# Kinetic and Thermodynamic Control of G-Quadruplex Polymorphism by $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$Cations 

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#### Abstract

G-Quadruplexes (G4s) are ubiquitous nucleic acid folding motifs that exhibit structural diversity that is dependent on cationic conditions. In this work, we exploit temperature-controlled single-molecule fluorescence resonance energy transfer (smFRET) to elucidate the kinetic and thermodynamic mechanisms by which monovalent cations ( $\mathrm{K}^{+}$and $\mathrm{Na}^{+}$) impact folding topologies for a simple G-quadruplex sequence ( $5^{\prime}-$ GGG-(TAAGGG) $3_{3}-3^{\prime}$ ) with a three-state folding equilibrium. Kinetic measurements indicate that $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$influence G 4 formation in two distinctly different ways: the presence of $\mathrm{Na}^{+}$modestly enhances an antiparallel G4 topology through an induced fit (IF) mechanism with a low  affinity ( $K_{\mathrm{d}}=228 \pm 26 \mathrm{mM}$ ), while $\mathrm{K}^{+}$drives G 4 into a parallel/hybrid topology via a conformational selection (CS) mechanism with much higher affinity $\left(K_{\mathrm{d}}=1.9 \pm 0.2 \mathrm{mM}\right)$. Additionally, temperature-dependent studies of folding rate constants and equilibrium ratios reveal distinctly different thermodynamic driving forces behind G 4 binding to $\mathrm{K}^{+}\left(\Delta H^{\circ}{ }_{\text {bind }}>0, \Delta S_{\text {bind }}^{\circ}>0\right)$ versus $\mathrm{Na}^{+}\left(\Delta H_{\text {bind }}^{\circ}<0, \Delta S_{\text {bind }}^{\circ}<0\right)$, which further illuminates the diversity of the possible pathways for monovalent facilitation of Gquadruplex folding.


## I. INTRODUCTION

G-Quadruplexes (G4s) represent ubiquitous nucleic acid secondary folding motifs that can form in G-rich DNA and RNA. ${ }^{1}$ Stable under both in vitro and in vivo conditions, G4s play important roles in numerous biological contexts, including replication, transcription, and chromosomal stabilization. ${ }^{2,3}$ Recently, G4s have drawn particular attention as potential drug targets for antiviral and anticancer therapies, ${ }^{3-6}$ and G4 folds have been incorporated as modular components in DNA nanostructures. ${ }^{7,8}$ A predictive understanding of the folding structure, kinetics, and thermodynamics of G4s in these diverse biological and biotechnological areas has proven to be elusive and requires a comprehensive knowledge of the underlying biophysics for nucleic acid folding.
In contrast with canonical nucleic acid duplex helical structures, G4s represent a highly structurally diverse secondary folding motif. ${ }^{9,10}$ The signature element of the G4 structure is the presence of G-tetrads, planar arrangements of four noncanonically base-paired (and noncontiguous) guanine residues, which stack in layers ( $n \approx 2$ or 3 ) to stabilize multiple G4 planes through adjacent $\pi-\pi$ interactions (Figure 1). The guanine residues in any single G4 plane can come from multiple nucleic acids as part of a higher-order molecular complex or from a single contiguous nucleic acid, in which case the G4 is termed a unimolecular fold. In such a unimolecular G4 fold, the four G-tracts (continuous G runs) in the G4 are spaced by short loop sequences (usually 2-3 nucleotides) to sterically allow the $G$-tracts to interact in a maximally


Figure 1. Crystal structure of a parallel G-quadruplex (G4). The three stacked G-tetrads are highlighted (green, red, and blue), and the two bound potassium ions are shown in purple (PDB 6IP3).
hydrogen-bonded plane. The loops flexibly permit adjacent G-tracts to have the same or opposite $5^{\prime}$ to $3^{\prime}$ orientations, which allows G4s to fold into one of many topologies, each defined by a specific combination of the G-tract polarities. ${ }^{11}$ G4 topologies are therefore classified as parallel (all strands sharing the same polarity), antiparallel (strands alternating polarities), or hybrid. Whether a G4 folds into a single topology or multiple, competing topologies (polymorphism)

[^0]
depends on many factors, including loop sequence. ${ }^{12,13}$ G4 structural diversity is further increased by the presence of partially folded intermediates such as G-triplexes (G3) or Gduplexes (G2), which can also be populated significantly and readily interconvert with fully folded G-quadruplexes. ${ }^{14,15}$

G4 folding is accompanied by obligate binding of cations to the central channel formed by the stacked G-tetrads. ${ }^{16}$ While this binding site is capable of binding polyvalent cations, it greatly favors monovalent cations, especially potassium $\left(\mathrm{K}^{+}\right)$. Importantly, the structure adopted by a G4 is regulated by the identity of its bound cation(s), which has been a subject of much research activity. ${ }^{16-18}$ For example, the human telomere G4 sequence tends to fold into a parallel or hybrid topology in $\mathrm{K}^{+}$solution and an antiparallel topology in $\mathrm{Na}^{+}$solution. ${ }^{9,20}$ The influence of ions on G4 folding has been studied extensively by bulk methods; ${ }^{10,12,13,21-28}$ however, the polymorphism of G4s can make such ensemble results difficult to interpret. Conversely, single-molecule experiments are more ideally suited to resolve topology-specific properties in multistate folding systems. ${ }^{29}$ In particular, single-molecule methods have been used to examine many aspects of G4 folding, ${ }^{30}$ including G4 folding kinetics ${ }^{31-35}$ and the temperature dependence of the folding equilibrium constants. ${ }^{36}$ However, temperature-dependent studies of the folding kinetics at the single-molecule level have yet to be reported, which in principle would provide the first access to the thermodynamics of the transition states for G4 formation.
As a focus of the present work, we use temperaturedependent single-molecule microscopy to investigate the influence of monovalent cations ( $\mathrm{K}^{+}$and $\mathrm{Na}^{+}$) on the kinetics and thermodynamics of G4 folding. Specifically, we study the folding of the $5^{\prime}$-GGG-(TAAGGG) $3_{3}-3^{\prime}$ sequence, which is known to form a polymorphic G4. ${ }^{34}$ We examine this system using fluorescence resonance energy transfer (FRET) to monitor the dynamics of G4 folding with a single-molecule FRET (smFRET) total internal reflection fluorescence (TIRF) microscope. ${ }^{37}$ Transitions between the polymorphic network of G4 states are observed in real time, from which we determine the equilibrium constants and rate constants for these transitions. By analyzing the dependence of such folding dynamics on cation concentration and temperature, we obtain the first quantitative insights into the kinetic mechanism for polymorphism control by $\mathrm{K}^{+}$and $\mathrm{Na}^{+}$as well as the thermodynamics of cation binding to G4.

## II. METHODS

G-Quadruplex FRET Construct. The single-molecule construct used in these studies consists of two annealed DNA oligomers. The first oligomer is a Cy3-labeled DNA Gquadruplex fold ( $5^{\prime}$-GGG-(TAAGGG) $3_{3}-3^{\prime}$ ) connected by eight thymine residues to an 18 -nucleobase duplex-forming domain (full sequence: $5^{\prime}$-TGGCGACGGCAGCGAGGC-T $8^{-}$ GGG-(TAAGGG) $3_{3}$-Cy3-3'). For control experiments, a nonfolding sequence was also utilized as the first oligomer ( $5^{\prime}-$ TGGCGACGGCAGCGAGGC-T ${ }_{8}$-GGTGAGTGGGAGT-GGTGTGTA-Cy3-3'). The second oligomer is complementary to the duplex-forming domain and is labeled with Cy 5 and biotin (5'-Cy5-GCCTCGCTGCCGTCGCCA-Biotin-3') for surface tethering by biotin-streptavidin interactions. ${ }^{38}$ Both oligomers are purchased in HPLC-purified form from Integrated DNA Technologies and used as is. (Company names listed herein are in the interest of completeness and do not reflect endorsement of a particular vendor.) The oligomers
are annealed by mixing $10 \mu \mathrm{M}$ aliquots ( $1 \mathrm{M}=1 \mathrm{~mol} / \mathrm{L}$ ) and heating to $85{ }^{\circ} \mathrm{C}$ in order to disrupt intramolecular basepairing before slowly cooling to room temperature at $1^{\circ} \mathrm{C} /$ $\min$. The annealed construct is used without purification, as constructs lacking the biotinylated strand do not adhere to the surface and constructs lacking the Cy3-labeled strand are inefficiently excited by the laser $\left(\varepsilon_{\mathrm{Cy5}} / \varepsilon_{\mathrm{Cy} 3}\right.$, the ratio of Cy 5 and Cy3 molar absorption coefficients, is $3 \%$ at 532 nm ).

Sample Preparation and Single-Molecule Microscopy. Sample preparation and single-molecule microscopy are carried out according to previously described protocols. ${ }^{39,40}$ In brief, a glass coverslip is cleaned by soaking for 24 h in acetone, followed by a 30 min treatment with UV-generated ozone. The coverslip surface is incorporated into a flow cell, where it is functionalized by sequential 10 min exposure to buffered solutions of (i) $10 \mathrm{mg} / \mathrm{mL}$ bovine serum albumin (BSA) with $1 \mathrm{mg} / \mathrm{mL}$ biotinylated BSA, (ii) $0.2 \mathrm{mg} / \mathrm{mL}$ streptavidin, and (iii) 150 pM smFRET construct. This process produces a surface decorated with approximately 1 smFRET construct per $10 \mu \mathrm{~m}^{2}$. The surface-tethered constructs are then imaged in 50 mM HEPES buffer ( pH 7.6 ) with an oxygen scavenging cocktail to extend the observation time ( 100 nM protocatechuate 3,4-dioxygenase; 5 mM protocatechuic acid) and 2 mM Trolox to increase fluorophore brightness by quenching fluorophore triplets to the ground state. ${ }^{41,42}$ Sodium and potassium levels are controlled by titration with NaCl and KCl solutions, respectively.

