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Toward a Digital Gene Response: RNA G-Quadruplexes with Fewer Quartets Fold with Higher Cooperativity

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Supporting Information

ABSTRACT: Changes in RNA conformation can alter gene expression. The guanine quadruplex sequence (GQS) is an RNA motif that folds in the presence of K^+ ions. Changes in the conformation of this motif could be especially important in regulating gene expression in plants because intracellular K^+ concentrations often increase during drought stress. Little is known about the folding thermodynamics of RNA GQS. We show here that RNA GQS with tracts containing three G's [e.g., $(GGGxx)_4$] have a modest dependence on the K^+ concentration, folding with no or even negative cooperativity (Hill coefficients ≤ 1), and are associated with populated folding intermediates. In contrast, GQS with tracts containing just two G's [e.g., $(GGxx)_4$] have a steep dependence on the K^+ concentration and fold with positive cooperativity (Hill coefficients of 1.7−2.7) without significantly populating intermediate states. We postulate that in plants, the more stable G3 sequences are largely folded even under unstressed conditions, while the less stable G2 sequences fold only at the higher K^+ concentrations associated with cellular stress, wherein they respond sharply to changing K^+ concentrations. Given the binary nature of their folding, G2 sequences may find application in computation with DNA and in engineering of genetic circuits.

A significant number of G-quadruplex sequences (GQS) are found in the nontelomeric regions of the genomes of many organisms. Recent studies have explored these sequences in humans,[1] chimps and mice,[2,3] prokaryotes including *Escherichia coli*[3,4] and *Saccharomyces cerevisiae*,[5] and the model plant species *Arabidopsis thaliana*.[6] The potential biological functions of GQS in humans include regulation of transcription by DNA GQS in promoter regions[7] and of translation by RNA GQS in 5′UTRs.[8,9] Plants are of particular interest for study of GQS, as intracellular K^+ concentrations can increase from ∼100 mM to as high as 600 mM under drought stress conditions.[10,11] Thermodynamic properties of GQS under such conditions, including ion binding and folding cooperativity, have not been elucidated.

Thermodynamics of folding in K^+ and Na^+ have been investigated for DNA GQS,[12−15] and a smaller amount of research on RNA GQS has been conducted. Studies have revealed that RNA GQS with short loops are more stable than equivalent DNA sequences. On the other hand, RNA GQS with long loops are less stable than their DNA counterparts, probably because of the parallel topology of RNA GQS.[16] Additionally, RNA GQS with three-G tracts, $(GGGxx)_4$ (herein termed "G3"), are more stable than those with two-G tracts, $(GGxx)_4$ (or "G2"), with equivalent loop ("xx") sequences.[6,9,16] While DNA GQS can fold into at least six possible topologies, encompassing antiparallel and parallel structures, RNA GQS almost always fold into a propeller-type parallel topology in which all of the bases adopt the *anti* conformation; this avoids the more restrictive antiparallel structures that require *syn* base conformations.[15,16] Indeed, circular dichroism (CD), NMR spectroscopy, and X-ray crystallography all reveal that model RNA oligonucleotides and long telomeric RNA prefer the parallel conformation.[9,16,17]

RNA and DNA GQS are stabilized by K^+ and Na^+. Dehydrated K^+ ions bind between quartets in a quadruplex structure, adopting nearly octahedral coordination, while dehydrated Na^+ ions bind within the plane of the quartet.[13,18] Thus, a G3 GQS can bind two K^+ or three Na^+, while a G2 GQS can bind only one K^+ or two Na^+, with additional ion interactions possible with the loop and backbone regions.[14,19]

Despite the above advances, there has been no systematic investigation of the cooperativity of GQS folding with respect to ion concentration. Folding cooperativity is important both chemically and biologically because it has the potential to influence ion response and thus gene regulation. In this work, we investigated RNA G-quadruplex folding as a function of G-tract length for various loop sequences using CD spectroscopy, nondenaturing polyacrylamide gel electrophoresis (native PAGE), and RNase T1 protection assays.

We began with a series of RNA GQS with related loop sequences and studied these by K^+ titrations using CD detection (Figure 1). These particular RNA oligonucleotides were chosen in part on the basis of RNAs encoded by *Arabidopsis* genes,[6,20] although some of these sequences can also be found in human and mouse (data not shown). GQS in which all of the loops have four nucleotides are termed "L444" and those with loops having nonuniform lengths as "L*xyz*" (e.g., "L221" for loops containing two, two, and one nucleotide). Explicit sequences are provided in Table S1 in the Supporting Information (SI). Data were fit to a two-state equation for the increase in ellipticity due to quartet formation as a function of K^+ concentration (eq S1 in the SI) to obtain $K^+_{1/2}$ and Hill coefficient (n) values. In general, smaller $K^+_{1/2}$ and larger n correspond to a more stable GQS, as shown by eq 1:[21]

