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 1 | 1TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 2 | 2TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 3 | 3TT |
| 5'-TGGTTTAGGGTTAGGGTTAGGG-3' | 0,1 | 01TT |
| 5'-TGGTTAGGGTTAGGGTTAGGG-3' | 0,2 | 02TT |
| 5'-TGGTTAGGGTTAGGGTTAGGG-3' | 0,3 | 03TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 1,2 | 12TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 1,3 | 13TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 2,3 | 23TT |
| 5'-AGGGTTAGGGTTAGGGTTAGGG-3' | 1,2,3 | 123TT |
| 5'-TGGTTTAGGGTTAGGGTTAGGG-3' | 0,1,2,3 | 0123TT |

Red indicates A to T conversion.

Figure 2. CD wavelength scans for the human telomeric G-quadruplex d[AGGG(TTAGGG)]$_3$ (red line) and the A to T converted loop sequences. (A) Sequences that possess single adenine to thymine base changes. (B) Sequences with multiple A to T modified loops.
thermal stability ($T_m = 62 \, ^\circ C$) relative to the wild type was observed. All of the transitions appeared to be monophasic and were characterized by sigmoidal curves. However, a closer examination of the baselines showed that there were differences for the single-loop and multiple-loop modified sequences. All of the single-loop modified sequences had severely sloping pretransition baselines, whereas the multiple-loop modifications exhibited minimal sloping effects observed for the baselines. There are a number of potential factors that may cause sloping pretransition baselines, such as a temperature dependence of the solvent, heat capacity effects, loss of base stacking interactions, or the presence of intermediates in the unfolding mechanism. The lack of clearly defined pre- and post-transition baselines is problematic for calculating thermodynamic

### Table 2. Summary of Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD) Thermal Denaturation of Telomeric G-Quadruplex (Sodium Form) Deoxyoligonucleotides Containing A to T Modifications in One or More Loops

| sequence | number of transitions | DSC$^a$ | CD$^c$ |
|----------|-----------------------|---------|--------|
|          |                       | $T_m$ (°C) | $\Delta H_{\text{fus}}$ (kcal/mol) | $\Delta G^\circ_{\text{[298K]}}$ (kcal/mol) | $T_m$ (°C) |
| wt G-quad | 2                     | 59 ± 0.8 | 37.4 ± 3.2 | 3.8 ± 0.2 | 58 ± 1.8 |
| 0T       | 2                     | 59 ± 1.8 | 36.1 ± 2.6 | 3.7 ± 0.3 | 53 ± 0.8 |
| 1TTT     | 2                     | 55 ± 0.6 | 35.7 ± 2.1 | 3.2 ± 0.2 | 53 ± 1.4 |
| 2TTT     | 2                     | 62 ± 1.2 | 34.3 ± 3.2 | 3.8 ± 0.3 | 62 ± 2.2 |
| 3TTT     | 2                     | 55 ± 1.3 | 34.6 ± 2.5 | 3.2 ± 0.4 | 54 ± 1.5 |
| 01TTT    | 1                     | 55 ± 1.5 | 19.2 ± 1.8 | 1.7 ± 0.1 | 52 ± 0.9 |
| 02TTT    | 1                     | 54 ± 0.6 | 23.5 ± 2.9 | 2.0 ± 0.3 | 53 ± 1.1 |
| 03TTT    | 1                     | 54 ± 1.1 | 21.6 ± 2.2 | 1.9 ± 0.1 | 51 ± 1.3 |
| 12TTT    | 1                     | 59 ± 2.0 | 32.3 ± 2.0 | 3.3 ± 0.2 | 58 ± 1.6 |
| 13TTT    | 1                     | 59 ± 1.4 | 32.1 ± 1.8 | 3.3 ± 0.4 | 57 ± 0.7 |
| 23TTT    | 1                     | 57 ± 1.6 | 30.0 ± 2.7 | 2.9 ± 0.3 | 59 ± 2.2 |
| 123TTT   | 1                     | 59 ± 1.1 | 28.0 ± 3.2 | 2.9 ± 0.2 | 58 ± 0.8 |
| 0123TTT  | 1                     | 47 ± 0.9 | 22.3 ± 2.2 | 1.5 ± 0.4 | 44 ± 1.1 |

