Recognition and Binding of Human Telomeric G-Quadruplex DNA by Unfolding Protein 1

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ABSTRACT: The specific recognition by proteins of G-quadruplex structures provides evidence of a functional role for *in vivo* G-quadruplex structures. As previously reported, the ribonucleoprotein, hnRNP Al, and it is proteolytic derivative, unwinding protein 1 (UP1), bind to and destabilize G-quadruplex structures formed by the human telomeric repeat d(TTAGGG)$_n$. UP1 has been proposed to be involved in the recruitment of telomerase to telomeres for chain extension. In this study, a detailed thermodynamic characterization of the binding of UP1 to a human telomeric repeat sequence, the d[AGGG(TTAGGG)$_3$] G-quadruplex, is presented and reveals key insights into the UP1-induced unfolding of the G-quadruplex structure. The UP1−G-quadruplex interactions are shown to be enthalpically driven, exhibiting large negative enthalpy changes for the formation of both the Na$^+$ and K$^+$ G-quadruplex−UP1 complexes (ΔH values of −43 and −19 kcal/mol,

respectively). These data reveal three distinct enthalpic contributions from the interactions of UP1 with the Na$^+$ form of G-quadruplex DNA. The initial interaction is characterized by a binding affinity of 8.5×10^8 M^{-1} (strand), 200 times stronger than the binding of UP1 to a single-stranded DNA with a comparable but non-quadruplex-forming sequence [4.1×10^6 M^{-1} (strand)]. Circular dichroism spectroscopy reveals the Na$^+$ form of the G-quadruplex to be completely unfolded by UP1 at a binding ratio of 2:1 (UP1:G-quadruplex DNA). The data presented here demonstrate that the favorable energetics of the initial binding event are closely coupled with and drive the unfolding of the G-quadruplex structure.
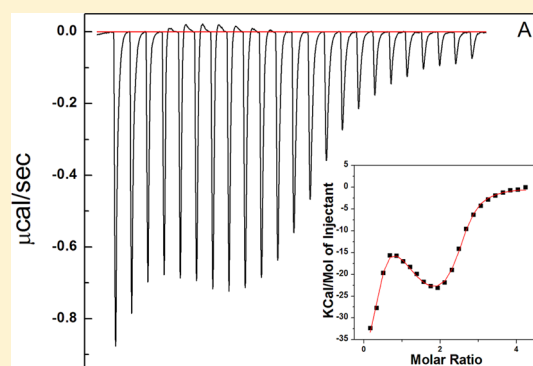
The human genome contains highly conserved repetitive G-rich sequences that are located in strategic regions of chromosomes and are important for biological functions such as transcriptional promoter regions and at the telomeric ends of eukaryotic chromosomes.[1−3] The human telomeric DNA at the terminal ends of chromosomes is a single-stranded region with a 3′-end overhang containing tandem repeats of d(TTAGGG)$_n$. This noncoding overhang is composed of ∼150−200 nucleotides and is thought to protect genomic DNA from end fusion and for the maintenance of chromosomal integrity during replication.[4,5] There is evidence to suggest that telomeres serve as a biological clock, determining the lifespan of the cell, and the loss of telomeric DNA upon replication ultimately leads to apoptosis as the telomeres become critically shortened.[6−8] Telomeric DNA has been implicated in cancer progression because of the upregulation of telomerase activity in cancer cells and subsequent lengthening of telomeres leading to cellular immortality as observed in malignant cells.[9−13] The nucleotide sequences that compose telomeric DNA have been shown to readily form G-quadruplex structural motifs *in vitro* and serve as novel targets for the development of new classes of anticancer agents.[14−19] The formation of higher-order DNA

structures within telomeric sequences lends confidence to the idea that these structures serve regulatory roles for telomere extension and maintenance.[17−20]

Several proteins and enzymes such as TRF2, POT1, and telomerase have been demonstrated to associate with telomeric DNA and exert a number of biological functions.[7,21,22] Telomerase is an enzyme composed of a reverse transcriptase and telomeric RNA transcript that function to elongate the repetitive sequence at the telomeric ends of the chromosomes.[13] Telomerase requires that the telomeric region be in the single-stranded conformation for binding and elongation to occur. The presence of the intramolecular folded G-quadruplex structural motif inhibits this interaction.[20] The discovery of proteins and helicases that specifically recognize and destabilize the G-quadruplex structural motif has strong implications on the dynamic nature and function of G-quadruplex structures within the genome.[21,22]

Destabilizing proteins unfold G-quadruplex DNAs in a nonenzymatic manner and do not require ATP hydrolysis for activity. Among these, hnRNP A1 is a member of a class of ribonucleoproteins that have been reported to be involved in RNA transport and alternative splicing and is closely associated with DNA polymerase transcripts.[23,24] hnRNP A1 contains two nucleic acid binding domains that strongly interact with either RNA or DNA sequences. Unwinding protein 1 (UP1) is a 196-amino acid proteolytic product of hnRNP A1 that retains the two nucleic acid binding domains.[25] UP1 has been shown to bind and destabilize G-quadruplex structures and potentially serve as a DNA chaperone responsible for the unfolding of the G-quadruplex structure into single-stranded DNA to facilitate the binding of telomerase for lengthening of the telomere.[26,27] In 2002, Fakuda and co-workers reported that UP1 binds to and destabilizes G-quadruplex structures in mouse minisatellite repeats as well as human telomeric DNA.[28] This work was further supported by Shamoo and co-workers in 2003.[29] In 1999, Ding and co-workers presented the structure of UP1 complexed with single-stranded DNA, providing a detailed characterization of the UP1−DNA complex. In this X-ray crystallographic structure, the protein formed a complex with short deoxyoligonucleotides (12-mers) with sequences consistent with those telomeric DNA regions in a single-stranded antiparallel conformation complexed with two UP1 proteins.[30] The two nucleic acid binding domains (BD1 and BD2) are composed of residues that participate in the formation of noncovalent interactions, including hydrogen bonding, stacking, van der Waals, and electrostatic interactions, with approximately four nucleotides [d(TAGG)] in the telomeric DNA sequence.[30]

It is clear that UP1 actively functions as a G-quadruplex-destabilizing protein; however, the nature through which UP1 recognizes the G-quadruplex structure and the unfolding mechanism(s) remain unknown. This work provides a detailed biophysical examination of the binding of UP1 to the human telomeric (Tel-22) G-quadruplex structural motif in both Na$^+$ and K$^+$ forms. These studies examine the energetics associated with formation of a complex between UP1 and human telomeric G-quadruplex DNA by isothermal titration calorimetry (ITC) and concomitantly follow the unfolding process upon complex formation by circular dichroism (CD) spectroscopy. These results provide a direct method for binding equilibrium measurements that results in an energetic characterization of the interaction and provide key insights into the recognition, affinity, and destabilization of G-quadruplex structures by UP1.