The prepared sample is observed with a through-objective TIRF microscope, ${ }^{43}$ which permits 532 nm excitation of Cy3 molecules constrained to within $\approx 100 \mathrm{~nm}$ of the surface. The fluorescence from the smFRET construct is collected and separated by dichroic mirrors into Cy 3 and Cy 5 channels before being directed onto an intensified charge-coupled device (CCD) camera operating at 10 frames per second. Movies are analyzed by software written in LabWindows/CVI, which identifies particle locations by thresholding and extracts raw donor and acceptor fluorescence trajectories by integration over the local $4 \times 4$ pixel neighborhood for each frame. Background fluorescence levels are determined by fits of particle point spread functions to a symmetric 2D Gaussian function (equal full width at half-maximum (fwhm) for $x$ and $y$ ), which permits calculation of background-corrected donor $(D(t))$ and acceptor $(A(t))$ signals. Time-dependent FRET trajectories are determined from the energy transfer efficiency, $E_{\mathrm{FRET}}(t)=A(t) /(A(t)+\gamma D(t))$, where $\gamma$ is the ratio of acceptor to donor detection efficiencies, which is determined to be nearly unity ( $\gamma=0.97$ ) via intensity changes during acceptor photobleaching. ${ }^{44}$ Temperature control is achieved through a thermoelectric element that is in thermal contact with the sample and capable of servoloop heating and cooling to within $0.1^{\circ} \mathrm{C}$ stability, as has been previously described. ${ }^{48}$

Hidden Markov Modeling. Rate constants for conformational transitions are determined by hidden Markov modeling analysis of the FRET trajectories. ${ }^{45}$ The model consists of $n$ states, each with a Gaussian-distributed FRET observable, $P\left(E_{i}, \sigma_{i}\right) \propto \exp \left[-\left(E-E_{i}\right) / 2 \sigma_{i}^{2}\right]$, where $E_{i}$ and $\sigma_{i}$ represent the FRET center and width of state $i$, respectively. Transitions between states occur according to first-order kinetics, as contained in the rate matrix $\mathbf{K}$, where $K_{i j}$ is the unimolecular rate constant for the transition from state $j$ to state $i$, and $K_{i i}=$ $-\Sigma_{j \neq i} K_{i j}$. The transition probability per frame is then calculated via the matrix exponential $\mathbf{T}=\exp \left(\mathbf{K} t_{\text {frame }}\right)$, where $t_{\text {frame }}$ is the experimental time ( 0.1 s ) between consecutive
frames. The likelihood function for a single trajectory is therefore described by the matrix product

$$
\begin{equation*}
L=\mathbf{1}^{T}\left[\prod_{i \geq 2} \mathbf{O}\left(E_{i}\right) \mathbf{T}\right] \mathbf{O}\left(E_{1}\right) \mathbf{p}_{\mathrm{eq}} \tag{1}
\end{equation*}
$$

where $\mathbf{p}_{\text {eq }}$ is the equilibrium probability vector $\left(K \mathbf{p}_{\text {eq }}=0\right), E_{i}$ is the $i$ th observed FRET value, $\mathbf{O}(E)$ is an $n \times n$ diagonal matrix with diagonal entries $O_{i i}(E)$ equal to the probability density of observing the FRET value $E$ in the $i$ th state, $\mathbf{1}^{T}$ is the row vector $(1,1, \cdots)$ of length equal to the number of states $n$, and the product is taken over all frames in the trajectory except the first. For multiple trajectories, the total likelihood function is the product of the single-trajectory likelihood functions ( $L_{\text {total }}$ $=\prod_{i} L_{i}$ ). The maximum likelihood estimate of $K_{i j}, E_{i}$, and $\sigma_{i}$ is obtained by performing a gradient ascent on $L_{\text {total }}$ using MATLAB, with bootstrapping analysis performed to estimate uncertainties. ${ }^{46}$ Each kinetic analysis includes 100-150 molecules and 2000-3000 observed transitions between FRET states.

Three-State Hill Fit. Population versus concentration data for the G4 are fit to a Hill model. The standard Hill equation describes ligand binding in systems with two distinguishable receptor configurations: a ligand-bound state and a ligand-free state. ${ }^{47}$ However, the G4 construct in this work has three observable states, so we instead used a modified Hill analysis in order to include all three states. The binding model consists of two competing ligand association processes, which convert a receptor $R$ into one of two distinguishable ligand-bound species $B_{1}$ or $B_{2}$, each potentially with its own stoichiometry ( $n_{1}$ and $n_{2}$ ) and dissociation constants ( $K_{\mathrm{d}, 1}$ and $K_{\mathrm{d}, 2}$ ):

1 :

$$
\begin{equation*}
R \xrightarrow{+n_{1} L} B_{1} \quad\left(K_{\mathrm{d}, 1}\right)^{n_{1}}=\frac{[R]}{\left[B_{1}\right]}[L]^{n_{1}} \tag{2}
\end{equation*}
$$

$2:$

$$
\begin{equation*}
R \xrightarrow{+n_{2} L} B_{2} \quad\left(K_{\mathrm{d}, 2}\right)^{n_{2}}=\frac{[R]}{\left[B_{2}\right]}[L]^{n_{2}} \tag{3}
\end{equation*}
$$

The above dissociation constant expressions can be readily manipulated to yield the fractional populations in each state as a function of ligand concentration:

$$
\begin{align*}
& \theta_{R}([L])=\frac{[R]}{[R]+\left[B_{1}\right]+\left[B_{2}\right]}=\frac{1}{1+\left(\frac{[L]}{K_{\mathrm{d}, 1}}\right)^{n_{1}}+\left(\frac{[L]}{K_{\mathrm{d}, 2}}\right)^{n_{2}}} \\
& \theta_{B_{1}}([L])=\frac{\left[B_{1}\right]}{[R]+\left[B_{1}\right]+\left[B_{2}\right]}=\frac{\left(\frac{[L]}{K_{\mathrm{d}, 1}}\right)^{n_{1}}}{1+\left(\frac{[L]}{K_{\mathrm{d}, 1}}\right)^{n_{1}}+\left(\frac{[L]}{K_{\mathrm{d}, 2}}\right)^{n_{2}}}  \tag{5}\\
& \theta_{B_{2}}([L])=\frac{\left[B_{2}\right]}{[R]+\left[B_{1}\right]+\left[B_{2}\right]}=\frac{\left(\frac{[L]}{K_{\mathrm{d}, 2}}\right)^{n_{2}}}{1+\left(\frac{[L]}{K_{\mathrm{d}, 1}}\right)^{n_{1}}+\left(\frac{[L]}{K_{\mathrm{d}, 2}}\right)^{n_{2}}} \tag{6}
\end{align*}
$$

This model is then applied to data for sodium and potassium titrations of the G-quadruplex, where $L=\mathrm{Na}^{+}$or $\mathrm{K}^{+}, R$ is the low FRET state, $B_{1}$ is the middle FRET state, and $B_{2}$ is the
high FRET state. Dissociation constants $\left(K_{\mathrm{d}, i}\right)$ and apparent stoichiometries $\left(n_{i}\right)$ are determined by simultaneously fitting all three populations to the Hill model using the weighted, nonlinear least-squares regression tools available in OriginPro.
van't Hoff and Arrhenius Analyses. Temperaturedependent equilibrium constants determined from hidden Markov modeling are then subjected to van't Hoff analysis, ${ }^{48,49}$ wherein the Gibbs free energy

$$
\begin{equation*}
\Delta G^{\circ}=\Delta H^{\circ}-T \Delta S^{\circ}=-R T \ln \left(K_{\mathrm{eq}}\right) \tag{7}
\end{equation*}
$$

is rewritten in van't Hoff form

$$
\begin{equation*}
\ln \left(K_{\mathrm{eq}}\right)=\frac{-\Delta H^{\circ}}{R}\left(\frac{1}{T}\right)+\frac{\Delta S^{\circ}}{R} \tag{8}
\end{equation*}
$$

and where $K_{\text {eq }}$ is the equilibrium constant, $R$ is the gas constant ( $8.314 \mathrm{~J} / \mathrm{mol} \mathrm{K}$ ), $T$ is the absolute temperature, $\Delta H^{\circ}$ is the reaction enthalpy, and $\Delta S^{\circ}$ is the reaction entropy. In accord with such a van't Hoff model, we assume $\Delta H^{\circ}$ and $\Delta S^{\circ}$ to be approximately constant over the explored temperature range and perform a linear least-squares fit of $\ln \left(K_{\text {eq }}\right)$ versus $1 / T$ to extract $\Delta H^{\circ}$ and $\Delta S^{\circ}$ via the slope and intercept, respectively. In analogous fashion, the temperature-dependent rate constants can be analyzed in the context of Kramers' theory ${ }^{50-52}$ to yield an Arrhenius-type expression

$$
\begin{equation*}
\ln (k / v)=\frac{-\Delta H^{\ddagger}}{R}\left(\frac{1}{T}\right)+\frac{\Delta S^{\ddagger}}{R} \tag{9}
\end{equation*}
$$

where $k$ is the rate constant, $\nu$ is the attempt frequency along the reaction coordinate, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the activation enthalpy and entropy, respectively. The attempt frequency is estimated as $1 \times 10^{6} \mathrm{~s}^{-1}$ from previous RNA folding studies, ${ }^{50,51}$ but its actual value is dependent on the structure of the free energy landscape. As a consequence, the activation entropy $\Delta S^{\ddagger}$ is only weakly (i.e., logarithmically) sensitive to $\nu$ and not determined absolutely; however, any differential change in this entropy $\left(\Delta \Delta S^{\ddagger}\right)$, for instance, due to change in ionic conditions, remains rigorously independent of the choice of $\nu$.

## III. RESULTS

The G-Quadruplex smFRET Construct Exhibits Multistate Folding. To probe the thermodynamics of Gquadruplex folding at the single-molecule level, we designed a fluorophore-labeled DNA construct for FRET microscopy (Figure 2A). The folding domain of the construct consists of a repeated six-nucleotide sequence ( $5^{\prime}$-TAAGGG- $3^{\prime}$ ), which forms a G-quadruplex with three stacked G-tetrads. This sequence is closely related to the human telomeric G4 sequence ( $5^{\prime}$-TTAGGG- $3^{\prime}$ ), differing only by a single nucleotide mutation in the loop (TAA versus TTA), which has been previously observed by Tippana et al. to increase polymorphism. ${ }^{34}$ The construct is labeled with the Cy3-Cy5 FRET pair ${ }^{42}$ such that folding of the G-quadruplex brings Cy3 and Cy 5 in closer proximity, increasing the energy transfer efficiency $\left(E_{\text {FRET }}\right)$ between the fluorophores. The full construct is surface-tethered by biotin-streptavidin interactions ${ }^{38}$ in order to enable the observation of the G-quadruplex in a single-molecule fluorescence microscope.