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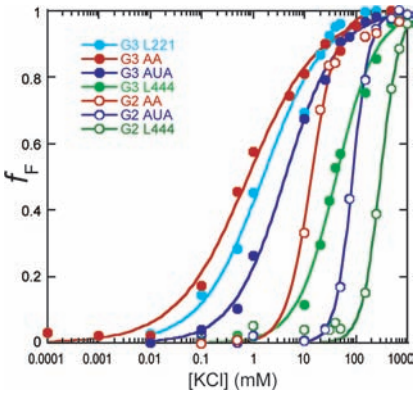

Figure 1. Fraction-folded plots for representative G2 (open symbols) and G3 (closed symbols) GQS from CD titrations. The data were fit to a two-state Hill equation (eq S1). $K^+_{1/2}$ and n values are provided in Table 1. See Figures S1 and S2 for CD spectra and additional fits.

$$\Delta G^\circ_{obs} = -nRT \ln([K^+]/[K^+_{1/2}]) \quad (1)$$

This equation corresponds to an *observed* free energy in K^+ that couples folding and ion-binding free energies.[22] For the RNA GQS tested herein, the $K^+_{1/2}$ values ranged from micromolar to high-millimolar, corresponding to a wide range of GQS stabilities (Table 1). In general, given identical loop sequences,

Table 1. GQS Folding Parameters[a]

GQS motif	$K^+_{1/2}$ (mM)[b]	n[c]	ΔG°_{obs} (kcal/mol)[d]
G3 A (tr1)[e]	0.003 ± 0.0007	1.0 ± 0.1	−6.2 ± 0.9
G3 A (tr2)[e]	25 ± 2	1.5 ± 0.2	−1.2 ± 0.1
G3 L221	1.4 ± 0.7	0.69 ± 0.03	−1.7 ± 0.4
G3 AA	0.7 ± 0.1	0.59 ± 0.08	−1.7 ± 0.2
G3 AUA	4.0 ± 0.8	0.85 ± 0.04	−1.6 ± 0.1
G3 L444	44 ± 3	1.1 ± 0.1	−0.5 ± 0.1
G2 A	0.8 ± 0.2	1.7 ± 0.4	−4.8 ± 2.5
G2 AA	14 ± 1	2.5 ± 0.4	−2.9 ± 0.4
G2 AUA	117 ± 28	2.7 ± 0.1	+0.25 ± 0.4
G2 L444	315 ± 3	2.2 ± 0.4	+1.5 ± 0.3

[a]GQS folding parameters were determined from CD titrations and fits of molar ellipticity data to eq S1, performed in triplicate. Values reported are averages ± standard deviations for the three titrations. [b]$K^+_{1/2}$ is the K^+ concentration required to fold half the RNA. [c]n is the Hill coefficient. [d]ΔG°_{obs} is the free energy of the folding transition at 20 °C, calculated at 100 mM K^+ using eq 1. [e]"tr$n$" identifies the folding transition when two or more transitions occurred.

G3 GQS have 7−30-fold lower $K^+_{1/2}$ values than the corresponding G2 GQS (Figure 1 and Table 1).

The dependence of the folding steepness on the K^+ concentration also differed with the motif. G3 GQS had n values ≤ ~1, while G2 GQS had n values of 1.7−2.7 (Table 1). For instance, G3L221, G3AA, and G3AUA folded with n = 0.69 ± 0.03, 0.59 ± 0.08, and 0.85 ± 0.04, respectively, and G3L444 had n = 1.1 ± 0.1. In contrast, G2A, G2AA, G2AUA, and G2L444, had significantly higher n values of 1.7 ± 0.4, 2.5 ± 0.4, 2.7 ± 0.1, and 2.2 ± 0.4, respectively. A larger Hill coefficient indicates a steeper dependence of the folding on the K^+ concentration (Figure 1), signifying increased folding cooperativity. Lower Hill coefficients, on the other hand, reflect no or negative folding cooperativity, possibly due to the presence of populated intermediates (see below).[23] Also, for some sequences, two transitions were clearly visible. For example, two separate folding transitions occurred for G3A (Figure S3 in the SI), with $K^+_{1/2}$ values of 3 μM and 25 mM (Table 1); clear identification of a folding intermediate in this instance supports the interpretation that the lower n values found for G3 sequences might be due to population of intermediates.