$^a$All deoxyoligonucleotides are in 10 mM BPES with 0.1 M NaCl at a pH of 7.0. $^b$Differential scanning calorimetry experiments were performed in 10 mM phosphate buffer (BPES) (pH = 7.0), 0.001 M disodium ethylenediaminetetraacetate (EDTA), and 0.1 M sodium chloride. For the DSC experiments, the DNA strand concentrations range from 175 to 225 μM. Experiments were conducted with a heating rate of 0.5 °C/min over a range from 10 to 100 °C. A minimum of five heating and cooling cycles was conducted for each sample to assess reversibility of the unfolding. $^c$Circular dichroism spectropolarimetry melts were performed in 10 mM phosphate buffer (BPES) (pH = 7.0), 0.001 M disodium EDTA, and 0.1 M sodium chloride at a DNA strand concentration of 3 μM in a 1 cm path length quartz cuvette. The temperature ranged from 10 to 90 °C at a rate of 1 °C/min. Measurements were taken at every 1 °C after a 1 min equilibration time. A minimum of three melts were performed per experiment and data averaged.
parameters by van’t Hoff analysis and may not represent the true enthalpy of the unfolding process.44–46

**Calorimetric Analysis of G-Quadruplex Unfolding.**

Differential scanning calorimetry (DSC) was used to measure the heat of unfolding for each of the G-quadruplex sequences. The advantage of using DSC is that the measurement is model independent and does not rely on van’t Hoff plots to determine the enthalpy of the transition from the folded to unfolded state. A biphasic transition profile was observed for the wild-type human telomeric sequence in 100 mM NaCl BPES buffer at pH 7.0. The observed transition (black line) is biphasic with a lower temperature transition at 39 °C and higher temperature transition at 59 °C. The observed data were fit to two transitions (red lines) and the total area under the curve integrated to provide the enthalpy of unfolding (∆H_{unfolding}).

**Figure 4.** DSC thermogram of the wild type human telomeric G-quadruplex DNA in 100 mM NaCl and 10 mM BPES buffer at pH 7.0. The observed transition (black line) is biphasic with a lower temperature transition at 39 °C and higher temperature transition at 59 °C. The observed data were fit to two transitions (red lines) and the total area under the curve integrated to provide the enthalpy of unfolding (∆H_{unfolding}).

transitions with melting T_m’s of 39 and 59 °C for the first and second transitions, respectively. Similar biphasic melting profiles have been observed for similar G-quadruplex sequences in K’ buffer.47–49 These data reveal the transition to be biphasic with a shoulder occurring prior to the larger transition that has a T_m of 59 °C. It is necessary to include two Gaussian curves (red lines) to deconvolute the raw data (black line) and achieve a suitable fit for the complete transition. The calorimetric enthalpy was calculated by integrating the area under the curve and was determined to be 37.4 kcal/mol for the unfolding direction. Thermograms for oligonucleotide sequences harboring both the single loop and multiple loop A to T modifications are shown in Figures 5 and 6, respectively. Melting temperatures and calorimetric enthalpies are calculated in a manner similar to the wild-type sequence and their results are reported in Table 2.