■ MATERIALS AND METHODS

Deoxyoligonucleotides and Sample Preparation. The 22-base human telomeric sequence 5′-d(AGGGTTAGGGTT-AGGGTTAGGG)-3′ (Tel-22), and the 22-mer non-G-quadruplex-forming control sequence (where the third G is mutated to an A) 5′-d(AGGATTAGGATTAGGATTAGGA)-3′ (Tel-22ss) were obtained from Midland Certified Reagents (Midland, TX). DNA samples were prepared by dissolving the deoxyoligonucleotides in Tris buffer [0.02 M Tris-HCl (pH 8.0) and 0.1 M NaCl or 0.1 M KCl], followed by an annealing process in which the DNA solution was heated to 85 °C and slowly cooled to 4 °C at a rate of 1.0 °C/min using an MJ Research thermocycler. Stock concentrations of DNAs were determined by the UV absorbance at 85 °C. Molar extinction coefficients (ε_{260}) for the sequences were 228500 M^{-1} cm^{-1} per

strand for Tel-22 and 240900 M^{-1} cm^{-1} per strand for Tel-22ss as determined by the nearest neighbor method.[31] The formation of the G-quadruplex structural motif and topologies were evaluated for both sequences at a concentration of 5 μM (strand) in 1 cm path length cells using an Aviv 400 CD spectrophotometer. Wavelength scans from 325 to 225 nm were examined for a positive ellipticity signal at 295 nm, which is the characteristic signature of antiparallel G-quadruplex DNA structure.[32,33]

Preparation of UP1. The cDNA of UP1 (amino acid residues 1−196 of hnRNP A1) was cloned into vector pET28-SMT3, resulting in an N-terminal SUMO tag and a six-His tag, and expressed in *Escherichia coli* BL21-Gold(DE3) cells. Cells were grown in Luria-Bertani medium for 3 h at 37 °C until an optical density at 600 nm of 0.6 was achieved. Induction using 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was performed followed by overnight incubation at 18 °C, and then the cells were harvested. The cells were washed with phosphate buffer (PBS) and resuspended in 5 mL of binding buffer [20 mM Tris (pH 8.0), 0.5 M NaCl, and 25 mM imidazole] per 100 mL of culture. The cells were disrupted by Emulsiflex-C3 cell homogenizer (Avestin Inc.) followed by centrifugation for 40 min at 16000 rpm and 4 °C. The supernatant was loaded on a 5 mL His-Trap column (GE-Health) and then eluted with buffer [20 mM Tris (pH 8.0), 0.5 M NaCl, and 500 mM imidazole]. The eluted protein was digested with SUMO protease and dialyzed overnight against 20 mM Tris (pH 8.0) and 0.5 M NaCl, followed by treatment with a second pass through the His-Trap column. The protein was further purified by being passed over a heparin column to exclude proteins that form complexes with nucleic acids. UP1 was then fractionated by gel filtration using a Superdex-75 16/60 column (GE Healthcare) and the concentration of UP1 determined by UV−visible spectroscopy.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were performed using a Microcal VP-ITC instrument (GE Healthcare, Piscataway, NJ) at 25 °C. All samples were thoroughly degassed while being stirred prior to use. Forward and reverse ITC titrations were conducted with both the G-quadruplex-forming (Tel-22) and non-quadruplex-forming 22-mer (Tel-22ss) deoxyoligonucleotides. In the forward titrations, the sample cell was filled to capacity with a dilute solution of DNA ([DNA] ≈ 5 μM strand) and titrated with UP1 protein (≈100−200 μM UP1). For the reverse ITC titrations, the sample cell was filled to capacity with a dilute solution of UP1 (≈5 μM UP1) and titrated with DNA at concentrations ranging from 50 to 100 μM (strand). A typical titration involved the injection of 25 (6−12 μL) aliquots of titrant with titrant injections made at 400 s intervals. The integrated heat data were corrected for the heat of dilution and blank effects and the corrected data fit for a binding model by nonlinear regression. The binding isotherms obtained in the forward titrations of the G-quadruplex-forming DNA were nonsigmoidal and could not be fit with any of the standard binding models incorporated into the Microcal Origin VP-ITC software. The binding isotherms obtained in the reverse titrations of the G-quadruplex-forming DNA as well as both the forward and reverse titrations for control DNA were sigmoidal and well fit with the standard one-site binding model incorporated into the Microcal Origin VP-ITC software.

It was apparent from the shape of the ITC isotherm that multiple events were present in the forward titrations for both the Na$^+$ and K$^+$ human telomere quadruplexes. The multiple-
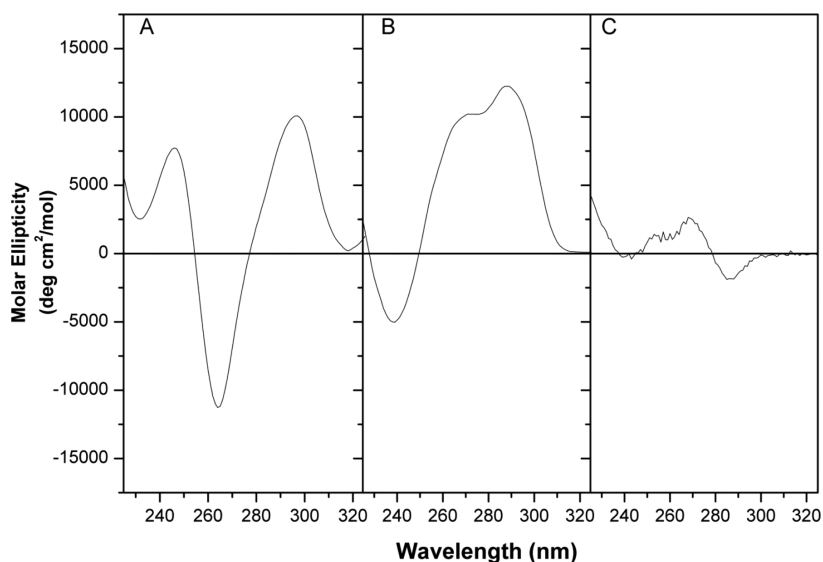
Figure 1. (A) Circular dichroism spectrum of the Na$^+$ form of the G-quadruplex formed by the Tel-22-mer deoxyoligonucleotide sequence. (B) Circular dichroism spectrum of the K$^+$ form of the G-quadruplex formed by the Tel-22-mer deoxyoligonucleotide sequence. (C) The control deoxyoligonucleotide (Tel-22ss) was designed to be unable to fold into the G-quadruplex structure wherein the third guanine in each of the four repeats was mutated to an adenine preventing the formation of the G-tetrad and the subsequent formation of the G-quadruplex structure. The CD spectrum reveals unstructured DNA with no positive ellipticity at 280 or 295 nm.