Low folding cooperativity is often associated with the presence of populated intermediates.[23] Herschlag and colleagues recently showed that biological macromolecule heterogeneity, which could include folding intermediates, decreases the bulk cooperativity parameter in comparison with single-molecule studies.[24] To investigate the Hill coefficient trend further, we used native PAGE to visualize intermediate structures. We evaluated RNA GQS on native gels with K^+ concentrations ranging from 0 to 100 mM. For G3 sequences, native gel traces showed a large number of bands per lane between the unfolded (no K^+) and folded (100 mM K^+) conditions (Figure 2A). For example, at 100 mM K^+,

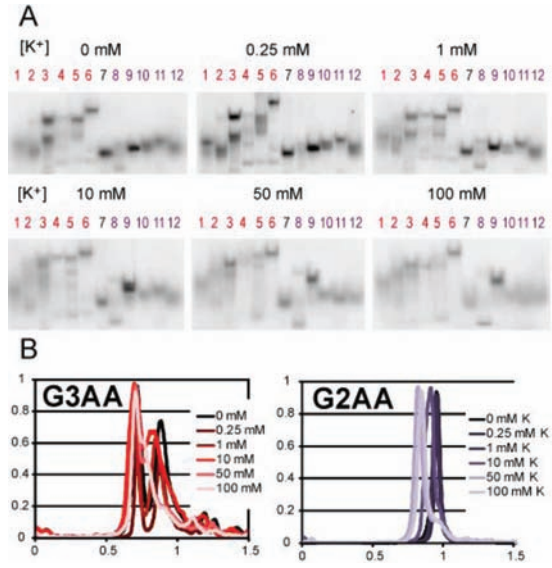

Figure 2. Native gels of RNA GQS oligonucleotides. (A) Native gel images at the indicated K^+ concentrations for G3 (red) and G2 GQS (purple). Lanes: (1) G3A, (2) G3L221, (3) G3AA, (4) G3AUA, (5) G3L444, (6) G3L444flank, (7) G2Amut, (8) G2A, (9) G2AA, (10) G2UA, (11) G2AUA, (12) G2L444. Full gel images are shown in Figure S5. (B) G3AA and G2AA native gel mobility traces for different K^+ concentrations (provided in legend) in gels and running buffer, normalized to the mobility of G2Amut, which does not form a GQS. Native gel mobility traces for the other GQS in (A) are shown in Figure S4.

G3AA traces (Figure 2A, lane 3) showed one main band, corresponding to a fully or mostly folded structure (Figure 2B, left). However, at low and intermediate K^+ concentrations of 0, 1, and 10 mM, intermediate bands migrated between the two main bands, most likely corresponding to intermediate structures (Figure 2A, B). In contrast, most of the G2 sequences exhibited only one band, with a smaller K^+-dependent spread in mobility relative to the G3 GQS (Figure 2A). For example, the G2AA traces (lanes 9) had sharp, resolved bands that decreased in mobility with K^+ concentration (Figure 2B, right). These differences as compared with G3 native gel behavior likely reflect a lack of intermediate structures in the folding of G2's.[25] Native gel and melt analyses

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showed no evidence of multimerization under the renaturation conditions and concentrations used (Figures S5, S6, and S9).

We conducted additional tests for folding intermediates. Structures of the folding intermediates were probed on a nucleotide level using RNase T1 protection assays (Figure 3).

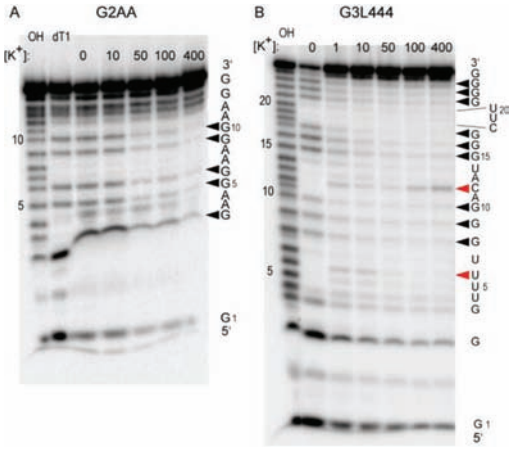

Figure 3. RNase T1 protection assays of (A) G2AA and (B) G3L444. Black arrows indicate protected nucleotides while red arrows show decreased protection for intermediate or final states as a function of K^+ concentration (mM). Quantification of these data is provided in Figure S7.

With G2AA, no protection was observed at low concentrations of K^+, with G's involved in the GQS structure protected only at higher (≥50 mM) K^+ concentrations (Figure 3a). We plotted normalized counts versus K^+ concentration for the RNase T1 assays and estimated a $K^+_{1/2}$ value of ~20 mM for G2AA (Figure S7A), in good agreement with the $K^+_{1/2}$ value of 14 mM found in the CD titrations (Table 1). Moreover, all of the G's showed an identical dependence on the K^+ concentration for protection, supportive of cooperative folding. For G3L444, most of the G residues (e.g., G15−17) were already largely protected at low (1 mM) K^+ concentrations (Figure 3 and Figure S7B), but two loop residues showed variable protection with increasing K^+ concentration: U6 was exposed at 1 and 10 mM K^+ and protected at 50 mM K^+, while C12 was slightly exposed at 1 mM and highly exposed above 50 mM K^+ (Figure S7B,C).[26] The $K^+_{1/2}$ values for C12 and U6 agree with that from the CD trace (44 mM) within a factor of 2 (Figure S7C). In sum, the K^+-dependent protection of G's seen in the T1 assays supports the presence of intermediate structures along the GQS folding pathway for G3 but not for G2.