The thermal unfolding of the sequences with A to T loop modifications revealed a that the low-temperature transition was reduced in magnitude for the single A to T loop modifications and was not observed at all for the multiple A to T loop modifications. The telomeric sequence where the S'-A base was converted to S'-T (0T) most resembled the wild-type and the additional single loop A to T change resulted in transition profiles that were broadened with only minor features present corresponding to the low-temperature transition. The sequences with multiple A to T loop modifications were all easily fit with a single transition and lacked any evidence of the low-temperature transition that was observed for the wild-type and single-loop modifications. Not only did the presence of the adenine bases contribute to the shape of the melting transition but also contributed to the calculated unfolding enthalpies. The unfolding enthalpies for the wild-type sequence and A to T loop-modified sequences are summarized in Table 2, which reveals that as the number of adenines modified to thymines is increased, the unfolding enthalpy decreases. When all of the adenines are converted to thymines (0123TTT sequence), the enthalpy of the transition was reduced from 37 kcal/mol (as observed for the wild-type sequence) to 22 kcal/mol for the 0123TTT sequence, a change in unfolding enthalpy of 15 kcal/mol. Hence, the adenines within the 5’T-TTA-3’ loops are demonstrated to play a significant role in stabilization of the G-quadruplex structure, presumably through adenine stacking interactions with the adjacent G-tetrads.

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Figure 5. Representative DSC thermograms for the single A to T base modifications of the telomeric G-quadruplex. The black lines represent the raw DSC data and the red lines represent the number of transitions. (A) Conversion of the 5'-A to 5'-T (0T); (B) A to T modification of loop 1 (1TTT); (C) A to T modification of loop 2 (2TTT); (D) A to T conversion of loop 3 (3TTT).

Figure 6. Representative DSC thermograms for multiple A to T loop conversions for the telomeric G-quadruplex sequence. The black lines represent the raw DSC data and the red lines represent the number of transitions. (A) wild type; (B) A to T modifications of loop 0 and loop 1 (01TTT); (C) A to T modifications of loop 0 and loop 2 (02TTT); (D) A to T modifications of loop 2 and loop 3 (23TTT); (E) A to T modifications of loop 1 and loop 2 (12TTT); (F) A to T modifications of loop 1 and loop 3 (13TTT); (G) A to T modifications of loop 2 and loop 3 (23TTT); (H) A to T modifications of loops 0, 1, and 2 (012TTT); and (I) A to T modifications of all adenines in the sequence to thymine (0123TTT).
modified sequence (0123TTT) possessing the lowest calculated free energy at 25 °C of 1.5 kcal/mol.

**Singular Value Decomposition of G-Quadruplex Unfolding.** In an effort to gain insights to the bimodal transition profile observed in the DSC thermogram for the wild-type human telomeric sequence, singular value decomposition (SVD) of three-dimensional CD melting data are utilized to determine the number of significant spectral species that are present during the unfolding of the wild-type sequence and the selected oligonucleotides with single or multiple A to T loop modifications (Figure 7). The number of significant spectral species are estimated by analyzing the different components of the $S$, $U \times S$, and $V$ matrices, calculating the autocorrelation coefficients of the $U$ and $V$ matrices, and analyses of the plots of difference matrices calculated with a succinct number of singular values. The $S$ matrix is evaluated by plotting the singular values versus the component number and counting the number of singular values that rise above the baseline. The $U \times S$ matrix is representative of the basis spectra, and the columns of the $U \times S$ matrix are plotted as a function of wavelength. Each trace in the plot depicts a component involved in the unfolding mechanism, and those arising above the baseline are considered spectrally significant. The $V$ matrix columns are plotted as a function of temperature, and similar to the $U \times S$ matrix, each trace represents a different component in the unfolding process. The components that give distinct traces without noise are considered spectrally significant. Autocorrelation coefficients are calculated for each column of both the $U$ and $V$ matrices, and values greater than 0.8 indicate that the respective component is spectrally significant. A final check to determine the significance of each spectral component is achieved by calculating difference matrices and evaluating them through a contour plot. If the difference plot is calculated with the same number of singular values as number of spectrally significant components, then the resulting contour plot will present as random noise. Difference plots that do not present as random noise are therefore calculated with the wrong number of singular values.