Temporal trajectories in a $100 \mathrm{mM} \mathrm{Na}{ }^{+}$buffer reveal multiple, distinct $E_{\text {FRET }}$ states which interconvert/equilibrate on time scales of 100 ms to seconds (Figure 2B). A histogram of $E_{\text {FRET }}$ values gathered from $N=83$ molecules is well-fit as a


Figure 2. (A) The G-quadruplex smFRET construct design that is labeled with Cy3 and Cy5 for FRET detection of the conformational dynamics. (B) A sample smFRET trajectory taken in a $100 \mathrm{mM} \mathrm{Na}^{+}$ buffer. The fitted state trajectory from hidden Markov modeling with the three states is overlaid in orange. (C) A FRET histogram (gray) aggregated from $N=83$ trajectories. Gaussian fits (solid lines) are used to determine the FRET efficiencies and the relative abundance of the three states. (F) Cartoons that indicate putative structures of the three states: the unfolded state $\left(E_{\text {FRET }}=0.45\right)$, the antiparallel G4 state $\left(E_{\text {FRET }}=0.73\right)$, and the parallel or hybrid G4 state $\left(E_{\text {FRET }}=\right.$ $0.97)$. See text for details.
sum of three Gaussian distributions $\left(E_{\text {low }}=0.45, E_{\text {middle }}=0.70\right.$, and $E_{\text {high }}=0.94$ ), which corresponds to three construct conformations with resolved interfluorophore distances (Figure 2C). Three distinct FRET states were also observed in the work of Tippana et al. on this sequence. ${ }^{34}$ To probe the identity of the FRET states, we first measured the FRET efficiency of a construct with a similar nucleotide composition but reordered it so that it cannot fold into a G4 or other
secondary structures (Figure 2D). The FRET efficiency of this ssDNA construct $\left(E_{\text {ssDNA }}=0.46\right)$ is in good agreement with that of the low FRET state $\left(E_{\text {low }}=0.45\right)$. Therefore, we assign the $E_{\text {low }}$ state as an unfolded G4 (U) in a single-stranded random coil configuration. The two higher FRET states are likely two different G4 topologies. To identify these states, we next introduce $\mathrm{K}^{+}$to the imaging solution, as $\mathrm{K}^{+}$is known to stabilize parallel or hybrid G4s and decrease the population of antiparallel configurations. ${ }^{10,16-18}$ In 15 mM K , the FRET distribution shifts to favor the $E_{\text {high }}$ state (Figure 2E) and reduces the population of the $E_{\text {low }}$ and $E_{\text {middle }}$ states. As a result, we assign the $E_{\text {high }}$ state as either a parallel G4 or a hybrid G4 (P) and the $E_{\text {middle }}$ state as an antiparallel G4 (A). All three putative structures (U, A, and P) are shown in Figure 2F.

Sodium Ions Indiscriminately Drive the Folding Equilibrium to the Antiparallel and Parallel/Hybrid Topologies. We first explored G-quadruplex folding as a function of $\mathrm{Na}^{+}$concentration at ambient temperature (23 ${ }^{\circ} \mathrm{C}$ ). The FRET histograms in Figure 3A reveal an overall shift from the unfolded state (U) to the antiparallel (A) and parallel/hybrid (P) G4 topologies with increasing $\left[\mathrm{Na}^{+}\right]$. Integration over these histogram peaks yields the subpopulation fractions in $\left[\mathrm{Na}^{+}\right]=50-300 \mathrm{mM}$ (Figure 3B), which can then be fit globally to a three-state Hill model (eqs 4-6) that explicitly includes all three interconversion routes. The leastsquares fits to such a multistate model are shown as solid lines in Figure 3B, yielding the stoichiometric coefficient $(\Delta n)$ for $\mathrm{Na}^{+}$binding and dissociation constants ( $K_{\mathrm{d}}$ ) for each interconversion pathway, as summarized in Table 1 (note that the cation binding stoichiometry reflects the change in the number of bound ions during folding, where it is possible that the unfolded state is already bound to one or more ions prior to folding). Interestingly, the unfolded state gains only one $\mathrm{Na}^{+}$ ion when folding into either G4 state ( $\Delta n_{\mathrm{U} \rightarrow \mathrm{A}} \approx \Delta n_{\mathrm{U} \rightarrow \mathrm{P}} \approx 0.87$ $\pm 0.09$ ), though $\mathrm{Na}^{+}$has a two-fold stronger affinity for antiparallel G4 ( $K_{\mathrm{d}}=132 \pm 9 \mathrm{mM}$ ) than for parallel/hybrid $\mathrm{G} 4\left(K_{\mathrm{d}}=228 \pm 26 \mathrm{mM}\right)$. Conversely, there is no difference within the experimental uncertainty in the number of $\mathrm{Na}^{+}$ions bound to the two G4 conformations. As a consequence of the A and P states both binding one $\mathrm{Na}^{+}$ion, the $\mathrm{A} \rightarrow \mathrm{P}$ equilibrium saturates to a fixed ratio at high $\left[\mathrm{Na}^{+}\right]$(Figure 3B). $\mathrm{Na}^{+}$clearly promotes G 4 polymorphism, with the G4 construct populating both the antiparallel and parallel/hybrid topologies, even at $\left[\mathrm{Na}^{+}\right]=300 \mathrm{mM}$.

Additional mechanistic insight can be obtained from the kinetic analysis of the $\mathrm{Na}^{+}$dependence of G-quadruplex folding events in the time domain. Rate constants for folding are determined by hidden Markov modeling (HMM) of smFRET trajectories ${ }^{45,53,54}$ using a three-state model which includes all six rate constants describing forward and reverse interconversion between each FRET pair (Figure 4A), with the rate constants for G4 folding as a function of $\left[\mathrm{Na}^{+}\right]=50-300 \mathrm{mM}$ summarized in Figure 4B. As a rigorous thermodynamic check on the validity of these extracted rate constants, we can calculate the free energy sum around the three-state cycle ( $\Delta G_{\text {cycle }}$ ) at each $\mathrm{Na}^{+}$concentration and plot the values in Figure 4C:

$$
\begin{equation*}
\Delta G_{\text {cycle }}=-k_{\mathrm{B}} T \ln \left(\frac{k_{\mathrm{U} \rightarrow \mathrm{~A}} k_{\mathrm{A} \rightarrow \mathrm{P}} k_{\mathrm{P} \rightarrow \mathrm{U}}}{k_{\mathrm{A} \rightarrow \mathrm{U}} k_{\mathrm{P} \rightarrow \mathrm{~A}} k_{\mathrm{U} \rightarrow \mathrm{P}}}\right) \tag{10}
\end{equation*}
$$

By detailed balance, the free energy sum around any cycle of states must equal zero. Indeed, the calculated $\Delta G_{\text {cycle }} / k_{B} T$


Figure 3. Influence of $\mathrm{Na}^{+}$on G 4 folding equilibrium. (A) FRET histograms and sample FRET trajectories at 50,100 , and 200 mM $\mathrm{Na}^{+}$. An example of a direct $\mathrm{A} \rightarrow \mathrm{P}$ transition is indicated by an asterisk. (B) The population of the three G4 conformational states in $\left[\mathrm{Na}^{+}\right]=50-300 \mathrm{mM}$. Fits to a three-state Hill model are shown as solid curves.
values vanish within uncertainty $\left( \pm 0.2 k_{\mathrm{B}} T\right)$ for all $\mathrm{Na}^{+}$ concentrations. In Figure 4B, the rate constants for the conversion of U to and from the G 4 conformations A and P have the largest magnitude, while transitions directly between the antiparallel (A) and parallel/hybrid (P) G4 topologies are a minor, though not entirely forbidden, kinetic process (Figure 3A).
To additionally explore the effects of the $\mathrm{Na}^{+}$cation on Gquadruplex folding, we have further developed an explicit sixstate kinetic model for $\mathrm{Na}^{+}$binding to the G-quadruplex (Figure 4D). The model consists of three DNA conformations,

Table 1. Results of Fitting G-Quadruplex Population Data vs $\left[\mathrm{Na}^{+}\right]$and $\left[\mathrm{K}^{+}\right]$to the Three-State Hill Model ${ }^{a}$

| Transition | Parameter | $\mathrm{Na}^{+}$ | $\mathrm{K}^{+}$ |
| :---: | :---: | :---: | :---: |
| $\mathrm{U} \rightarrow \mathrm{A}$ | $\Delta n$ | $0.87 \pm 0.09$ | $-0.05 \pm 0.05$ |
|  | $K_{\mathrm{d}}(\mathrm{mM})$ | $132 \pm 9$ | - |
| $\mathrm{U} \rightarrow \mathrm{P}$ | $\Delta n$ | $0.87 \pm 0.09$ | $0.62 \pm 0.05$ |
|  | $K_{\mathrm{d}}(\mathrm{mM})$ | $228 \pm 26$ | $1.9 \pm 0.2$ |
| $\mathrm{~A} \rightarrow \mathrm{P}$ | $\Delta n$ | $0.00 \pm 0.09$ | $0.67 \pm 0.07$ |
|  | $K_{\mathrm{d}}(\mathrm{mM})$ | - | $2.9 \pm 0.3$ |

${ }^{a} \mathrm{U}$ is the unfolded state (low $E_{\text {FRET }}$ ), A is the antiparallel G4 state (intermediate $E_{\text {FRET }}$ ), and P is the parallel/hybrid G4 state (high $E_{\text {FRET }}$ ). Uncertainties are reported as standard errors of the mean. For processes with Hill coefficients ( $\Delta n$ ) equal to zero within uncertainty, dissociation constants $\left(K_{\mathrm{d}}\right)$ are not reported.
each with ligand-free and ligand-bound forms, which topologically generate a triangular prism (hence the "Toblerone" model moniker). In accordance with the $1: 1 \mathrm{Na}^{+}$binding stoichiometry from the Hill fit of the population data (Table 1 ), the kinetic model includes a single $\mathrm{Na}^{+}$ion in each ligandbound state. Ligand binding kinetics are modeled to be much faster than subsequent G4 conformational dynamics, ${ }^{16,17,55}$ which allows all ligand binding steps to be treated as a quasiequilibrium governed by a single binding constant

$$
\begin{equation*}
K_{\mathrm{d}, i}=\frac{\left[C_{i}\right]}{\left[C_{i} L\right]}[L] \tag{11}
\end{equation*}
$$

where $i$ labels the conformational state (unfolded $U$, antiparallel G4 A, or parallel/hybrid G4 P), $C_{i}$ is the ligandfree conformation, $C_{i} L$ is the ligand-bound conformation, $L$ is the ligand (in this case, $\mathrm{Na}^{+}$), and the square brackets indicate concentration of the enclosed species. The fraction of ligandbound molecules is therefore

$$
\begin{equation*}
\theta_{\mathrm{bound}, i}=\frac{\left[C_{i} L\right]}{\left[C_{i}\right]+\left[C_{i} L\right]}=\frac{[L]}{[L]+K_{\mathrm{d}, i}} \tag{12}
\end{equation*}
$$

Transitions between conformations are governed by firstorder rate constants, depending on ligand occupancy:

$$
\begin{align*}
& C_{i} \xrightarrow{k_{i, \text { apo }}} C_{j}  \tag{13}\\
& C_{i} L \xrightarrow{k_{i j, \text { holo }}} C_{j} L \tag{14}
\end{align*}
$$

where $k_{i j, \text { apo }}$ and $k_{i, j, \text { holo }}$ are rate constants for the transition from state $i$ to state $j$ in the ligand-free ("apo") and ligand-bound ("holo") forms, respectively. The observed rate constants $k_{i j, o b s}$ for transitions between conformational states are populationweighted averages of the apo and holo rate constants:

$$
\begin{equation*}
k_{i j, \text { obs }}=k_{i j, \text { apo }}\left(\frac{K_{\mathrm{d}, i}}{[L]+K_{\mathrm{d}, i}}\right)+k_{i j, \text { holo }}\left(\frac{[L]}{[L]+K_{\mathrm{d}, i}}\right) \tag{15}
\end{equation*}
$$

For the six-state kinetic model in Figure 4D, eq 15 forms a system of six equations, where the number of free parameters in the model can be reduced to 11 by imposing detailed balance constraints $\left(\Delta G_{\text {cycle }}=0\right)$ on all cycles. The resulting constrained equations are then globally fit to the kinetic data in Figure 4B, as summarized by the solid line fits.

