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Letter

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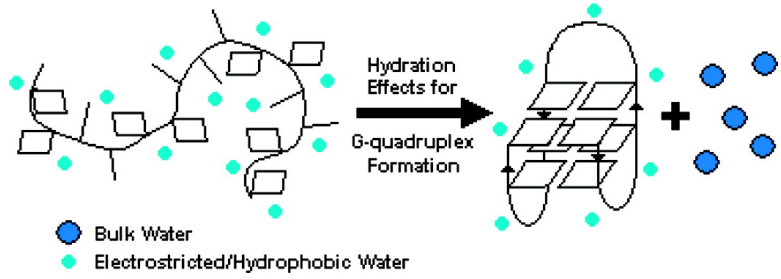

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Energetic and Hydration Contributions of the Removal of Methyl Groups From Thymine to Form Uracil in G-Quadruplexes

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A combination of spectroscopic and calorimetric techniques is used to investigate the unfolding of two G-quadruplexes: d($G_2U_2G_2UGUG_2U_2G_2$), *G2-U*, and d($G_2T_2G_2TGTG_2T_2G_2$), *G2*. The comparisons of their thermodynamic data allow us to elucidate the role of methylation on the energetic and hydration properties accompanying their stable formation. The favorable formation of each G-quadruplex results from the characteristic enthalpy−entropy compensation, uptake of ions, and release of water molecules. The loops of *G2-U* and *G2* contribute favorably to their formation, and the absence of methyl groups stabilizes the G-quadruplex. The unfolding of *G2-U* produces a larger ΔV, indicating a difference in the hydration states of the two oligonucleotides, while the opposite signs between $\Delta\Delta G°$ with the $\Delta\Delta V$ suggest that the differential hydration reflects structural, or hydrophobic, water is involved in the unfolding of G-quadruplexes.

Methylation of macromolecules plays a significant role in signaling, imprinting, and gene expression.[1] One of the simplest chemical modifications of a molecule is the addition of a methyl group. The methyl groups of DNA duplex or triple helices offset the hydrophilic−hydrophobic balance, increasing overall stability.[2] Telomeres protect the ends of chromosomes[3] and are required for the proper replication and segregation of eukaryotic chromosomes.[4] Telomeric DNA consists of clusters of guanine and thymine residues on the 3′ overhang strands, forming G-quadruplexes.[5] Recently, due to their multiple roles *in vivo*,[6] G-quadruplexes have been considered as targets for drug therapy in cancer research.[7]

In this work, we used a combination of UV and circular dichroism spectroscopies and differential scanning and pressure perturbation calorimetries, to elucidate the role of methylation on the energetic and hydration properties accompanying the formation of G-quadruplexes. The unfolding of the G-quadruplex with sequence d($G_2U_2G_2UGUG_2U_2G_2$), *G2-U*, is investigated, and the results are compared with those of the thrombin aptamer, d($G_2T_2G_2TGTG_2T_2G_2$), *G2*, that has been characterized by a variety of biophysical techniques.[8] In the six dT → dU substitutions in the loops of *G2-U*, all six methyl groups of the thymines are absent, magnifying the demethylation effects of a small molecule. The location of the loops at the surface of these molecules makes it ideal to investigate the physical behavior of G-quadruplexes toward the solvent water. Furthermore, *G2* and *G2-U* have four runs of two guanines forming one G-quartet stack. This avoids the formation of aggregates and/or multiple conformations of telomere model sequences with guanine runs containing a higher number (≥3) of guanines.

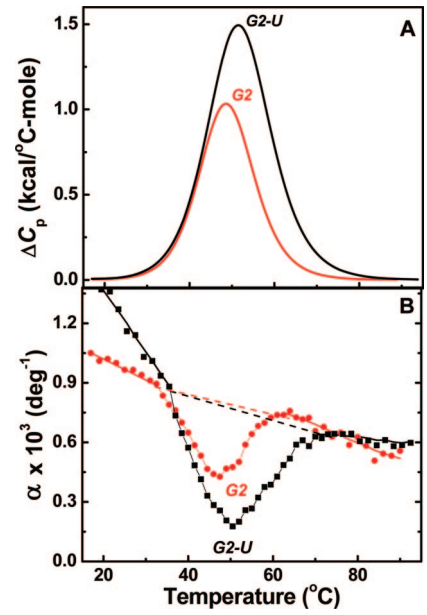

Figure 1. Calorimetric analysis of G-quadruplexes. (A) Typical DSC thermograms of G-quadruplexes. (B) Corresponding PPC curves of G-quadruplexes. All experiments were carried out in 10 mM Cs-HEPES buffer, 50 mM KCl at pH 7.5, at total strand concentrations of 1.1 mM for *G2* and 0.7 mM for *G2-U*.

The correlation of the thermodynamic and hydration data reveals, due to both higher stacking contributions of the loops and higher removal of structural water, that the absence of methyl groups from the loops of the thrombin aptamer (*G2*) increases its stability.