event titrations were fit using a model developed by a combination of the mass balance and equilibrium constant expressions expanded to n-independent site binding expressions. Lewis and co-workers previously reported the details regarding the development of the fitting algorithm used to describe the complex ITC binding isotherms obtained in the forward titrations.[34,35] The forward titration of the non-quadruplex-forming sequence and the reverse titrations for all three systems, Tel-22 (Na$^+$), Tel-22 (K$^+$), and Tel-22ss, were sigmoidal and described with a simple one-site binding model.

Circular Dichroism Titrations. Examination of changes in structural features of the human telomere G-quadruplex resulting from UP1 binding were performed using CD spectrophotometry. CD titrations were conducted using an AVIV 400 CD spectrophotometer (Aviv, Inc.). Experiments were performed in 1 cm path length rectangular cells at 25 °C. Wavelength scans were conducted from 225 to 325 nm at every nanometer with an averaging time of 2 s. Initial concentrations of the Tel-22-mer quadruplex DNAs were 5 μM (strand) and titrated with the UP1 protein. After each addition of UP1, the sample was allowed to equilibrate for 10 min while being constantly stirred. Contributions of the buffer were subtracted from each wavelength scan and the data normalized to molar ellipticity (degrees square centimeter per mole) to account for dilution effects of the initial DNA concentration in the cell.

■ RESULTS

Characterization of G-Quadruplex Structure by Circular Dichroism Spectroscopy. Before investigating the interactions of UP1 with G-quadruplex DNA, we found it was necessary to characterize the structure of each of the deoxyoligonucleotide sequences. CD provides a powerful tool for detecting the presence of the G-quadruplex structural motif.[32,33] The G-quadruplex structural motifs exhibit characteristic positive ellipticity at wavelengths between 280 and 300 nm, primarily because of the stacking interactions of the guanine bases in the G-tetrads.

The human telomere base sequence (Tel-22) prepared in Na$^+$ buffer forms a G-quadruplex structure with the characteristic CD spectrum shown in Figure 1A. The Na$^+$ form G-quadruplex is characterized by strong positive maxima at 295 and 245 nm and a minimum at 265 nm. In contrast, this same Tel-22 G-quadruplex prepared in K$^+$ buffer reveals a markedly different CD spectrum as shown in Figure 1B with a positive ellipticity at 295 nm but with a broad shoulder at 265 nm. There are multiple conformations that could give rise to a number of strand orientations; however, there are no determining factors to unambiguously define parallel and/or antiparallel strand orientations based on CD measurements alone.[32−34] However, the unique nature of the G-quadruplexes in either Na$^+$ or K$^+$ environments as reflected by the CD spectra provides a valuable tool for assessing the presence and disappearance (unfolding) of the Na$^+$ or K$^+$ form of the G-quadruplex structural motif. The control deoxyoligonucleotide (Tel-22ss) was designed to be unable to fold into the G-quadruplex structure and remain in a random coil wherein the third guanine in each of the four repeats was mutated to an adenine to prevent the formation of the G-tetrad and the subsequent formation of the G-quadruplex structure. The CD spectrum reveals an unstructured DNA with no positive ellipticity at 280 or 295 nm as shown in Figure 1C.

The thermodynamic stabilities of the Na$^+$ and K$^+$ forms of the Tel-22 G-quadruplexes were examined by CD melting studies. The molar ellipticities for each of the DNA structural motifs were monitored as a function of temperature, and the resulting changes in the CD signal at 295 nm revealed sigmoidal melting curves. The melting temperatures, T_m, were derived from the midpoint of these transitions and are shown in Figure 2. The Na$^+$ form of the Tel-22-mer G-quadruplex exhibits a T_m of 59 °C in 100 mM NaCl and 0.01 M Tris buffer (pH 8). In contrast, the K$^+$ form of the G-quadruplex was markedly more stable than the Na$^+$ form. The Tel-22 (K$^+$) G-quadruplex exhibited a T_m of 68 °C in 100 mM KCl buffer and 0.01 M Tris (pH 8.0), unfolding at a temperature
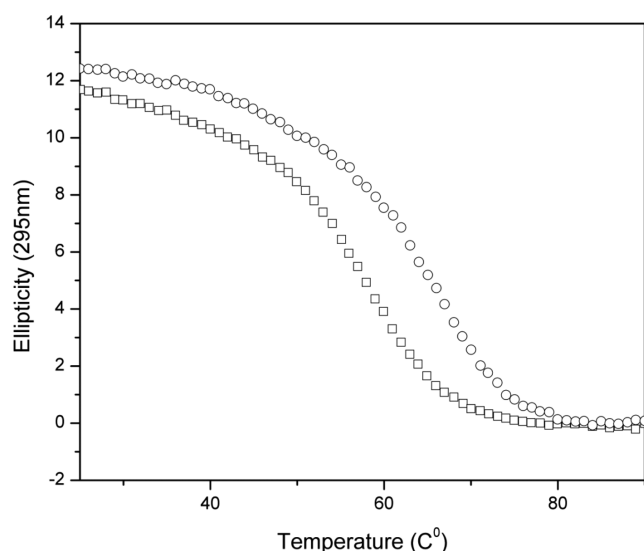
Figure 2. Circular dichroism spectropolimetry melts of Tel-22 (Na$^+$) (□) and Tel-22 (K$^+$) (○). Melting studies were conducted in 0.01 M Tris-HCl (pH 8.0), 0.001 M EDTA, and either 0.1 M NaCl or 0.1 M KCl. The change in ellipticity was monitored at 295 nm from 10 to 90 °C. The melting temperature (T_m) for each of the G-quadruplexes was calculated as the first derivative of the melting curve and found to be 59 °C for Tel-22 (Na$^+$) and 68 °C for Tel-22 (K$^+$).

approximately 10 °C higher than the unfolding temperature of its Na$^+$ counterpart.[36−40]

Binding and Unfolding of Telomeric G-Quadruplex DNA by UP1. Previous studies have reported that UP1 binds and unfolds G-quadruplex structures;[28,29] however, these studies provide limited information regarding the energetics associated with the interaction beyond an estimated equilibrium constant. The studies presented here examine the fundamental thermodynamic properties associated with the binding and unfolding of human telomeric G-quadruplex DNA by directly measuring the binding enthalpy (ΔH_{bind}) upon complex formation utilizing isothermal titration calorimetry (ITC) and CD spectroscopy and allow us to derive relevant thermodynamic parameters that provide a more direct understanding of the binding mechanism(s) associated with complex formation and unfolding.