The results of the six-state fit indicate that $\mathrm{Na}^{+}$promotes G4 folding through a "bind-then-fold" mechanism: $\mathrm{Na}^{+}$binds to the unfolded (U) state, which subsequently folds into a G4 (A


Figure 4. Influence of $\mathrm{Na}^{+}$on G 4 folding kinetics. (A) Left: a structure of the hidden Markov model (HMM) that is used to fit the smFRET trajectories in order to obtain the G4 folding rate constants. Right: the orientation of the three-state cycle used to calculate the free energy change around the loop, $\Delta G_{\text {cycle }}=\Delta G_{U \rightarrow P}+\Delta G_{P \rightarrow A}+\Delta G_{A \rightarrow U}$. (B) The rate constants for G 4 folding in $50-300 \mathrm{mM} \mathrm{Na}^{+}$. Fits to the six-state $\mathrm{Na}^{+}$binding model that is depicted in (D) are shown as solid curves. (C) A plot of $\Delta G_{\text {cycle }}$ at each $\left[\mathrm{Na}^{+}\right]$. To satisfy detailed balance conditions, $\Delta G_{\text {cycle }}$ must equal zero. (D) A six-state "Toblerone" connectivity model for the influence of $\mathrm{Na}^{+}$on G 4 folding kinetics. The dominant pathways through this network are shown as red arrows, which reflect a "bind-then-fold" mechanism.
or P) topology (Figure 4D, pathways highlighted in red). ${ }^{56-58}$ There are several key indicators in support of this mechanism. First, the forward folding rate constants $k_{\mathrm{U} \rightarrow \mathrm{A}}$ and $k_{\mathrm{U} \rightarrow \mathrm{P}}$ both vanish at low $\left[\mathrm{Na}^{+}\right]$(Figure 4B, filled red and blue symbols, respectively), which implies that folding to either of the G4
topologies without $\mathrm{Na}^{+}$is, evidently, impossible ( $k_{\mathrm{UA}, \mathrm{apo}}=$ $k_{\mathrm{UP}, \text { apo }}=0 \mathrm{~s}^{-1}$ ). Additionally, $k_{\mathrm{U} \rightarrow \mathrm{A}}$ and $k_{\mathrm{U} \rightarrow \mathrm{P}}$ saturate at high $\left[\mathrm{Na}^{+}\right]\left(k_{\mathrm{UA}, \text { holo }}=0.7 \pm 0.3 \mathrm{~s}^{-1}, k_{\mathrm{UP}, \text { holo }}=1.0 \pm 0.4 \mathrm{~s}^{-1}\right)$, which indicates that binding of $\mathrm{Na}^{+}$to the G 4 is driven to completion. However, there is also a weaker dependence of the unfolding rate constants $k_{\mathrm{A} \rightarrow \mathrm{U}}$ and $k_{\mathrm{P} \rightarrow \mathrm{U}}$ on [ $\mathrm{Na}^{+}$], so other pathways besides the induced fit ("bind-then-fold") pathway must be permitted to a lesser degree.
$\mathrm{K}^{+}$Has a Much Higher Affinity Than $\mathrm{Na}^{+}$for the GQuadruplex and Exclusively Promotes the Parallel/ Hybrid Topology. Formation of G-quadruplexes is known to be more sensitive to $\mathrm{K}^{+}$than to $\mathrm{Na}^{+}$, which we next explore in order to measure the specific effects $\mathrm{K}^{+}$versus $\mathrm{Na}^{+}$has on G 4 folding (Figure 5). Interestingly, $\mathrm{K}^{+}$drives the conformational equilibrium of the G4 smFRET construct exclusively toward the parallel/hybrid G4 (P) topology (Figure 5A), in essence, by depleting both the unfolded (U) and antiparallel G4 (A) states. Furthermore, this dramatic increase in folding occurs at only a few millimolar $\mathrm{K}^{+}$concentrations, in contrast to the $100-$ fold higher concentrations of $\mathrm{Na}^{+}$that is required to achieve folding (Figure 3B). Thus, the G4 construct clearly has a much higher affinity for $\mathrm{K}^{+}$than $\mathrm{Na}^{+}$, though only in achieving the parallel/hybrid G4 conformation. Fitting the full suite of the $\mathrm{K}^{+}$-dependent population data (Figure 5B) to a three-state Hill model quantitatively confirms these qualitative observations, as summarized in Table 1. In this case, there is no $\mathrm{K}^{+}$uptake during the transition from the unfolded state to the antiparallel $\mathrm{G} 4\left(\Delta n_{\mathrm{U} \rightarrow \mathrm{A}}=-0.05 \pm 0.05\right)$, which indicates that $\mathrm{K}^{+}$does not participate in this process. Meanwhile, approximately one cation binds during folding into the parallel/hybrid G4 $\left(\Delta n_{\mathrm{U} \rightarrow \mathrm{P}}=0.62 \pm 0.05\right)$, with a $\mathrm{K}^{+}$binding affinity $\left(K_{\mathrm{d}}\left(\mathrm{K}^{+}\right)\right.$ $=1.9 \pm 0.2 \mathrm{mM})$ that is two orders of magnitude greater than that of $\mathrm{Na}^{+}\left(K_{\mathrm{d}}\left(\mathrm{Na}^{+}\right)=228 \pm 26 \mathrm{mM}\right)$ to achieve the same conformational state. The Hill coefficient being less than unity indicates that the parallel/hybrid G4 forms to a limited degree in the absence of the $\mathrm{K}^{+}$cation, which is consistent with the observed formation of the parallel/hybrid G4 in an $\mathrm{Na}^{+}$-only buffer (Figure 3). We note that the $\mathrm{K}^{+}$binding affinity reported here is in good agreement with previous bulk studies by Largy et al. $\left(K_{\mathrm{d}}\left(\mathrm{K}^{+}\right)=1-5 \mathrm{mM}\right)$, which were also performed in $\mathrm{Na}^{+}$rich solutions. ${ }^{28}$ This agreement between fluorophore-labeled G4s and fluorophore-free G4s is evidence that the presence of the Cy3 and Cy5 fluorophores does not significantly perturb the G4 folding equilibrium, or at least cation binding to the G4, despite the fact that cyanine-based dyes are known to readily stack on G-tetrads. ${ }^{59}$ By driving the G4 construct exclusively to the parallel/hybrid topology, $\mathrm{K}^{+}$thereby exerts a much greater restriction over G4 polymorphism than $\mathrm{Na}^{+}$.

Similar to the analysis of the $\mathrm{Na}^{+}$-dependent G4 folding kinetics described above, smFRET trajectories are subjected to hidden Markov modeling in order to extract rate constants as a function of $\left[\mathrm{K}^{+}\right]$(Figure 5C). As before, we can rigorously confirm these kinetic results by verifying that the rate constants explicitly obey a detailed balance $\left(\Delta G_{\text {cycle }}=0\right)$ at all $\mathrm{K}^{+}$ concentrations (Figure 5D). Five of the six folding rate constants show little to no dependence on the $\mathrm{K}^{+}$ concentration, while the rate constant for unfolding from the parallel/hybrid G4 to the unfolded state ( $k_{\mathrm{P} \rightarrow \mathrm{U}}$ ) is strongly suppressed by the presence of $\mathrm{K}^{+}$. To explore the mechanistic implications of these data, the folding rate constants are fit to the six-state kinetic model (eq 15) and overlaid on Figure 5C as solid lines. The fit correctly predicts that $k_{\mathrm{P} \rightarrow \mathrm{U}}$ goes to zero in the high $\left[\mathrm{K}^{+}\right]$limit $\left(k_{\text {PT,holo }}=0.01 \pm 0.03 \mathrm{~s}^{-1}\right)$, which is


Figure 5. Influence of $\mathrm{K}^{+}$on G4 folding. (A) FRET histograms and sample FRET trajectories in $100 \mathrm{mM} \mathrm{Na}^{+}$with 0 , 5 , and $15 \mathrm{mM} \mathrm{K}{ }^{+}$. An example of a direct $A \rightarrow P$ transition is indicated by an asterisk. (B) State populations and (C) folding rate constants determined in $100 \mathrm{mM} \mathrm{Na}{ }^{+}$ with $0-20 \mathrm{mM} \mathrm{K}^{+}$. Data are fit to a three-state Hill model in (B) and a six-state ligand binding model in (C). (D) The calculation of $\Delta G_{\text {cycle }}$ at each $\mathrm{K}^{+}$concentration to confirm that the rate constants satisfy detailed balance conditions. (E) A six-state model of $\mathrm{K}^{+}$binding to G 4 . Red arrows indicate the highest flux pathways (a "fold-then-bind" mechanism).
consistent with a conformational selection (CS, "fold-thenbind") model in which the apo DNA prefolds to a transient parallel/hybrid G4 state before binding to $\mathrm{K}^{+}$with high affinity $\left(K_{\mathrm{d}, \mathrm{A}}=2.6 \pm 1.2 \mathrm{mM}\right)$ in order to stabilize the parallel/hybrid configuration. The $\mathrm{K}^{+}$-induced folding pathways (red, highlighted in Figure 5E) contrast strongly with those predicted from the induced fit (IF, "bind-then-fold") mechanism seen in the $\mathrm{Na}^{+}$-stabilized formation (see Figure 4D) of the G4 quadruplex and originate from a much stronger and topologyspecific binding interaction.