The unfolding of each quadruplex was initially characterized by temperature-dependent UV spectroscopy. The UV spectra of each molecule at low and high temperatures and the corresponding UV melting curves at 297 nm are shown in

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TABLE 1: Thermodynamic Profiles for the Folding of G-Quadruplexes[a]

T_M (°C)	ΔH_{cal} (kcal)	$\Delta G°_{20}$ (kcal)	$T\Delta S_{cal}$ (kcal)	ΔC_p (cal-°C^{-1})	Δn_W (mol)	Δn_{K^+} (mol)	ΔV (cm^3)
			G2-U				
53.9	–39.0	–4.0	–35.0	–850	+11	–0.7	+27
(56.4)	(–36.8)	(–4.1)	(–32.7)	–850	(+9)	(–0.6)	
			G2				
48.1	–22.4	–2.0	–20.4	+540	+9	–0.5	+18
(52.6)	(–23.7)	(–2.3)	(–21.4)	+540	(+8)	(–0.5)	

[a] All experiments were performed in 10 mM Cs-Hepes buffer at pH 7.5 and 50 mM KCl or 100 mM KCl (these values in parentheses). All parameters are reported per mole of G-quadruplex. Experimental errors are shown in parentheses: T_M (±0.5 °C), ΔH_{cal} (±4%), $\Delta G°_{20}$ (±6%), $T\Delta S$ (±4%), ΔC_p (±35%), Δn_W (±10%), Δn_{K^+} (±7%), ΔV (±15%).

Supporting Information Figures S1A and B, respectively. The resulting sigmoidal curves of each G-quadruplex show monophasic transitions with transition temperatures (T_M's) above 48 °C and hypochromicities of 26% (*G2*) and 40% (*G2-U*). The T_M remains constant as the strand concentration is increased 20-fold (Supporting Information Figure S1C), confirming their intramolecular formation. The CD spectra of each molecule as a function of temperature are shown in Supporting Information Figure S2A. *G2-U* has spectral characteristics similar to *G2*,[8] forming a G-quadruplex in the "chair" conformation.[9] The corresponding CD melting curves at 291 nm (Supporting Information Figure S2B) show hypochromicities of 78% (*G2*) and 87% (*G2-U*), also confirming their monophasic unfolding. The main observation from these optical melting curves is the larger hypochromicity values for *G2-U*, 14% (UV melts) and 9% (CD melts). One possible explanation is that base−base stacking interactions are optimized in *G2-U* by a quality layer of water molecules, as will be discussed below.

The calorimetric unfolding of each molecule is shown in Figure 1A. Analysis of these thermograms yielded standard thermodynamic profiles for the formation of each G-quadruplex (Table 1). *G2-U* has a higher T_M, by ∼4 °C,[8e] and its higher stability at 20 °C (by −2.0 kcal/mol) results from the characteristic compensation of a more favorable enthalpy (−17.4 kcal/mol) and unfavorable entropy contributions (−15.4 kcal/mol). These parameters correspond to the stabilizing effect of removing six methyl groups from *G2*. Favorable enthalpy contributions in the folding of a G-quadruplex include the stacking of two G-quartets, stacking of the loops against the G-quartet stack, base−base stacking in the loops, and hydration effects from the immobilization of electrostricted water and/or release of structural water, while the unfavorable entropy contributions include the ordering of the oligonucleotide into a G-quadruplex, and the putative immobilization of ions and water molecules. DSC curves were obtained at several salt concentrations to determine, indirectly, heat capacity effects, ΔC_p's, from the slope of the lines of the ΔH_{cal} vs T_M plots (Supporting Information Figure S3); see Table 1. Positive heat capacity effects indicate *G2* exposes hydrophobic groups to the solvent at high temperatures. However, if the assumption is made that their random coil states are similar, the actual difference is 6 Cs and 12 Hs out of a total of nearly 500 atoms. The negative ΔC_p of *G2-U* (by −1.4 kcal/°C-mol) indicates that its folded state has a lower heat capacity value, suggesting that *G2-U* is excluding more water molecules. This is due perhaps to a slightly larger apparent molar volume; i.e., *G2-U* interacts better with the solvent, changing its number of hydrating water molecules.

The differential binding of water molecules (Δn_W) and counterions (Δn_{K^+}) for the helix−coil transition of each G-quadruplex were determined from the DSC and UV melting curves as a function of osmolyte and salt concentrations (data not shown), respectively.[10] The Δn_W and Δn_{K^+} values are shown in Table 1; the folding of each quadruplex is accompanied by a release of water molecules (positive Δn_W) and a similar uptake of ions (negative Δn_{K^+}). The larger release of water molecules from *G2-U* indicates a more hydrated folded state, despite its smaller apparent molar volume.