For the unfolding of the wild-type telomeric G-quadruplex sequence in a Na⁺ environment, the SVD data reveal a minimum of three spectral species, as shown in Figure 8. The autocorrelation values for the first three columns of both the $U$ and $V$ matrices are larger than 0.8, which is also indicative of the presence of three spectrally significant species. A summary of the singular values and the $U$ and $V$ matrix autocorrelation values for the wild-type G-quadruplex sequence is provided in Table 3. The difference matrix plots for the wild-type sequence calculated with three singular values resulted in contour plots of random noise, whereas plots created with one, two, or four singular values yielded contour plots with defined contour lines (data not shown). Hence, these data are indicative of a minimum of three spectrally significant species in the unfolding of the wild-type human telomeric sequence and may account for the bimodal transition profile observed in the DSC thermogram for the wild-type human telomeric sequences. A summary of these data for all of the G-quadruplex A to T loop modified sequences is presented in Table 4. For all sequences harboring A to T loop modifications, a minimum of four spectrally significant species were found in all cases with three exceptions; the 1TTT, 12TTT, and 23TTT sequences were shown to have a minimum of four spectrally distinct species.

### CONCLUSIONS

The work described in this manuscript specifically focuses on the role that the adenine bases within the conserved 5′-TTA-3′-loops play with respect to the G-quadruplex structural stability. Using CD spectropolarimetry and calorimetric methods, the adenines in each of the loop sequences were systematically probed for their energetic contributions exerted through base stacking and/or hydrogen bonding within the G-quadruplex structure(s). Considerable work has been focussed on the effects of altering the base composition and/or length of the TTA loop sequences with alternate base substituions, nonbase linkers, and/or and length on G-quadruplex stabilities. Mergny and co-workers examined 27 different G-tract sequences with differing loop length(s) and sequence(s) and concluded that all of the sequences examined could form stable G-quadruplex structures; however, those in potassium were more stable than those in sodium environments. In cases of single-loop bases, pyrimidines were preferred over adenine. Under sodium conditions, the folding of the G-quadruplex structures was diverse and sequence dependent, whereas in potassium, the folding topology was observed to be all parallel. These studies demonstrated that when guanine was used to replace adenine in the loops, none of the single base substitutions hindered the formation of antiparallel quadruplexes in sodium. However, the effects of such a substitution did differ, depending on the loop where the substitution occurred. This finding may be a result of G-slippage in the G-tetrad formation. The length of the loops was also varied. Neidle and co-workers demonstrated that parallel stranded intramolecular quadruplexes are the only possible fold if the loop is composed of a single residue. In the case of two or more residues comprising the loop, both parallel and antiparallel folded structures can form with similar free energies. Fox and co-workers demonstrate the bases between the G-tracts significantly affect quadruplex stability; TTA to AAA abolishes G-quadruplex formation. Hence, the loop regions of the G-quadruplex forming sequences are shown to play a critical role in the type of fold and structural stability of the G-quadruplex. The conservancy the 5′-TTA-3′ loop sequence for all loops in the human telomeric DNA and the role that each of the loops exert in the diversity of structures...
and stabilities may play a significant role in the biology of the telomeric region of the chromosome.\textsuperscript{56,57}

The energetic basis for the low-temperature transition observed for the DSC profiles is complex. One possibility that may explain the observed biphasic melting profile could be the presence of multiple conformers and/or intermediate species within the solution that thermally denature independent of each other. The sodium form of the human telomeric G-quadruplex has been shown to form both the “chair” and/or “basket” folding topologies in solution.\textsuperscript{2,20,24} It is possible that the conversion of an A to T in one or more of the loop regions may influence the folding topologies such that the G-quadruplex structure formed will favor one or the other folding topologies.