Temperature Dependence of G-Quadruplex Folding Equilibria and Kinetics. Temperature-controlled singlemolecule experiments can be used to deconstruct the overall free energy of G-quadruplex folding into enthalpic and
entropic contributions. ${ }^{49}$ The temperature dependence of the folding rate constants and equilibrium constants in 100 mM $\mathrm{Na}^{+}$is captured in Figure 6 over a temperature range of 13-29 ${ }^{\circ} \mathrm{C}$. The rate constant ( $y$-axis) scale in Figure 6 is logarithmic, which highlights the exponential dependence of the rate constants on temperature. To the best of our knowledge, this is the first time smFRET has been used to examine temperaturedependent kinetics for a nonbinary system with multiple ( $\geq 3$ ) conformations. First, we observe from the $y$-axis scale that the majority of the conformational transitions involve the unfolded (U) state (Figure 6A,B, left panels), with direct folding and unfolding between the antiparallel G4 (A) and parallel/hybrid G4 (P) being minor kinetic processes at all temperatures (Figure 6C, left). Second, we observe the equilibrium







Figure 6. The temperature dependence of G4 folding rate constants (left panels) and equilibrium constants (right panels) in $100 \mathrm{mM} \mathrm{Na}^{+}$for (A) unfolded $\rightarrow$ antiparallel G4, (B) unfolded $\rightarrow$ parallel/hybrid G4, and (C) antiparallel G4 $\rightarrow$ parallel/hybrid G4 transitions. The solid lines indicate Arrhenius and van't Hoff fits for the rate constants and equilibrium constants, respectively, which are used to extract the enthalpy and entropy change for each process.
constants for folding from the U state to both G 4 states $\left(K_{\text {eq, UA }}\right.$ and $K_{\text {eq,UP }}$ ) decrease at high temperature, which demonstrates that the overall folding process is enthalpically favorable. The kinetic origin of this behavior is confirmed by examining the corresponding rate constants: the forward rate constants ( $k_{U \rightarrow \mathrm{~A}}$ and $k_{\mathrm{U} \rightarrow \mathrm{P}}$ ) are largely temperature insensitive, which indicates a negligible activation enthalpy, while the reverse rate constants ( $k_{\mathrm{A} \rightarrow \mathrm{U}}$ and $k_{\mathrm{P} \rightarrow \mathrm{U}}$ ) strongly increase with temperature, which is indicative of an enthalpic barrier for unfolding.
A more quantitative analysis can be carried out by fitting the rate constants and equilibrium constants to Arrhenius and van't Hoff models, respectively, from which overall and activation enthalpies ( $\Delta H^{\circ}, \Delta H^{\dagger}$, from slopes) and entropies ( $\Delta S^{\circ}, \Delta S^{\ddagger}$, from intercepts) are determined. The thermodynamic values obtained from such three-state fits can, in turn, be used to construct 3D folding energy landscapes (see Figure 7), which depict the enthalpy $(\Delta H)$, entropy $(-T \Delta S)$, and free energy $(\Delta G)$ contributions for each of the G-quadruplex conformations and intervening transition states. The enthalpic landscape (Figure 7A) reveals that both folding processes (U $\rightarrow \mathrm{A}$ (red) and $\mathrm{U} \rightarrow \mathrm{P}$ (blue)) are enthalpically favorable $\left(\Delta H_{\mathrm{UA}}^{\circ}=-66 \pm 7 \mathrm{~kJ} / \mathrm{mol}, \Delta H_{\mathrm{UP}}^{\circ}=-84 \pm 9 \mathrm{~kJ} / \mathrm{mol}\right)$ with a negligible barrier $\left(\Delta H^{\ddagger}{ }_{\mathrm{UA}}=-4 \pm 6 \mathrm{~kJ} / \mathrm{mol}, \Delta H_{\mathrm{UP}}^{\ddagger}=-13 \pm 6\right.$ $\mathrm{kJ} / \mathrm{mol}$ ). Meanwhile, the entropy landscape (Figure 7B) indicates that folding is entropically costly ( $\Delta S_{\text {UA }}^{\circ}=-240 \pm$ $20 \mathrm{~J} / \mathrm{mol} \mathrm{K}, \Delta S_{\mathrm{UP}}^{\circ}=-300 \pm 30 \mathrm{~J} / \mathrm{mol} \mathrm{K}$ ), with an entropic penalty of $\approx 50 \%$ being achieved upon reaching the transition state $\left(\Delta S^{\ddagger}{ }_{\text {UA }}=-140 \pm 20 \mathrm{~J} / \mathrm{mol} \mathrm{K}, \Delta S_{\mathrm{UP}}^{\ddagger}=-170 \pm 20 \mathrm{~J} / \mathrm{mol}\right.$ K ). The overall free energy changes for folding (Figure 7C) are
relatively small in comparison to the individual contributions from the enthalpy $(\Delta H)$ and entropy $(-T \Delta S)$. Indeed, as an example of enthalpy-entropy compensation, these two contributions cancel nearly quantitatively to yield small overall free energy changes while still maintaining large free energy transition state barriers. ${ }^{60,61}$

Thermodynamics of G-Quadruplex Folding at Elevated Monovalent Cation Concentrations. To probe the effects of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$cations on folding thermodynamics, we have repeated these temperature-dependent studies of the Gquadruplex under multiple cation conditions. The temperature dependence for G-quadruplex folding under ambient (100 $\mathrm{mM}) \mathrm{Na}^{+}$, high $(200 \mathrm{mM}) \mathrm{Na}^{+}$, and $100 \mathrm{mM} \mathrm{Na}{ }^{+}$with 4 mM $\mathrm{K}^{+}$are shown in Figure 8, where the latter two data sets can be compared to the first in order to isolate the impact due to $\mathrm{Na}^{+}$ versus that due to $\mathrm{K}^{+}$, respectively. Arrhenius and van't Hoff analyses are performed on each of the data sets, with the fitted entropic and enthalpic parameters for the three cationic conditions $\left(100 \mathrm{mM} \mathrm{Na}^{+}, 200 \mathrm{mM} \mathrm{Na}\right.$, and $100 \mathrm{mM} \mathrm{Na}^{+}$ with $4 \mathrm{mM} \mathrm{K}{ }^{+}$) shown side by side in Table 2.

The dependence of the folding enthalpies and entropies on cation concentration can be used to determine the thermodynamic "signature" (i.e., the sign of $\Delta \Delta H^{\circ}$ and $\Delta \Delta S^{\circ}$ ) of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$binding. For example, we first consider the change in the folding enthalpy of the unfolded to the parallel/hybrid G4 transition upon an increase in $\left[\mathrm{Na}^{+}\right]$from 100 to 200 mM , defined as

$$
\begin{equation*}
\Delta \Delta H_{\mathrm{UP}}^{\circ}\left(\mathrm{Na}^{+}\right) \equiv \Delta H_{\mathrm{UP}}^{\circ}\left(200 \mathrm{mM} \mathrm{Na}{ }^{+}\right)-\Delta H_{\mathrm{UP}}^{\circ}\left(100 \mathrm{mMNa}{ }^{+}\right) \tag{16}
\end{equation*}
$$



Figure 7. Folding landscape for G4 in $100 \mathrm{mM} \mathrm{Na}^{+}$, as determined by van't Hoff and Arrhenius analyses. (A) The enthalpy $(\Delta H)$ of each stable conformation (black points) and transition state (light gray points), defined relative to the unfolded state, U. (B) The same as (A), but for the enthalpic contribution to the free energy $(-T \Delta S)$ evaluated at 300 K . (C) The same as (A), but for the free energy ( $\Delta G$ $=\Delta H-T \Delta S)$ evaluated at 300 K .

The results in Table 2 show that this quantity is positive $\left(\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{Na}^{+}\right)=40 \pm 11 \mathrm{~kJ} / \mathrm{mol}\right)$. Because $\mathrm{Na}^{+}$interacts with the G4 construct through a "bind-then-fold" mechanism, $\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{Na}^{+}\right)$is opposite in sign to the enthalpy of $\mathrm{Na}^{+}$ binding, $\Delta H_{\text {bind,Na, }}^{\circ}$ as depicted in Figure 9A. Therefore, since $\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{Na}^{+}\right)>0$, we can infer that $\mathrm{Na}^{+}$binding is enthalpically favorable ( $\Delta H_{\text {bind, } \mathrm{Na}}^{\circ}<0$ ). We also observe
that the $\mathrm{Na}^{+}$-induced change in the folding entropy for this process is positive $\left(\Delta \Delta S^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{Na}^{+}\right)=140 \pm 40 \mathrm{~J} / \mathrm{mol} \mathrm{K}\right)$. Thus, by a similar logic, $\mathrm{Na}^{+}$binding must result in a loss of entropy $\left(\Delta S^{\circ}{ }_{\text {bind, } \mathrm{Na}}<0\right)$. Together, $\Delta H_{\text {bind, } \mathrm{Na}}^{\circ}$ and $\Delta S_{\text {bind, } \mathrm{Na}}^{\circ}$ indicate that $\mathrm{Na}^{+}$binding is consistent with the formation of energetically stable contacts with the unfolded G4 at the entropic cost of restricting the translational freedom of $\mathrm{Na}^{+}$ and/or the conformational flexibility of the unfolded DNA.

Similarly, the thermodynamics of $\mathrm{K}^{+}$binding to the parallel/ hybrid G4 can be determined by examining the differential folding enthalpy $\left(\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{K}^{+}\right)\right)$and entropy $\left(\Delta \Delta S_{\mathrm{UP}}^{\circ}\left(\mathrm{K}^{+}\right)\right)$ upon the addition of $\mathrm{K}^{+}$, in this case defined as

$$
\begin{align*}
\Delta \Delta H_{\mathrm{UP}}^{\circ}\left(\mathrm{K}^{+}\right) \equiv & \Delta H_{\mathrm{UP}}^{\circ}\left(100 \mathrm{mM} \mathrm{Na}^{+}, 4 \mathrm{mM} \mathrm{~K}^{+}\right) \\
& -\Delta{H^{\mathrm{UP}}}_{\circ}\left(100 \mathrm{mM} \mathrm{Na}^{+}\right) \tag{17}
\end{align*}
$$

and

$$
\begin{align*}
\Delta \Delta S_{\mathrm{UP}}^{\circ}\left(\mathrm{K}^{+}\right) \equiv & \Delta S_{\mathrm{UP}}^{\circ}\left(100 \mathrm{mMNa}^{+}, 4 \mathrm{mM} \mathrm{~K}^{+}\right) \\
& -\Delta{S_{\mathrm{UP}}^{\circ}\left(100 \mathrm{mM} \mathrm{Na}^{+}\right)}_{\circ} \tag{18}
\end{align*}
$$

In contrast to interactions mediated by $\mathrm{Na}^{+}, \mathrm{K}^{+}$interacts with the G-quadruplex through a "fold-then-bind" mechanism and, as a result, $\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{K}^{+}\right)$and $\Delta \Delta S^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{K}^{+}\right)$have the same sign as $\Delta H^{\circ}{ }_{\text {bind, }, \mathrm{K}}$ and $\Delta S^{\circ}{ }_{\text {bind, } \mathrm{K}}$, respectively (Figure 9B). The observed $\Delta \Delta H^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{K}^{+}\right)$and $\Delta \Delta S^{\circ}{ }_{\mathrm{UP}}\left(\mathrm{K}^{+}\right)$are both positive, which indicates that $\mathrm{K}^{+}$binding is enthalpically unfavorable $\left(\Delta H_{\text {bind,K }}^{\circ}>0\right)$ and entropically favorable $\left(\Delta S_{\text {bind,K }}^{\circ}>0\right)$. Interestingly, the signs of these thermodynamic parameters are exactly opposite to those of $\mathrm{Na}^{+}$binding, a fact that serves to emphasize the disparate natures of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$binding to the G-quadruplex construct.