Forward ITC titrations are conducted by placing the G-quadruplex DNA in the sample cell and UP1 in the injection syringe. The results of the binding of UP1 to the Tel-22 (Na$^+$) G-quadruplex are shown in Figure 3A and summarized in Table 1. Panels A and B of Figure 3 depict the raw ITC data for the binding of UP1 to the Na$^+$ and K$^+$ forms of G-quadruplex DNAs, respectively. The insets of panels A and B of Figure 3 represent the integration of the measured heats associated with complex formation for each injection. The bottom panels of Figure 3 (C and D) show the CD spectra for the titrations of UP1 into the DNA solution at specific molar ratios that coincide with the ITC titrations. For the Na$^+$ form of G-quadruplex DNA, the binding isotherm shown in Figure 3A (inset) reveals a complex binding process, indicative of three events. The solid line drawn through the data points (in red) represents the best fit to the data by a three-event model and results in three distinct enthalpic components of the interaction.[35] At low UP1 concentrations (initial injections), K_1 is estimated to be 8.5 × 10^8 M^{-1} (strand). Two additional equilibrium constants, K_2 and K_3, are determined to be 6.1 ×

10^7 and 6.3 × 10^6 M^{-1} (strand), respectively. The overall stoichiometry for binding of UP1 to the Tel-22 Na$^+$ G-quadruplex was determined to be 2 protein units per G-quadruplex DNA. Binding enthalpies for the three events observed for the Na$^+$ form of the G-quadruplex were determined to be ΔH_1 (−45.4 kcal/mol), ΔH_2 (−6.3 kcal/mol), and ΔH_3 (−29.6 kcal/mol), indicative of a highly enthalpically favored binding for event 1, a markedly reduced favorable enthalpy for event 2, and favored enthalpically driven binding for event 3.

To aid in the interpretation of the complexity of the observed binding isotherm, CD experiments that allowed us to monitor the structural characteristics and/or changes of the G-quadruplex by observing the changes in the molar ellipticity of the G-quadruplex DNAs as a function of UP1 binding were conducted in parallel. The results are presented in Figure 3C for the Na$^+$ form and Figure 3D for the K$^+$ form of Tel-22 G-quadruplex DNA. The decrease in ellipticity at 295 nm is indicative of UP1-mediated unfolding of the G-quadruplex structure. A complete unfolding of the Na$^+$ form of the G-quadruplex is achieved by a molar ratio of 2:1 (UP1:G-quadruplex). On the basis of these results, we conclude that the third binding event that is observed in the isotherm corresponds to the interaction of an additional UP1 molecule with the unfolded G-quadruplex. Therefore, the enthalpic contributions to the first two events must be a combination of binding of UP1 to the G-quadruplex structure (i.e., recognition) and the subsequent unfolding of the G-quadruplex. The sigmoidal portion of the binding isotherm is indicative of the second UP1 molecule interacting with the unfolded G-quadruplex sequence.

The binding of UP1 to the more stable Tel-22 K$^+$ G-quadruplex results in a binding isotherm with features markedly different from those observed for the Na$^+$ form of the G-quadruplex, as shown in Figure 3B. The UP1-induced unfolding of the K$^+$ G-quadruplex was found to be incomplete even at ratios of >4:1 (UP1:G-quadruplex) as shown by the CD spectra in Figure 3D. The binding isotherm for the interaction of UP1 with the K$^+$ form of the G-quadruplex could be adequately described using a two-event binding model with estimates of K_1 and K_2 of 4.3 × 10^7 and 1.7 × 10^6 M^{-1}, respectively. The enthalpy of the first event for the interaction of UP1 with the K$^+$ form of the G-quadruplex (ΔH_1) was significantly reduced and determined to be −23.4 kcal/mol. The enthalpy change for the second event was also less favorable with a ΔH_2 of −7.8 kcal/mol; however, the saturation stoichiometry for the K$^+$ form of the G-quadruplex remained 2.0 (UP1 per G-quadruplex).

The control deoxyoligonucleotide (Tel-22ss) was designed by replacing the d(TTAGGG) repeat with d(TTAGGA), resulting in a deoxyoligonucleotide that is comparable in sequence but incompatible for folding into a G-quadruplex structure under Na$^+$ or K$^+$ buffer conditions. Binding of UP1 to this single-stranded deoxyoligonucleotide under Na$^+$ buffer conditions is shown in Figure 4 and reveals UP1 to bind to the single-stranded DNA in a single event with a binding affinity of 4.1 × 10^6 M^{-1} and an enthalpy change (ΔH) of −37.0 kcal/mol and a stoichiometry of 2:1 (UP1:G-quadruplex strand).