Pressure perturbation calorimetry (PPC) is used to determine the unfolding volume of each G-quadruplex, shedding light on the hydration effects accompanying the absence of methyl groups. In a PPC experiment, one follows the temperature dependence of the coefficient of thermal expansion (α) as a function of temperature. The magnitudes of α and of the slopes of the pre- and post-transition baselines (Supporting Information Table S1) provide insight into the type of chemical groups that are exposed to the solvent throughout the transition. The PPC curves of *G2* and *G2-U* are shown in Figure 1B. At low temperatures, the positive values of α and the negative slope of the baselines indicate the exposure of hydrophilic groups (sugar−phosphate backbone) to the solvent by their folded states; however, *G2* has a decreased α value coupled with a less negative slope due to the additional exposure of the aliphatic methyl groups. This suggests *G2-U* is slightly more hydrophilic. At higher temperatures, due to the additional exposure of aromatic groups of the nucleobases, the post-transition baselines have lower α values and slopes close to zero. Overall, the random coil states are more or less similar; however, the slope of *G2* is still slightly more negative due to additional aliphatic contributions of the methyl groups. The helix−coil transitions are shown as negative peaks with T_M's similar to the T_M's of the other unfolding curves. Integration and analysis[11] of these peaks yielded ΔV_U's of −27 cm^3/mol (*G2-U*) and −18 cm^3/mol (*G2*), revealing a volume contraction of the system as the temperature is increased, and if voids do not exist, these volume contractions correspond to changes in their hydration state;[12] i.e., due to a release of water molecules, the unfolding of a G-quadruplex is accompanied by a decrease in its volume of hydration. This result is in excellent agreement with the water releases measured with the osmotic stress technique; i.e., ΔV_U and Δn_W have similar signs.

The thermodynamic contributions of the loops are determined by subtracting the thermodynamic contributions of a single G-quartet stack[8b] from the thermodynamic profiles of each G-quadruplex. The loops of *G2-U* contributed a $\Delta\Delta G°$ term of −1.9 kcal/mol (−1.7 kcal/mol more favorable than *G2*), a favorable enthalpy of −22.2 kcal/mol (−13.1 kcal/mol higher than *G2*), and an unfavorable entropy contribution of −20.3 kcal/mol (−11.3 kcal/mol less favorable than *G2*), confirming that the loops are stacked against the G-quartets, and additional stacking contributions are present among the loop bases. However, the values in parenthesis are similar to those obtained earlier for the absence of six methyl groups,[13] invoking hydration differences in these loops. To determine the type of water that is involved in the absence of methyl groups from these loops,

we use the signs of the differential thermodynamic parameters with the sign of $\Delta\Delta V_U$.[12b,14] The negative signs of the $\Delta\Delta G°$ (−2.0 kcal/mol) and $\Delta\Delta H-\Delta(T\Delta S)$ compensation (−14.6 kcal/mol) terms are opposite to the positive sign of the $\Delta\Delta V_U$ (+9 cm^3/mol) term. This indicates that the absence of methyl groups involves structural or hydrophobic water molecules, consistent with the nature of the interaction of methyl groups with water. Furthermore, the correlation of this $\Delta\Delta V_U$ with the $\Delta\Delta n_W$ (+2 mol water/mol) yields qualitatively a molar volume of 13.5 mL/mol for water immobilized by polar and nonpolar groups. This value is smaller, by 2 mL/mol, than the molar volume of electrostricted water.[12a]

The overall results can be summarized as follows: The removal of the methyl groups from the thymines of the thrombin aptamer to form uracils yielded a more stable G-quadruplex (*G2-U*) with a lower hydration level. This is explained in terms of several contributions, i.e., improved stacking, release of structural water molecules, and small uptake of counterions, resulting in a better interaction of *G2-U* with the solvent water. These effects may be specific for G-quadruplexes but are consistent with previous reports of other nucleic acid systems. For instance, it was shown earlier[15a] that the higher stability of an RNA oligonucleotide duplex, relative to DNA, or DNA/RNA hybrid duplexes with similar sequence (actually the hybrids had only two dT → rU substitutions), is due to favorable base−base stacking contributions (higher folding enthalpies). However, the DNA duplex is more hydrated, while the other three duplexes show similar hydration. This is contrary to our findings and may be explained in terms of hydration differences between the B- and A-conformations of the oligonucleotides, i.e., hydration differences in their sugar puckering. Furthermore, the placement of dT → dU substitutions in DNA homoduplexes or triplexes yielded helices with lower stability due to lower stacking contributions.[15b,c] However, the former helices are well hydrated due to their B-conformation and formation of single (duplex) and double chains (triplex) of thymine methyl groups in their major groove.

In conclusion, the favorable formation of a G-quadruplex results from the characteristic compensation of a favorable enthalpy and unfavorable entropy contributions, uptake of ions, and release of water molecules. Their loops contribute with favorable stacking (enthalpy) contributions to their stable formation. Contrary to what is observed with other DNA structures, the presence of the thymine methyl groups destabilizes the G-quadruplex. One explanation for this effect is the immobilization of structural or hydrophobic water by these methyl groups and/or the lower hydration level of the quadruplex, due to a higher content of guanines and the absence of well-defined grooves.

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Supporting Information Available: UV and CD curves, heat capacity data, and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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