## IV. DISCUSSION

In this work, we have used the $5^{\prime}$-GGG-(TAAGGG) $3_{3}-3^{\prime}$ DNA G-quadruplex (TAA-G4) as a model system for understanding the thermodynamic influence of monovalent cations $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$on G-quadruplex folding. The TAA-G4 sequence is similar to the human telomere G-quadruplex repeat ( $5^{\prime}$-TTAGGG$3^{\prime}$ ), with enhanced polymorphism due to the $T \rightarrow$ A mutation in the loop sequence. ${ }^{34}$ The TAA-G4 is incorporated into a DNA construct designed for single-molecule FRET (smFRET) microscopy. The observed smFRET data reveal that the TAAG4 populates three interconverting FRET states (Figure 2), a finding that is in agreement with previous single-molecule measurements made by Tippana et al. on a similar TAA-G4 construct. ${ }^{34}$ From the smFRET trajectories, equilibrium constants and rate constants for transitions among the three states can be measured, with the dependence of these kinetic quantities obtained as a function of cation concentration and temperature.

We assigned structures to the three FRET states (low, middle, and high) as an unfolded state (U), an antiparallel G4 state (A), and a parallel or hybrid G4 state (P), respectively. Unfortunately, we are unable to further dissect the observed G4 folds by smFRET alone, such as distinguishing between chair versus basket antiparallel states or between parallel versus $3+1$ hybrid versus $1+3$ hybrid states. We note that the order of the A and P FRET efficiencies ( $E_{\text {antiparallel }}<E_{\text {parallel }}$ ) is the reverse of that observed by Tippana et al. for the same G4 sequence. ${ }^{34}$ However, the Tippana et al. smFRET construct incorporated the G4 fold that is directly adjacent to duplex DNA, whereas the G4 in our construct is connected to single-


Figure 8. Temperature response of G4 folding under multiple cationic conditions ( $100 \mathrm{mM} \mathrm{Na}^{+}$in black, $200 \mathrm{mM} \mathrm{Na}^{+}$in red, and $100 \mathrm{mM} \mathrm{Na}^{+}$ with $4 \mathrm{mM} \mathrm{K} \mathrm{K}^{+}$in blue) for the three G4 dynamic processes: (A) unfolded $\rightarrow$ antiparallel G 4 , (B) unfolded $\rightarrow$ parallel/hybrid G4, and (C) antiparallel G4 $\rightarrow$ parallel/hybrid G4. Arrhenius plots for the forward and reverse rate constants are shown in the left and middle panels, respectively, while van't Hoff plots for the equilibrium constants are shown in the right panels.

Table 2. Thermodynamic Parameters for G-Quadruplex Folding Determined by van't Hoff and Arrhenius Analyses of Temperature-Dependent Kinetic Measurements at Multiple Cation Concentrations (Figure 8) ${ }^{a}$

|  |  | $100 \mathrm{mM} \mathrm{Na}^{+}$ | $200 \mathrm{mM} \mathrm{Na}{ }^{+}$ | $100 \mathrm{mM} \mathrm{Na}^{+}, 4 \mathrm{mM} \mathrm{K}^{+}$ |
| :---: | :---: | :---: | :---: | :---: |
| $\Delta H(\mathrm{~kJ} / \mathrm{mol})$ |  |  |  |  |
| Unfolded $\rightarrow$ G4 (Antiparallel) | $\Delta H^{\circ}{ }_{\text {TA }}$ | $-66 \pm 7$ | $-16 \pm 9^{*}$ | $-12 \pm 14^{*}$ |
|  | $\Delta H^{\ddagger}{ }_{\text {TA }}$ | $-4 \pm 6$ | $29 \pm 6^{*}$ | $8 \pm 6$ |
|  | $\Delta H^{\dagger}{ }_{\text {AT }}$ | $62 \pm 2$ | $45 \pm 7^{*}$ | $20 \pm 13^{*}$ |
| Unfolded $\rightarrow$ G4 (Parallel/Hybrid) | $\Delta H^{\circ}{ }_{\text {TP }}$ | $-85 \pm 9$ | $-44 \pm 7^{*}$ | $-56 \pm 6^{*}$ |
|  | $\Delta H^{\ddagger}{ }_{\text {TP }}$ | $-13 \pm 6$ | $21 \pm 4^{*}$ | $18 \pm 4^{*}$ |
|  | $\Delta H^{\ddagger}{ }_{\mathrm{PT}}$ | $72 \pm 7$ | $66 \pm 6$ | $74 \pm 4$ |
| G4 (Antiparallel) $\rightarrow$ G4 (Parallel/Hybrid) | $\Delta H^{\circ}{ }_{\text {AP }}$ | $-34 \pm 40$ | $-40 \pm 25$ | $-29 \pm 24$ |
|  | $\Delta H^{\ddagger}{ }_{\text {AP }}$ | $9 \pm 23$ | $28 \pm 19$ | $-5 \pm 18$ |
|  | $\Delta H^{\ddagger}{ }_{\text {PA }}$ | $43 \pm 33$ | $68 \pm 15$ | $25 \pm 16$ |
| $\Delta S(\mathrm{~J} / \mathrm{mol} \mathrm{K})$ |  |  |  |  |
| Unfolded $\rightarrow$ G4 (Antiparallel) | $\Delta S^{\circ}{ }_{\text {TA }}$ | $-240 \pm 20$ | $-50 \pm 30^{*}$ | $-50 \pm 50^{*}$ |
|  | $\Delta S^{\ddagger}{ }_{\text {TA }}$ | $-140 \pm 20$ | $-30 \pm 20^{*}$ | $-100 \pm 20$ |
|  | $\Delta S^{\ddagger}{ }_{\text {AT }}$ | $90 \pm 10$ | $30 \pm 20^{*}$ | $-60 \pm 40^{*}$ |
| Unfolded $\rightarrow$ G4 (Parallel/Hybrid) | $\Delta S^{\circ}{ }_{\text {TP }}$ | $-300 \pm 30$ | $-150 \pm 20^{*}$ | $-190 \pm 20^{*}$ |
|  | $\Delta S^{\ddagger}{ }_{\text {TP }}$ | $-170 \pm 20$ | $-50 \pm 10^{*}$ | $-70 \pm 10^{*}$ |
|  | $\Delta S^{\ddagger}{ }_{\text {PT }}$ | $130 \pm 20$ | $100 \pm 20$ | $120 \pm 10$ |
| G 4 (Antiparallel) $\rightarrow$ G4 (Parallel/Hybrid) | $\Delta S^{\circ}{ }_{\text {AP }}$ | $-110 \pm 140$ | $-140 \pm 80$ | $-90 \pm 80$ |
|  | $\Delta S_{\mathrm{AP}}^{\ddagger}$ | $-100 \pm 80$ | $-40 \pm 70$ | $-160 \pm 60$ |
|  | $\Delta S^{\ddagger}{ }_{\text {PA }}{ }^{\text {a }}$ | $10 \pm 110$ | $100 \pm 50$ | $-60 \pm 50$ |

${ }^{a}$ Uncertainties are reported as standard errors of the mean. Asterisks indicate statistical significance (evaluated at the $p=0.01$ level) of the parameters when compared to the $100 \mathrm{mM} \mathrm{Na}{ }^{+}$case.


Figure 9. Determination of cation binding thermodynamics. (A) For $\mathrm{Na}^{+}$, which follows a "bind-then-fold" model, the measured enthalpy/ entropy $(\Delta H / S)$ at low $\left[\mathrm{Na}^{+}\right]$contains contributions from both $\mathrm{Na}^{+}$ binding $\left(\Delta H / S_{\text {bind }}\right)$ and the conformational change $\left(\Delta H / S_{\text {conf }}\right)$. However, at high $\left[\mathrm{Na}^{+}\right]$, the unfolded conformation saturates with $\mathrm{Na}^{+}$, and $\Delta H / S$ reflects only $\Delta H / S_{\text {conf. }}$ Therefore, $\Delta \Delta H / S=\Delta H /$ $S\left(\right.$ high $\left.\left[\mathrm{Na}^{+}\right]\right)-\Delta H / S\left(\right.$ low $\left.\left[\mathrm{Na}^{+}\right]\right)$has the opposite sign of $\Delta H /$ $S_{\text {bind }}$. (B) For $\mathrm{K}^{+}$, which operates by a "fold-then-bind" mechanism, the differential enthalpy/entropy $\Delta \Delta H / S=\Delta H / S\left(\right.$ high $\left.\left[\mathrm{K}^{+}\right]\right)$$\Delta H / S\left(\right.$ low $\left.\left[\mathrm{K}^{+}\right]\right)$has the same sign as $\Delta H / S_{\text {bind }}$.
stranded DNA. Indeed, our observed FRET orderings agree with that of Ray et al., who examined G4s flanked by singlestranded DNA. ${ }^{62}$ Clearly, the folding geometry of Gquadruplexes, and thus the ordering of the FRET states, can be quite sensitive to the local sequence environment.
Monovalent cations drive the TAA-G4 folding equilibrium from the unfolded state into the antiparallel and parallel/ hybrid G4 states in a cation-specific fashion. $\mathrm{K}^{+}$exclusively promotes the parallel/hybrid configuration (Figure 5A,B), while $\mathrm{Na}^{+}$permits both configurations with a small preference for the antiparallel topology (Figure 3). Furthermore, concentration-dependent kinetics (Figures 4C and 5C) reveal that $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$promote G-quadruplex folding through distinctly different kinetic mechanisms. For $\mathrm{Na}^{+}$, the cation binds to the unfolded state before the ligand-bound complex folds into one of the G-quadruplex configurations (Figure 4D, a "bind-then-fold" mechanism). In contrast, the TAA-G4 sequence must first fold into the parallel/hybrid G4 topology before $\mathrm{K}^{+}$will bind to form a stabilized complex (Figure 5E, a "fold-then-bind" mechanism). The fact that these two monovalent cations act on the TAA-G4 via such different mechanisms reinforces how responsive G-quadruplexes are to their environment or, in this case, cation size. We present a simplified, combined kinetic model of $\mathrm{Na}^{+}$- and $\mathrm{K}^{+}$-induced
folding of G-quadruplexes in Figure 10. In this model, $\mathrm{Na}^{+}$ binding is a prerequisite for unfolded to quadruplex transitions,


Figure 10. Proposed scheme of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$binding to TAA-G4.
after which the parallel/hybrid configuration of the G4 can subsequently bind to a $\mathrm{K}^{+}$ion, perhaps by replacing $\mathrm{Na}^{+}$(as shown in the figure) or by attaching to an alternative binding site. It is possible and even likely that the identified $\mathrm{Na}^{+}$ binding site has a similar affinity for other monovalent cations including $\mathrm{K}^{+}$, but the much higher affinity of the $\mathrm{K}^{+}$-specific binding site means that our $\mathrm{K}^{+}$titrations saturate before lower affinity binding to the triplex can be observed.