Under conditions where multiple binding sites or multiple equilibria exist, unusual binding isotherm shapes are often encountered in the forward titrations. In such cases, the ITC experiment can be designed so that the ligand and target molecules are reversed, and in this case, DNA in the injection
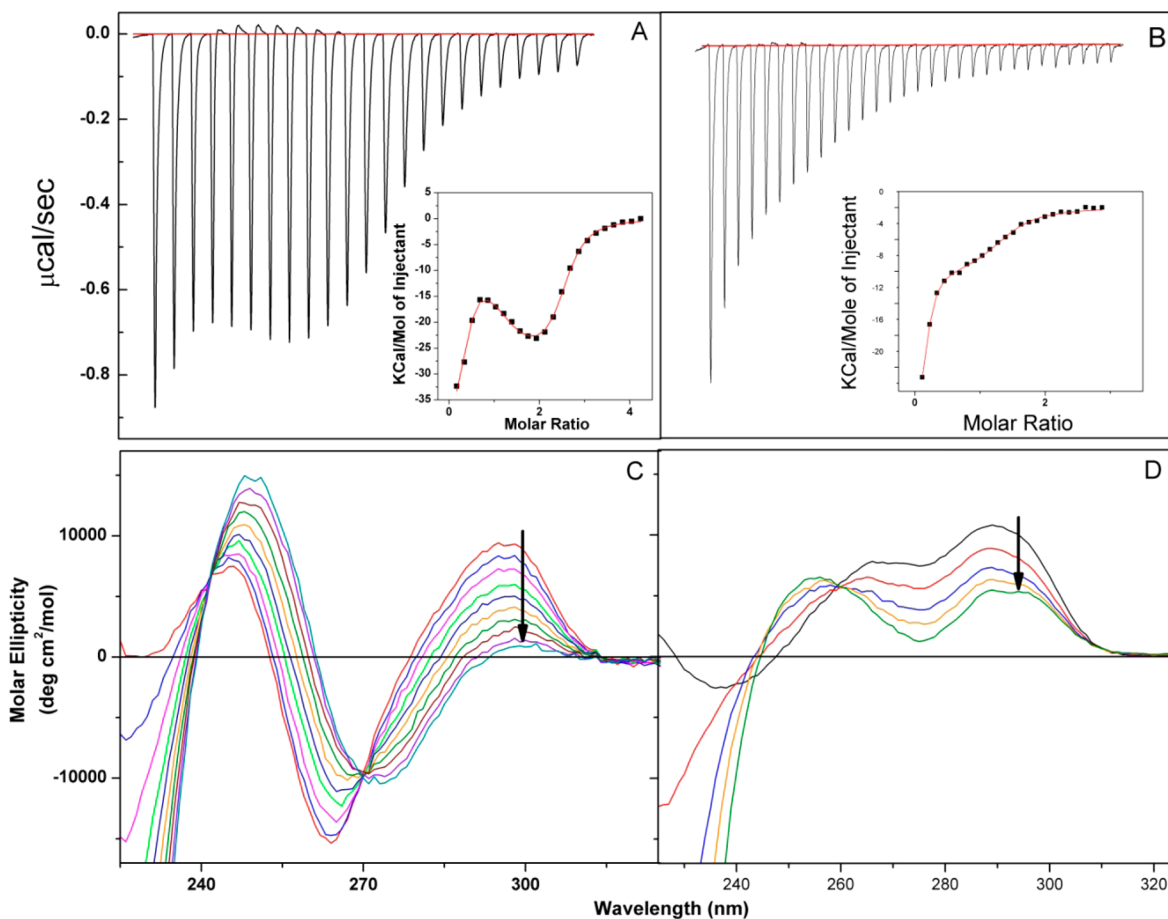
Figure 3. Isothermal titration calorimetry (ITC) binding isotherms for the interactions of UP1 with the Tel-22 (Na$^+$ form) G-quadruplex (A) and the Tel-22 (K$^+$ form) G-quadruplex (B). Both the raw heat rate and the integrated heat data are shown along with the nonlinear regression fit (red). The titrations were conducted in Tris buffer [0.02 M Tris-HCl (pH 8.0) and 0.1 M NaCl (Na$^+$ form) or KCl (K$^+$ form)] at 25 °C. The solid lines (red) drawn through the data points in the insets represent the best fits for a three-event model (Na$^+$ form) in panel A and a two-event model (K$^+$ form) in panel B. Thermodynamic parameters for these binding processes are listed in Table 1. The bottom panels (C and D) show the concomitant CD spectra for the titration of the Tel-22 G-quadruplex with UP1 (the Na$^+$ and K$^+$ forms for panels C and D, respectively). In Panels C and D, the molar ratio (r) of UP1 to G-quadruplex spans the range of 0–2.5. The black arrows represent the directionality of the change in ellipticity at 295 nm with increasing concentrations of UP1.

Table 1. Thermodynamic Parameters Derived from the Nonlinear Least-Squares Fit of the Isothermal Titration Calorimetry Binding Studies in Which UP1 Was Titrated into G-Quadruplex DNA

	n_{tot}	no. of events	n	K_{eq} (M^{-1})	ΔG (kcal/mol of protein)^a	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)^b
Tel-22 (Na$^+$)	2	3	0.2	$8.5 \times 10^8 \pm 1.9$	-12.1 ± 0.2	-45.4 ± 2.1	33.2 ± 2.1
			0.8	$8.4 \times 10^7 \pm 3.7$	-10.6 ± 0.2	-7.3 ± 1.9	-3.5 ± 1.7
			1.2	$6.3 \times 10^6 \pm 0.2$	-9.3 ± 0.1	-29.6 ± 0.9	20.0 ± 0.9
Tel-22 (K$^+$)	2	2	0.2	$2.8 \times 10^7 \pm 2.2$	-10.4 ± 0.5	-23.4 ± 3.0	15.1 ± 2.5
			1.8	$1.3 \times 10^6 \pm 0.6$	-8.5 ± 0.4	-7.8 ± 1.0	-0.6 ± 1.3
Tel-22ss	2	1	2	$4.1 \times 10^6 \pm 1.9$	-9.0 ± 0.1	-37.0 ± 1.2	98.2 ± 1.5

^{a}The Gibbs free energy was calculated from the relation $\Delta G° = -RT \ln K$. ^{b}The entropy ($-T\Delta S°$) was calculated from the rearrangement of the equation $\Delta G° = \Delta H° - T\Delta S°$ to $-T\Delta S° = \Delta G° - \Delta H°$.

syringe is titrated into UP1 in the sample cell. We refer to this experimental method as a "reverse" ITC titration.[41–43] For simple binding interactions, it is expected that the shape of the binding isotherm should remain consistent after the direction of the titration is reversed. However, in more complex cases as observed in Figure 3, a binding isotherm consisting of multiple events may be simplified into an isotherm that is an approximate average of all events as shown in Figure 5. In Figure 5A, the Tel-22 Na$^+$ G-quadruplex is titrated into UP1. Having a large excess of UP1 in the sample cell results in the