Table 3. Summary of Singular Values and Autocorrelation Data for the Wild-type Human Telomeric G-Quadruplex DNA Sequence

| order | singular value | \( U \)-correlation | \( V \)-correlation |
|-------|---------------|-----------------|-----------------|
| 1     | 288.5280      | 0.978185        | 0.970595        |
| 2     | 88.5680       | 0.984135        | 0.969943        |
| 3     | 25.3149       | 0.991713        | 0.907941        |
| 4     | 6.3224        | 0.719899        | −0.117919       |

Table 4. Singular Value Decomposition and Autocorrelation Data for the Wild-type Human Telomeric G-Quadruplex DNA Sequence and Selected Sequences Having A to T Modifications in One or More Loops

| sequence   | \( S \) matrix | \( U \times S \) matrix | \( V \) matrix | autocorrelation coefficient | difference matrix | minimum spectral species |
|------------|---------------|------------------------|---------------|-----------------------------|-------------------|-------------------------|
| wt G-quad  | 3             | 3                      | 3             | 3                           | 3                 | 3                       |
| 0T         | 3             | 4                      | 3             | 3                           | 4                 | 3                       |
| 1TTT       | 4             | 4                      | 4             | 4                           | 4                 | 4                       |
| 2TTT       | 4             | 3                      | 3             | 3                           | 3                 | 3                       |
| 3TTT       | 4             | 4                      | 3             | 4                           | 3                 | 3                       |
| 01TTT      | 4             | 3                      | 3             | 3                           | 3                 | 3                       |
| 02TTT      | 4             | 3                      | 3             | 3                           | 3                 | 3                       |
| 03TTT      | 4             | 3                      | 3             | 3                           | 3                 | 3                       |
| 12TTT      | 5             | 4                      | 4             | 4                           | 4                 | 4                       |
| 13TTT      | 5             | 4                      | 4             | 4                           | 4                 | 4                       |
| 23TTT      | 4             | 4                      | 3             | 3                           | 3                 | 3                       |
| 123TTT     | 4             | 4                      | 3             | 3                           | 3                 | 3                       |
| 0123TTT    | 4             | 4                      | 3             | 3                           | 3                 | 3                       |
(basket or chair) conformation. However, if this were the case, we would not expect the \( T_m \) of the low-temperature transition to deviate as much as 15 °C, as observed in the case of the 2TTT loop. The \( T_m \) of the low-temperature transitions that are apparent in the wild-type and single-loop conversions appear to be linked to the high temperature transition and were never clearly resolved with a baseline between the transitions. Furthermore, we are not aware of any published biphasic DSC thermograms of G-quadruplexes that have been resolved by stabilizing one of the transitions relative to the other using variations in cation concentration, scan rates, or sequence variations.26,28,60,61

An additional possibility for the biphasic profile is the presence of intermediates in the unfolding pathway. In 2011, Chaires and co-workers proposed that the human telomeric G-quadruplex does not unfold in a two-state manner, suggesting that there may be significantly populated intermediates states.26,37 Our analyses of three-dimensional CD data by SVD reveal that all A to T loop-altered sequences unfold along a pathway containing at least three spectral species. Our results are consistent with previous G-quadruplex folding studies wherein magnetic tweezers were used to reveal the presence of intermediate species along the unfolding pathway of the human telomere G-quadruplex DNA in the presence of Na+.62 SVD analyses of CD data collected as a function of temperature for the human telomere G-quadruplex in the presence of K’ has also been previously reported. This study also revealed the presence of an intermediate species along the unfolding pathway of the G-quadruplex.37