Titrations of $\mathrm{K}^{+}$and $\mathrm{Na}^{+}$indicate that the G4 construct binds to a single cation during folding (Figures 3 and 5). This observation is surprising in light of G4 crystal structures, which routinely indicate that G4s with three tetrads, as studied here, bind to two monovalent cations. ${ }^{16}$ One explanation for this disagreement is that the low FRET state is not fully unfolded, but rather a partially folded G4 intermediate that is already bound to one cation. If that is the case, the cation binding observed in this work is, in fact, the recruitment of the second cation that is required for G4 folding. Promising G4 intermediates to fill this role include a G-duplex, a G-triplex, or a two-tetrad structure, each of which has been previously shown to bind to a single cation. ${ }^{14,28}$ However, the existence of such structures as metastable G4 intermediates is disputed, ${ }^{63}$ and more work is required to elucidate this issue. Nevertheless, the observed cation binding stoichiometry in this work provides further evidence of a folding intermediate with a single bound cation, though such a structure must be noncompact in order to have a FRET efficiency that is indistinguishable from a fully unfolded structure (Figure 2D).

To explore the influence of $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$on the thermodynamics of TAA-G4 folding, temperature-dependent experiments have been pursued to obtain the folding enthalpies and entropies as a function of cation concentration. By considering the changes in the thermodynamic variables upon an increase in $\left[\mathrm{Na}^{+}\right]$or $\left[\mathrm{K}^{+}\right]$, we can infer the thermodynamic impact of cation binding. Interestingly, the folding thermodynamics ( $\Delta H^{\circ}$ and $\Delta S^{\circ}$ ) of the TAA-G4 construct respond similarly to the addition of either 100 mM $\mathrm{Na}^{+}$or $4 \mathrm{mM} \mathrm{K}{ }^{+}$, as summarized in Table 2. However, because $\mathrm{Na}^{+}$and $\mathrm{K}^{+}$interact with the TAA-G4 through different mechanisms ("bind-then-fold" versus "fold-then-bind"), we can conclude that the signs of the enthalpy and entropy changes for $\mathrm{Na}^{+}$binding are opposite to those of $\mathrm{K}^{+}$binding (Figure 9). Specifically, $\mathrm{Na}^{+}$binding to the U conformation is driven by enthalpically favorable interactions $\left(\Delta H_{\text {bind, } \mathrm{Na}}^{\circ}<0\right)$ that outweigh a loss of entropy $\left(\Delta S^{\circ}{ }_{\text {bind }, \mathrm{Na}}<0\right)$, perhaps due to a conformational restriction upon ion binding. In contrast, $\mathrm{K}^{+}$
binds to the parallel/hybrid G4 topology in an entropically driven process $\left(\Delta S_{\text {bind, } \mathrm{K}}^{\circ}>0\right)$ that occurs at an enthalpic cost $\left(\Delta H^{\circ}{ }_{\text {bind, } K}>0\right)$. One explanation of this entropically driven binding is that $\mathrm{K}^{+}$replaces a loosely fitting $\mathrm{Na}^{+}$in the binding pocket in order to form a more compact G-quadruplex, resulting in the expulsion of surface-bound water molecules. Such a release of water molecules would be entropically favorable, yet energetically costly, due to the breaking of hydrogen bonds between the water molecules and the nucleic acid. This hypothesis of G-quadruplex compaction and water release upon $\mathrm{K}^{+}$binding is generally supported in the literature with studies of the ion-dependent folding volumes for the Gquadruplex, ${ }^{64,65}$ though one study reports that $\mathrm{K}^{+}$produces larger G4 volumes than $\mathrm{Na}^{+} .{ }^{23}$ Clearly, further study is needed to elucidate this feature of G4 folding, such as with pressuredependent single-molecule folding experiments. ${ }^{66}$

## V. CONCLUSIONS

In this work, we explored the influence of monovalent cations on G-quadruplex folding through single-molecule temperaturecontrolled kinetic measurements. With the novel use of smFRET to measure the temperature-dependent folding kinetics on a system with more than two FRET conformations, we observe three-state G-quadruplex folding ascribed to conformational transitions between an unfolded state and two G-quadruplex topologies. Concentration-dependent measurements show that the $\mathrm{Na}^{+}$binds to the unfolded state and remains bound while G-quadruplex topologies are sampled. In contrast, $\mathrm{K}^{+}$binds exclusively to the parallel/hybrid Gquadruplex conformation and has a binding affinity approximately 100 times larger than that of $\mathrm{Na}^{+}$. The thermodynamics of $\mathrm{Na}^{+}$binding are enthalpically driven ( $\Delta H^{\circ}{ }_{\text {bind, } \mathrm{Na}}<0$, $\Delta S^{\circ}{ }_{\text {bind, } \mathrm{Na}}<0$ ), while the thermodynamics of $\mathrm{K}^{+}$binding are entropically driven ( $\Delta H^{\circ}{ }_{\text {bind, } \mathrm{K}}>0, \Delta S^{\circ}{ }_{\text {bind, } \mathrm{K}}>0$ ), likely due to G4 compaction and concomitant water release.

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## Notes

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## REFERENCES

(1) Dolinnaya, N. G.; Ogloblina, A. M.; Yakubovskaya, M. G. Structure, properties, and biological relevance of the DNA and RNA G-quadruplexes: Overview 50 years after their discovery. Biochemistry (Moscow) 2016, 81, 1602-1649.
(2) Rhodes, D.; Lipps, H. J. G-quadruplexes and their regulatory roles in biology. Nucleic Acids Res. 2015, 43, 8627-8637.
(3) Varshney, D.; Spiegel, J.; Zyner, K.; Tannahill, D.; Balasubramanian, $S$. The regulation and functions of DNA and RNA G-quadruplexes. Nat. Rev. Mol. Cell Biol. 2020, 21, 459-474.
(4) Zhao, C.; Qin, G.; Niu, J.; Wang, Z.; Wang, C.; Ren, J.; Qu, X. Targeting RNA G-quadruplex in SARS-CoV-2: A promising therapeutic target for COVID-19? Angew. Chem., Int. Ed. 2021, 60, 432-438.
(5) Neidle, S. Quadruplex nucleic acids as novel therapeutic targets. J. Med. Chem. 2016, 59, 5987-6011.
(6) Banerjee, N.; Panda, S.; Chatterjee, S. Frontiers in G-Quadruplex therapeutics in cancer: Selection of small molecules, peptides and aptamers. Chem. Biol. Drug Des. 2022, 99, 1-31.
(7) Yatsunyk, L. A.; Mendoza, O.; Mergny, J.-L. Nano-oddities": Unusual nucleic acid assemblies for DNA-based nanostructures and nanodevices. Acc. Chem. Res. 2014, 47, 1836-1844.
(8) Rajendran, A.; Endo, M.; Hidaka, K.; Lan Thao Tran, P.; Mergny, J.-L.; Sugiyama, H. Controlling the stoichiometry and strand polarity of a tetramolecular G-quadruplex structure by using a DNA origami frame. Nucleic Acids Res. 2013, 41, 8738-8747.
(9) Harkness, R. W.; Mittermaier, A. K. G-quadruplex dynamics. Biochim. Biophys. Acta, Proteins Proteomics 2017, 1865, 1544-1554.
(10) Burge, S.; Parkinson, G. N.; Hazel, P.; Todd, A. K.; Neidle, S. Quadruplex DNA: sequence, topology and structure. Nucleic Acids Res. 2006, 34, 5402-5415.
(11) Ma, Y.; Iida, K.; Nagasawa, K. Topologies of G-quadruplex: Biological functions and regulation by ligands. Biochem. Biophys. Res. Соттии. 2020, 531, 3-17.
(12) Grün, J. T.; Schwalbe, H. Folding dynamics of polymorphic Gquadruplex structures. Biopolymers 2022, 113, No. e23477.
(13) Nguyen, T. Q. N.; Lim, K. W.; Phan, A. T. Folding kinetics of G-quadruplexes: Duplex stem loops drive and accelerate Gquadruplex folding. J. Phys. Chem. B 2020, 124, 5122-5130.
(14) Cerofolini, L.; Amato, J.; Giachetti, A.; Limongelli, V.; Novellino, E.; Parrinello, M.; Fragai, M.; Randazzo, A.; Luchinat, C. G-triplex structure and formation propensity. Nucleic Acids Res. 2014, 42, 13393-404.
(15) Hou, X.-M.; Fu, Y.-B.; Wu, W.-Q.; Wang, L.; Teng, F.-Y.; Xie, P.; Wang, P.-Y.; Xi, X.-G. Involvement of G-triplex and G-hairpin in the multi-pathway folding of human telomeric G-quadruplex. Nucleic Acids Res. 2017, 45, 11401-11412.
(16) Bhattacharyya, D.; Mirihana Arachchilage, G.; Basu, S. Metal cations in G-quadruplex folding and stability. Front. Chem. 2016, 4, 38.
(17) Largy, E.; Mergny, J.-L.; Gabelica, V. Role of alkali metal ions in G-quadruplex nucleic acid structure and stability. In The Alkali Metal Ions: Their Role for Life; Sigel, A., Sigel, H., Sigel, R. K. O., Eds.; Springer International Publishing, Cham, 2016, p 203-258.
(18) Nishio, M.; Tsukakoshi, K.; Ikebukuro, K. G-quadruplex: Flexible conformational changes by cations, pH , crowding and its applications to biosensing. Biosens. Bioelectron. 2021, 178, 113030.
(19) Parkinson, G. N.; Lee, M. P. H.; Neidle, S. Crystal structure of parallel quadruplexes from human telomeric DNA. Nature 2002, 417, 876-880.
(20) Luu, K. N.; Phan, A. T.; Kuryavyi, V.; Lacroix, L.; Patel, D. J. Structure of the human telomere in K+ solution: An intramolecular (3