multievent reaction proceeding to completion with the formation of the UP1-saturated unfolded complex with each injection. As the free UP1 concentration is decreased, endothermic peaks that are indicative of unfolding are observed. The binding constant, K_{eq}, of 7.4×10^7 M^{-1} (DNA strand) is estimated by fitting a single-site model to the data shown in Figure 6A. This is an approximate value for the average of the association constants as derived from the forward ITC titration. Similarly, the ΔH of binding (-17.4 kcal/mol) for the reverse ITC titration is in good agreement with the
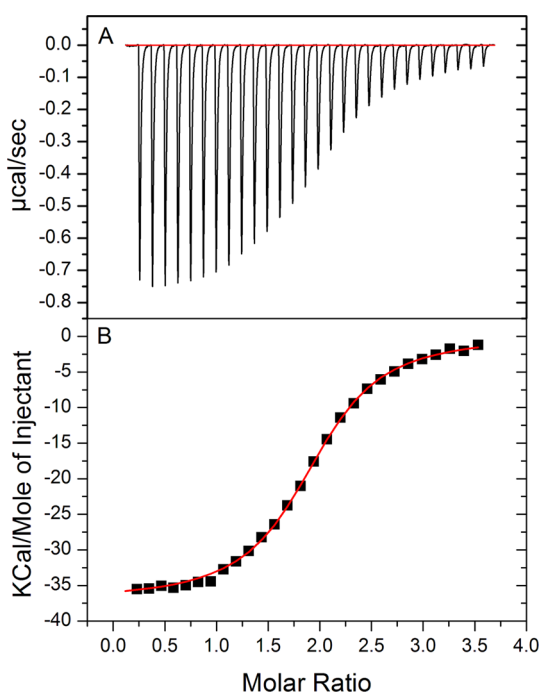
Figure 4. (A) Isothermal titration binding isotherm or the titration of Tel-22ss with UP1. Experiments were performed in Tris buffer [0.02 M Tris-HCl (pH 8.0) and 0.1 M KCl] at 25 °C. (B) Integration of the raw data to provide the binding enthalpy. The solid lines drawn through the data points represent best fits of a nonlinear least-squares model from which thermodynamic binding parameters can be estimated as described in the footnotes of Table 1.

approximate average of the three individual components as estimated from the forward ITC titration. Figure 5B shows the results of the reverse titration for the interaction of the K⁺ form of the G-quadruplex with UP1. A binding affinity of 2.6×10^7 M⁻¹ (DNA strand) and an enthalpy change (ΔH) of −9.5 kcal/mol were determined. The values for the thermodynamic parameters for the reverse titrations are listed in Table 2.

■ DISCUSSION

This work describes the energetics of binding of UP1 with two distinct G-quadruplex conformations, Tel-22 (Na⁺ form) and Tel-22 (K⁺ form), and a control single-stranded nucleic acid sequence (Tel-22ss) that has a similar sequence but is unable to fold into a G-quadruplex. Earlier studies by Fiset and Chabot as well as Zhang and co-workers reported UP1 to bind with high affinity to d(TTAGGG)$_n$-containing sequences that could form G-quadruplex structures, resulting in the destabilization of the G-quadruplex structure.[26,27] Fakuda and co-workers reported that UP1 could bind and unfold G-quadruplex structures formed by the sequence d(GGCAG)$_n$ and by the human telomeric sequence d(TTAGGG)$_n$.[28,44] Shamoo and co-workers reported that substitution of 2-aminopurine, nebularine, or 7-deazaquanine for the first guanine residue in the sequence d(TAGGG)$_n$ was poorly tolerated and greatly reduced the binding affinity of UP1.[29] These results, in conjunction with the crystal structure reported by Ding and co-workers, led to the emergence of a consensus binding sequence for UP1 of d(nYAGn), where Y is either a thymine or cytosine residue, and is stabilized by hydrogen bonding, base stacking, and hydrophobic effects.[29,30]