Fluorescence studies have also reported that both Na’ and K’ forms of the G-quadruplexes have populated intermediate states and/or rapid equilibrium between multiple conformers.63,64 Specifically, single molecule FRET studies have been performed on the human telomeric G-quadruplex in the presence of Na’ that have shown the existence of four populated species during G-quadruplex folding including an antiparallel basket, and antiparallel chair, an unfolded single strand, and a transient state that could include a G-triplex or an (2 + 2) antiparallel structure.65,66 It is safe to assume that the CD techniques used in this study are not sensitive enough to discern between two G-quadruplex structures that are energetically similar. In this case, fast conversion between the antiparallel chair and basket conformations would not be observable in the SVD analysis of the oligonucleotide sequences harboring the A to T modified loops and they would appear as one significant spectral species. Indeed, intermediates encountered by the CD experiments may be calorimetrically silent; either the intermediate concentrations are too low to detect or they have enthalpies that are indistinguishable. We speculate that the biphasic melting profiles of the human telomeric sequence used for this study are due to the presence of unfolding intermediates and are characterized by the loss the enthalpic contributions of the loop base stacking interactions represented by the low-temperature transition.

The single loop A to T modifications significantly reduced the magnitude of the low temperature transition, whereas conversions from A to T of multiple loop sequences resulted in a complete loss of the low-temperature transition. Conversion of the S'-terminal (i.e., S'-A) to S’-T had only a minimal effect on the biphasic profile and closely resembled the wild-type sequence. The unfolding of a G-quadruplex structure may involve a process wherein the A bases within the loop begin to denature prior to the denaturation of the G-tetrad core. The disruption of adenine–guanine stacking interactions and/or hydrogen bonding between the bases in the loops would have enthalpic consequences and could be observed by DSC. It is important to distinguish that neither the low- or high-temperature transitions of the denaturing profile represents the intermediate structure. Instead, the initial unfolding step may involve loop destabilization (low-temperature transition), followed by the breaking of the G-tetrad core of the G-quadruplex structure (high-temperature transition). The oligonucleotides harboring multiple A to T loops modifications have are observed to have only high-temperature transitions, yet could not be adequately fit with a simple two-state model. This suggests that these G-quadruplex structures unfold by a similar mechanism involving initial loop destabilization followed by the denaturation of the G-tetrad at higher temperatures. Low-temperature transitions are no longer apparent because the adenine–guanine base stacks that are responsible for the low-temperature transition are no longer present in the sequence. The results of this study demonstrate that the adenines within the G-quadruplex loops play a significant role in influencing the stability and energetics of the G-quadruplex structure. Additionally, these results support previous published work that suggests G-quadruplex thermodynamic stability is a combination of loop sequence effects and G-tetrad formation.

### EXPERIMENTAL SECTION

**Oligonucleotide Preparation and Nomenclature for A to T Conversion in G-Quadruplex Sequences.** Synthetic oligonucleotides with the selected adenine to thymine base changes were purchased from Midland Certified Reagents (Midland, TX). The deoxyoligonucleotides were dissolved in 100 mM NaCl BPES buffer (0.01 M NaH2PO4, 0.01 M Na2HPO4 and 0.001 M EDTA) and allowed to equilibrate overnight at 4 °C hironimus prior to their use; stock solutions were filtered using 0.45 μm Millipore syringe filters, and the concentrations were determined by UV–visible spectrophotometry at 90 °C to ensure that any secondary structure was fully denatured. The molar extinction coefficients were calculated using the nearest neighbor method, as previously described.67 The DNA solutions were then annealed at a cooling rate of 0.5 °C/min using a MJ Research Mini-Cycler (Bio-Rad Laboratories). Confirmation that each of the DNA sequences was in the G-quadruplex conformation was carried out by circular dichroism (CD) spectropolarimetry, scanning from 225 to 325 nm and observing the presence of a positive ellipticity at 295 nm.

Changes to the loop sequences were designed such that the A residue(s) were sequentially replaced with T residues, starting at the S’ end (referred to as loop 0) and at each of the subsequent loops (loops 1–3). The sequence changes and nomenclature used to distinguish each of them are illustrated in Table 1.