+ 1) G-quadruplex scaffold. J. Am. Chem. Soc. 2006, 128, 9963-9970.
(21) Lane, A. N.; Chaires, J. B.; Gray, R. D.; Trent, J. O. Stability and kinetics of G-quadruplex structures. Nucleic Acids Res. 2008, 36, 5482-5515.
(22) Chalikian, T. V.; Liu, L.; Macgregor, R. B. Jr. Duplex-tetraplex equilibria in guanine- and cytosine-rich DNA. Biophys. Chem. 2020, 267, 106473.
(23) Li, Y. Y.; Dubins, D. N.; Le, D. M. N. T.; Leung, K.; Macgregor, R. B. The role of loops and cation on the volume of unfolding of Gquadruplexes related to HTel. Biophys. Chem. 2017, 231, 55-63.
(24) Majhi, P. R.; Qi, J.; Tang, C.-F.; Shafer, R. H. Heat capacity changes associated with guanine quadruplex formation: An isothermal titration calorimetry study. Biopolymers 2008, 89, 302-309.
(25) Green, J. J.; Ying, L.; Klenerman, D.; Balasubramanian, S. Kinetics of unfolding the human telomeric DNA quadruplex using a PNA trap. J. Am. Chem. Soc. 2003, 125, 3763-7.
(26) Laouer, K.; Schmid, M.; Wien, F.; Changenet, P.; Hache, F. Folding dynamics of DNA G-quadruplexes probed by millisecond temperature jump circular dichroism. J. Phys. Chem. B 2021, 125, 8088-8098.
(27) Hatzakis, E.; Okamoto, K.; Yang, D. Thermodynamic stability and folding kinetics of the major G-quadruplex and its loop isomers formed in the nuclease hypersensitive element in the human c-Myc promoter: Effect of loops and flanking segments on the stability of parallel-stranded intramolecular G-quadruplexes. Biochemistry 2010, 49, 9152-9160.
(28) Largy, E.; Marchand, A.; Amrane, S.; Gabelica, V.; Mergny, J.-L. Quadruplex turncoats: Cation-dependent folding and stability of quadruplex-DNA double switches. J. Am. Chem. Soc. 2016, 138, 2780-2792.
(29) Sustarsic, M.; Kapanidis, A. N. Taking the ruler to the jungle: Single-molecule FRET for understanding biomolecular structure and dynamics in live cells. Curr. Opin. Struct. Biol. 2015, 34, 52-59.
(30) Maleki, P.; Budhathoki, J. B.; Roy, W. A.; Balci, H. A practical guide to studying G-quadruplex structures using single-molecule FRET. Molecular genetics and genomics: MGG 2017, 292, 483-498.
(31) Budhathoki, J. B.; Maleki, P.; Roy, W. A.; Janscak, P.; Yodh, J. G.; Balci, H. A comparative study of G-quadruplex unfolding and DNA reeling activities of human RECQ5 helicase. Biophys. J. 2016, 110, 2585-2596.
(32) Aznauryan, M.; Sondergaard, S.; Noer, S. L.; Schiott, B.; Birkedal, V. A direct view of the complex multi-pathway folding of telomeric G-quadruplexes. Nucleic Acids Res. 2016, 44, 11024-11032. (33) Noer, S. L.; Preus, S.; Gudnason, D.; Aznauryan, M.; Mergny, J. L.; Birkedal, V. Folding dynamics and conformational heterogeneity of human telomeric G-quadruplex structures in $\mathrm{Na}+$ solutions by single molecule FRET microscopy. Nucleic Acids Res. 2016, 44, 46471.
(34) Tippana, R.; Xiao, W.; Myong, S. G-quadruplex conformation and dynamics are determined by loop length and sequence. Nucleic Acids Res. 2014, 42, 8106-8114.
(35) Jansson, L. I.; Hentschel, J.; Parks, J. W.; Chang, T. R.; Lu, C.; Baral, R.; Bagshaw, C. R.; Stone, M. D. Telomere DNA G-quadruplex folding within actively extending human telomerase. Proc. Natl. Acad. Sci. U. S. A. 2019, 116, 9350-9359.
(36) Arns, L.; Knop, J.-M.; Patra, S.; Anders, C.; Winter, R. Singlemolecule insights into the temperature and pressure dependent conformational dynamics of nucleic acids in the presence of crowders and osmolytes. Biophys. Chem. 2019, 251, 106190.
(37) Lerner, E.; Cordes, T.; Ingargiola, A.; Alhadid, Y.; Chung, S.; Michalet, X.; Weiss, S. Toward dynamic structural biology: Two decades of single-molecule Forster resonance energy transfer. Science 2018, 359, 288.
(38) Smith, C. L.; Milea, J. S.; Nguyen, G. H. Immobilization of nucleic acids using biotin-strept(avidin) systems. In Immobilisation of DNA on Chips II; Wittmann, C., Ed.; Springer, Berlin, 2005, p 63-90.
(39) Nicholson, D. A.; Sengupta, A.; Sung, H.-L.; Nesbitt, D. J. Amino acid stabilization of nucleic acid secondary structure: Kinetic insights from single-molecule studies. J. Phys. Chem. B 2018, 122, 9869-9876.
(40) Nicholson, D. A.; Sengupta, A.; Nesbitt, D. J. Chiralitydependent amino acid modulation of RNA folding. J. Phys. Chem. B 2020, 124, 11561-11572.
(41) Aitken, C. E.; Marshall, R. A.; Puglisi, J. D. An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. Biophys. J. 2008, 94, 1826-35.
(42) Ha, T.; Tinnefeld, P. Photophysics of fluorescent probes for single-molecule biophysics and super-resolution imaging. Annu. Rev. Phys. Chem. 2012, 63, 595-617.
(43) Axelrod, D. Total internal reflection fluorescence microscopy. In Methods in Cell Biology, 1st ed.; Elsevier Inc., 2008, Vol. 89, p 169221.
(44) Roy, R.; Hohng, S.; Ha, T. A practical guide to single-molecule FRET. Nat. Methods 2008, 5, 507-16.
(45) Chodera, J. D.; Noe, F. Markov state models of biomolecular conformational dynamics. Curr. Opin. Struct. Biol. 2014, 25, 135-144.
(46) Hanson, S. M.; Ekins, S.; Chodera, J. D. Modeling error in experimental assays using the bootstrap principle: Understanding discrepancies between assays using different dispensing technologies. J. Comput. Aided Mol. Des 2015, 29, 1073-1086.
(47) Leipply, D.; Draper, D. E. Dependence of RNA tertiary structural stability on $\mathrm{Mg}^{2+}$ concentration: Interpretation of the Hill equation and coefficient. Biochemistry 2010, 49, 1843-1853.
(48) Mikulecky, P. J.; Feig, A. L. Heat capacity changes associated with nucleic acid folding. Biopolymers 2006, 82, 38-58.
(49) Holmstrom, E. D.; Nesbitt, D. J. Biophysical insights from temperature-dependent single-molecule Forster resonance energy transfer. Annu. Rev. Phys. Chem. 2016, 67, 441-65.
(50) Hori, N.; Denesyuk, N. A.; Thirumalai, D. Frictional effects on RNA folding: Speed limit and Kramers turnover. J. Phys. Chem. B 2018, 122, 11279-11288.
(51) Truex, K.; Chung, H. S.; Louis, J. M.; Eaton, W. A. Testing Landscape Theory for Biomolecular Processes with Single Molecule Fluorescence Spectroscopy. Phys. Rev. Lett. 2015, 115, 018101.
(52) Dupuis, N. F.; Holmstrom, E. D.; Nesbitt, D. J. Tests of Kramers' Theory at the Single-Molecule Level: Evidence for Folding of an Isolated RNA Tertiary Interaction at the Viscous Speed Limit. J. Phys. Chem. B 2018, 122, 8796-8804.
(53) McKinney, S. A.; Joo, C.; Ha, T. Analysis of single-molecule FRET trajectories using hidden Markov modeling. Biophys. J. 2006, 91, 1941-51.
(54) Lee, T. H. Extracting kinetics information from single-molecule fluorescence resonance energy transfer data using hidden Markov models. J. Phys. Chem. B 2009, 113, 11535-42.
(55) Deng, H.; Braunlin, W. H. Kinetics of sodium ion binding to DNA quadruplexes. J. Mol. Biol. 1996, 255, 476-483.
(56) Leulliot, N.; Varani, G. Current topics in RNA-protein recognition: Control of specificity and biological function through induced fit and conformational capture. Biochemistry 2001, 40, 79477956.
(57) Boehr, D. D.; Nussinov, R.; Wright, P. E. The role of dynamic conformational ensembles in biomolecular recognition. Nat. Chem. Biol. 2009, 5, 789-796.
(58) Du, X.; Li, Y.; Xia, Y. L.; Ai, S. M.; Liang, J.; Sang, P.; Ji, X. L.; Liu, S. Q. Insights into protein-ligand interactions: Mechanisms, models, and methods. Int. J. Mol. Sci. 2016, 17, 144.
(59) Nanjunda, R.; Owens, E. A.; Mickelson, L.; Alyabyev, S.; Kilpatrick, N.; Wang, S.; Henary, M.; Wilson, W. D. Halogenated pentamethine cyanine dyes exhibiting high fidelity for G-quadruplex DNA. Bioorg. Med. Chem. 2012, 20, 7002-11.
(60) Dunitz, J. D. Win some, lose some: Enthalpy-entropy compensation in weak intermolecular interactions. Chem. Biol. 1995, 2, 709-712.
(61) Fox, J. M.; Zhao, M.; Fink, M. J.; Kang, K.; Whitesides, G. M. The molecular origin of enthalpy/entropy compensation in biomolecular recognition. Annu. Rev. Biophys. 2018, 47, 223-250.
(62) Ray, S.; Bandaria, J. N.; Qureshi, M. H.; Yildiz, A.; Balci, H. Gquadruplex formation in telomeres enhances POT1/TPP1 protection against RPA binding. Proc. Natl. Acad. Sci. U. S. A. 2014, 111, 2990-5.
(63) Sponer, J.; Islam, B.; Stadlbauer, P.; Haider, S. Chapter Seven Molecular dynamics simulations of G-quadruplexes: The basic principles and their application to folding and ligand binding. In Annual Reports in Medicinal Chemistry; Neidle, S., Ed.; Academic Press, 2020, Vol. 54, p 197-241.
(64) Hellman, L. M.; Rodgers, D. W.; Fried, M. G. Phenomenological partial-specific volumes for G-quadruplex DNAs. Eur. Biophys. J. 2010, 39, 389-396.
(65) Knop, J.-M.; Patra, S.; Harish, B.; Royer, C. A.; Winter, R. The deep sea osmolyte trimethylamine N -oxide and macromolecular crowders rescue the antiparallel conformation of the human telomeric G-quadruplex from urea and pressure stress. Chem. Eur. J. 2018, 24, 14346-14351.
(66) Sung, H. L.; Nesbitt, D. J. Single-molecule kinetic studies of DNA hybridization under extreme pressures. Phys. Chem. Chem. Phys. 2020, 22, 23491-23501.


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