Until now, there has been limited information regarding the energetic forces that characterize the binding and unfolding of the telomeric G-quadruplex by UP1. The focus of this work is to provide a thermodynamic description of the interaction of UP1 with Tel-22 G-quadruplex structural motif. The sequences and G-quadruplex structures used for our study all contain the d(TAG) binding site but differ in folding topology. The G-quadruplex structures and stabilities were characterized by CD spectroscopy to demonstrate that the 22-nucleotide sequence (Tel-22) was folded into G-quadruplex structures under the Na⁺ and K⁺ buffer conditions that were used and exhibited thermal stabilities and folding topologies comparable to those previously published.[38,39] The greater stability of the Tel-22 K⁺ G-quadruplex is indicated by an increase in the melting temperature of 10 °C versus that of the Tel-22 Na⁺ G-quadruplex. This increase in stability is partially responsible for the limited unfolding of the Tel-22 K⁺ G-quadruplex upon binding of UP1. In the forward ITC titrations, small aliquots of protein were injected into a large excess of nucleic acid. The binding isotherms for these experiments are indicative of multiple enthalpic contributions and reveal a complex binding isotherm for the Na⁺ and K⁺ G-quadruplexes. The unusual shapes of the isotherms that are observed for the binding of UP1 to the Tel-22 Na⁺ G-quadruplex solutions (at molar ratios of <1:1) suggest two overlapping events with enthalpy changes that are opposite in sign (i.e., exothermic and endothermic) are simultaneously occurring. The observation of endothermic peaks ($\Delta H > 0$) at the early stages of the titration for the Tel-22 Na⁺ G-quadruplex (shown in peaks 4−9 in Figure 3A) suggests that there is an entropically favored process associated with either the binding of UP1 or the unfolding of the quadruplex structure upon binding of UP1. The complex shape that is observed for the complete binding isotherm results from the heat change being the sum of the three enthalpy changes composed of the binding of 2 mol of UP1 and unfolding of the G-quadruplex structure. Concomitant CD experiments demonstrate the unfolding of the Tel-22 Na⁺ G-quadruplex to be largely complete after a molar ratio of 1:1 (UP1:G-quadruplex) is reached. Binding of the second mole of UP1 to the UP1−Tel-22 Na⁺ G-quadruplex complex is accompanied by only small changes in the CD spectrum. We postulate a thermodynamic model to describe the interaction of UP1 with the Na⁺ form of the Tel-22 G-quadruplex to include three events: (1) the initial binding interaction of 1 mol of UP1 with the G-quadruplex, (2) the coupling of the UP1 binding energy to the UP1-induced unfolding of the G-quadruplex structure, and (3) the binding of the second UP1 molecule to the unfolded G-quadruplex. The thermodynamic model includes the free energy change (ΔG_i), the enthalpy change (ΔH_i), and the entropy change ($-T\Delta S_i$) parameters for the three overlapping events. The first event corresponds to the recognition and binding of UP1 to the Tel-22 G-quadruplex without significant unfolding. The third event corresponds most closely to the binding of UP1 to unstructured (i.e., unfolded) DNA. From analyses of the ITC data, the binding affinity for the first event (K_1) is estimated to be 8.5×10^8 M⁻¹ while the affinity for the third event is approximately 2 orders of magnitude lower ($K_3 = 6.3 \times 10^6$ M⁻¹). UP1 clearly exhibits preferential binding to the G-quadruplex structure as compared with its binding to unfolded or unstructured DNA. The change in binding enthalpy for the first event is far more favorable when compared with the change in the binding enthalpy for the
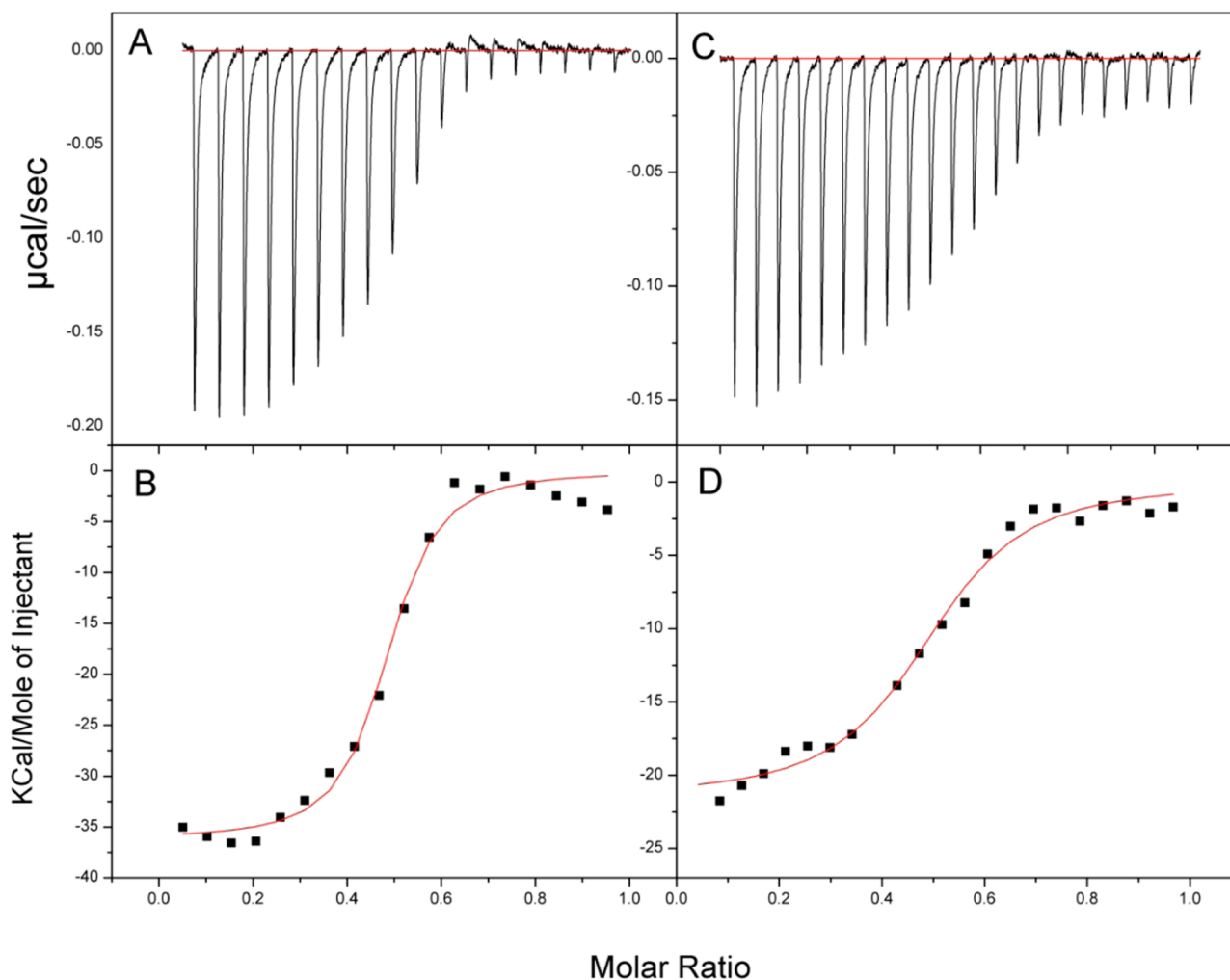
Figure 5. Reverse ITC titration of Tel-22 G-quadruplex DNA titrated into an excess of UP1. Experiments were performed in Tris buffer [0.02 M Tris-HCl (pH 8.0) and either 0.1 M NaCl (A and B) or 0.1 M KCl (C and D)] at 25 °C. Both the raw heat data and the integrated heat data are shown along with the nonlinear regression fits (red) to the data. The raw data in panels A (Na$^+$) and B (K$^+$) show that a large excess of UP1 in the sample cell results in driving the multievent reaction(s) to completion, and with each injection, there are both binding and unfolding of all G-quadruplex DNA that is added.

third event (ΔH_1 = −45.4 kcal/mol, and ΔH_3 = −29.6 kcal/mol).

Our interpretation of the complex isotherm for the interaction of UP1 with the Na$^+$ form of the Tel-22 G-quadruplex is illustrated by the Hess's law diagram that is presented in Figure 6. The second event is a composite of UP1 quadruplex recognition binding and unfolding of the G-quadruplex. In effect, the UP1 quadruplex binding enthalpy change, ΔH_1, is coupled to the quadruplex unfolding enthalpy change, ΔH_{unfold}, and ΔH_2 corresponds approximately to the summation of the favorable enthalpy change for binding the first mole of UP1 to the G-quadruplex with the unfavorable enthalpy change for the unfolding process. Using this model, the following equations for the overall interaction of 2 mol of UP1 with 1 mol of the Tel-22 G-quadruplex

$$\Delta H_2 = \Delta H_1 + \Delta H_{unfold} \tag{1}$$

$$\Delta H_{rev} = \Delta H_1 + \Delta H_{unfold} + \Delta H_3 \tag{2}$$

$$\Delta H_{rev} = \Delta H_2 + \Delta H_3 \tag{3}$$

and the measured values of ΔH_1, ΔH_3, and ΔH_{rev} listed in Tables 1 and 2, we can estimate the value of the enthalpy

change for UP1-induced unfolding of the Tel-22 quadruplex (ΔH_{unfold}) to be 38.1 kcal/mol. The enthalpy change (ΔH_{DSC}) for the unfolding of the Na$^+$ Tel-22 G-quadruplex was also measured by DSC (data not shown) and found to be 39.6 kcal/mol, in excellent agreement with the value for ΔH_{unfold} as calculated from the ITC data and the Hess's law analysis. One caveat would be that ΔH_{unfold} is the apparent enthalpy change for the unfolding of the G-quadruplex with 1 mol of bound protein (UP1−Tel-22) while ΔH_{DSC} is the measured enthalpy change for unfolding of the G-quadruplex in the absence of any bound UP1 (Tel-22).