**Circular Dichroism Spectropolarimetry.** Circular dichroism (CD) melting studies were conducted using an AVIV 400 CD spectropolarimeter (AVIV Biomedical, Inc., Lakewood, NJ) at a strand concentration of 3 μM in a 1 cm path length quartz cuvette. The deoxyoligonucleotide solutions were allowed to equilibrate in the cuvette, with stirring for 15 min prior to each measurement. The thermal difference spectra revealed the largest change at 295 nm for all sequences, and the CD signal at 295 nm was monitored as a function of temperature. The
temperature ranged from 10 to 90 °C at a rate of 1 °C/min. Measurements were taken at every 1 °C after a 1 min equilibration time. A buffer melting baseline was subtracted from each spectrum and data normalized to molar ellipticity. The melting temperature ($T_m$) was defined as the midpoint of the transition calculated by the first derivative of the sigmoidal curve. Model-dependent van’t Hoff analyses of the melting curves were not performed due to the presence of sloping pretransition baselines and undetermined heat capacity changes upon melting of the G-quadruplex structures.

**Differential Scanning Calorimetry.** The heat associated with the unfolding of the G-quadruplex was measured using a Microcal VP-DSC (Malvern Instruments, Northampton, MA), at strand concentrations ranging from 175 to 225 μM. Experiments were conducted with a heating rate of 0.5 °C/min over a range from 10 to 100 °C. A minimum of five heating and cooling cycles was conducted for each sample to assess reversibility of the unfolding. Buffer versus buffer scans were conducted in an identical manner for the subtraction of the transition calculated by the thermodynamic temperature, and $\Delta S$ is the change in entropy.

SVD was performed on each data matrix using Matlab software (MathWorks, Natick, MA) by applying the following equation

\[
SVD(A) = USV
\]

where $A$ equals the three-dimensional $i \times j$ data matrix, $U$ equals the resulting matrix containing the basis spectra, $S$ equals a diagonal matrix containing singular values, and $V$ equals a matrix containing the amplitude vectors. Autocorrelation of $U$ and $V$ matrices were performed using the following equation

\[
C(X_i) = \sum (X_{i,j}) (X_{i, j+1})
\]

where $\Delta C_p$ is the change in heat capacity, $T$ is the thermodynamic temperature, and $T_m$ is the midpoint of the transition defined as the melting temperature ($T_m$) and corresponds to a population of 50% folded and 50% unfolded G-quadruplex structure.\(^{18,28}\)

**Singular Value Decomposition Analysis.** CD melting data were collected over a range of wavelengths (320–220 nm) and used to create a three-dimensional melting profile for analysis by singular value decomposition (SVD).\(^{28}\) Melting profiles were collected every 2 °C from 4 to 90 °C with a temperature slope of 1 °C/min. Three data points were collected every nanometer from 320 to 220 nm for each degree and averaged. Samples were allowed to equilibrate for 1 min at each temperature prior to data collection. The resulting data were arranged in a $i \times j$ matrix, with $j$ being the number of different temperature scans collected over $i$, number of wavelengths. SVD was performed on each data matrix using Matlab software (MathWorks, Natick, MA) by applying the following equation

\[
D = A - USV_T
\]

**Author Information**

**Corresponding Authors**

*E-mail: batucker@uab.edu. Phone: (205) 934-4747, (205) 975-0670 (B.A.T.).

*E-mail: Jason.Hudson@adfs.alabama.gov. Phone: (205) 934-4747, (205) 982-9292 ext 243 (J.S.H.).

*E-mail: dgraves@uab.edu. Phone: 205-915-9673. Fax: 205-934-2543. Department of Chemistry, University of Alabama at Birmingham, 901 14th Street South, Birmingham, Alabama 35294, United States (D.G.).

**ORCID***

Edwin Lewis: 0000-0003-4432-1482

Eugenia Kharlampieva: 0000-0003-0227-0920

David Graves: 0000-0002-0000-287X

**Notes**

The authors declare no competing financial interest.

**Acknowledgments**

This work was supported by a grant NSF-DMR 1608728 (E.K.).

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