The complete binding isotherm observed for the interaction of UP1 with the K$^+$ form of the Tel-22 G-quadruplex is simpler in that only two events are required to model the shape of the isotherm. None of the individual integrated heats contained the endothermic "hook". The absence of the endothermic hook is attributed to the fact that the Tel-22 K$^+$ G-quadruplex is only partially unfolded by the binding of UP1 or the kinetics for the unfolding results in a more complete overlap of the UP1 binding event. CD studies reveal that the Tel-22 K$^+$ G-quadruplex never achieved complete unfolding, even at molar ratios as high as 4:1 (UP1:G-quadruplex). The binding affinity
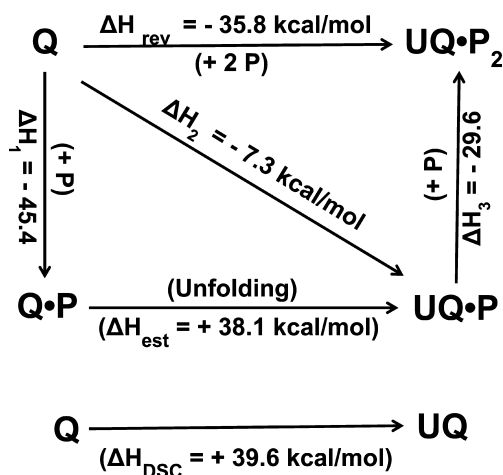
Figure 6. Hess's law description of the energetics of UP1 binding and unfolding of the Na$^+$ form of the Tel-22 G-quadruplex DNA structure. The favorable binding enthalpy (ΔH_1) of -45.4 kcal/mol reflects the initial binding of UP1 to the G-quadruplex DNA. This is coupled with the energetically unfavorable UP1-induced unfolding of the G-quadruplex enthalpy change of 38.1 kcal/mol, resulting in an overall favorable driving force (ΔH_2) of -7.3 kcal/mol.

for the first event ($K_1 = 1.7 \times 10^6$ M^{-1}) was markedly lower than that observed for the Na$^+$ G-quadruplex and exhibited a reduced binding enthalpy change ($\Delta H_1 = -26$ kcal/mol). The K$^+$ form of the Tel-22 G-quadruplex is a more stable structure, having a melting temperature of 68 °C, and the energetics for the UP1 interaction appear to be insufficient to destabilize the G-quadruplex structure completely. The fact that the UP1−Tel-22 K$^+$ G-quadruplex binding isotherm is described well by only two events is the result of the partial unfolding. The thermodynamic parameters for event 1 most closely describe the binding of the first mole of UP1 to the Tel-22 K$^+$ G-quadruplex without significant unfolding. The thermodynamic parameters for event 2 are the combination of the binding and partial unfolding with the unfolding continuing to saturation. This idea is supported by a recently published report that makes a similar observation for the binding and unfolding of the K$^+$ conformation.[40] The authors examined the bound conformation of the potassium form of the G-quadruplex with a single-molecule fluorescence technique and observed that the oligonucleotide was still in a compacted state after complex formation and not fully unfolded.

Via comparison of the interactions of UP1 with the Na$^+$ and K$^+$ Tel-22 G-quadruplexes, several differences are noted. First, the Na$^+$ G-quadruplex is almost completely unfolded upon binding of 1 mol of UP1/mole of DNA, whereas the K$^+$ G-quadruplex is only partially unfolded at saturation. Second and more importantly, UP1 exhibits Tel-22 G-quadruplex structural recognition in that the binding constant is on the order of 10^8 for Tel-22-Na$^+$, 10^7 for Tel-22-K$^+$, and 10^6 for Tel-22ss. Finally,

the enthalpy change for the binding of the first UP1 to the Tel-22 Na$^+$ form is large enough to drive the complete unfolding of the G-quadruplex, whereas the enthalpy change for the binding of UP1 to the K$^+$ Tel-22 G-quadruplex is insufficient to drive complete unfolding of the G-quadruplex structure. Obviously, the biological activity of UP1 could be caused at least in part by these energetic differences.

A model for understanding the recognition of the d(TTA-GGG) sequence by UP1 was proposed by Shamoo et al.,[29] and it was noted that the base composition, stereochemistry, and steric properties of the bases making up the 5′-TAG-3′ nucleic acid sequence are critical for UP1 binding and even slight modifications to this sequence are detrimental to the binding affinity. The NMR solution structure of the G-quadruplex DNA reported by Wang and Patel[45] shows adenine and guanine stacking interactions between the loop sequence and the adjacent guanine in the sequence that forms one the four guanines in the G-tetrad. The steric properties of the bases in the single-stranded conformation may be such that strong interactions of UP1 with the d(nTAGn) sequence are not formed, thus reducing the binding affinity and binding enthalpy change for the control single-stranded sequence used in this study.

The results presented here represent the first examination of the energetic characteristics of the binding and unfolding of G-quadruplex DNA by UP1. Our results provide key insights into the enthalpic contributions to the binding and coupling of the binding enthalpy to the unfolding interaction. We have found the binding and unfolding of the Tel-22 G-quadruplex structure by UP1 to be influenced by both the structure and stability of the deoxyoligonucleotide. Because the G-quadruplex unfolding process is noncatalytic, the energy requirement for unfolding is directly coupled to the binding energetics. The recognition aspects and the exact mechanism by which UP1 facilitates G-quadruplex unfolding remain speculative and difficult to interpret based on these data alone; however, additional research is underway to determine recognition determinants, binding energetics, and further insights into the unfolding mechanism.

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Notes

The authors declare no competing financial interest.

Table 2. Thermodynamic Parameters Derived from the Best Fit Line of the Isothermal Titration Binding Studies in Which G-Quadruplex DNA Was Titrated with UP1

	n	K_a (mol^{-1})	ΔG (kcal/mol)^a	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)^b
Tel-22 (Na$^+$)	0.5 ± 0.01	3.3×10^7 ± 0.8	−10.2 ± 0.1	−35.8 ± 3.5	25.6 ± 3.6
Tel-22 (K$^+$)	0.5 ± 0.02	2.6×10^7 ± 0.8	−10.1 ± 0.1	−18.9 ± 0.1	8.8 ± 0.2

^{a}The Gibbs free energy was calculated from the relation $\Delta G° = -RT \ln K$. ^{b}The entropy ($-T\Delta S°$) was calculated from the rearrangement of the relation $\Delta G° = \Delta H° - T\Delta S°$ to $-T\Delta S° = \Delta G° - \Delta H°$.

■ ABBREVIATIONS

hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; UP1, unfolding protein 1; Tel, human telomere; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; CD, circular dichroism.

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