A simple model was developed to illustrate possible folding schemes for G2 and G3 GQS. On the basis of native gels and T1 protection assays, G3 GQS are proposed to populate intermediate structures. In one scenario, a K^+ ion can bind between the upper two or lower two quartets (intermediates in Figure 4A). A given GQS could be largely trapped in such an intermediate structure, with binding of the second K^+ ion hindered by electrostatic repulsion with the first. Similar ion-repulsive behavior leads to partial K^+ occupancy of K^+ ion channels.[27] At higher K^+ concentrations, a second ion could bind, and the GQS would fully form, leading to protection of all G's from RNase T1 cleavage and to the second transition. A related possibility is a two-quartet intermediate having a given G-tract slipped out of register (not shown). Folding intermediates are not consistent with G2 GQS folding (Figure 4B).

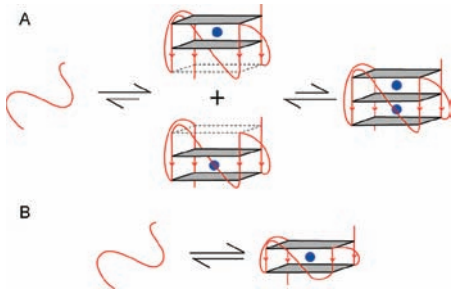

Figure 4. Models for RNA GQS folding for (A) G3 and (B) G2 GQS. Circles represent K^+ ions. Boxes indicate quartets.

To test the stability of these potential intermediates, we made a series of mutants in the second tract of G's in G3AA and G2AA, changing single G's to A's. These sequences were tested for formation and stability by assaying for inverse melting at 295 nm, a hallmark of GQS.[28] G3 sequences were less sensitive to the G-to-A mutation than G2 sequences (Table S2 and Figure S8). In particular, all three G3AA mutants retained a clear inverse melt at 295 nm with strong hyperchromicity, especially the AGG and GGA mutants, which melted at ~55 °C. In contrast, neither of the G2AA mutants melted above ~10 °C, if at all. The greater sensitivity of G2 sequences to mutation supports greater folding cooperativity, in line with the Hill constants, native gel mobility, and RNase T1 results and consistent with the absence of populated intermediates as depicted in Figure 4B.

The physical meaning of the Hill coefficients arrived at herein is important to consider.[22] The GQS samples for K^+ titrations were prepared by first dialyzing against LiCl to neutralize the backbone but prevent GQS folding, as previously described[6] (also see the SI). It is clear that the Hill coefficient for folding does not directly indicate the number of ions bound in the quartets. For example, the Hill coefficients for the G2 sequences were 2.3−2.7, yet only one ion can bind between the two quartets. It is possible that the value reflects *all* ions taken up in the folding transition, which could include loop-bound and diffuse ions via exchange of Li^+. In the case of the G3 sequences, a Hill coefficient near unity could reflect the number of ions going from the intermediate, which has loop-bound and diffuse ions, to the final folded state. Despite these complexities,[22] the Hill equation provides a phenomenologically and physiologically important description of how the RNA folds in the presence of increasing K^+: it describes where the folding transition is centered (i.e., the $K^+_{1/2}$ value) and how steep it is with respect to K^+ concentration (i.e., the n value). During stress, K^+ concentrations within plant cells can increase from ~100 mM to as high as ~600 mM.[11]

To visualize the dependence of the *observed* free energy on the K^+ concentration, we plotted ΔG°_{obs} versus K^+ concentration (Figure 5). The x intercept for each line is the $K^+_{1/2}$ value. For a plant under well-watered conditions (cellular K^+ concentration between 75 and 125 mM),[10,29] the G3 GQS, which have $K^+_{1/2}$ values of <50 mM, have the intrinsic ability to form stable quadruplex structures, as indicated by the negative ΔG°_{obs} values under these conditions (Figure 5, "normal"). In contrast, less stable GQS such as the G2 sequences do not fold as strongly under such conditions. However, they may form under water-limiting conditions, wherein cellular K^+ concentrations can reach 600 mM, as shown by the fact that their ΔG°_{obs} curves cross the x axis (i.e., $\Delta G^\circ_{obs} = 0$) in the "drought

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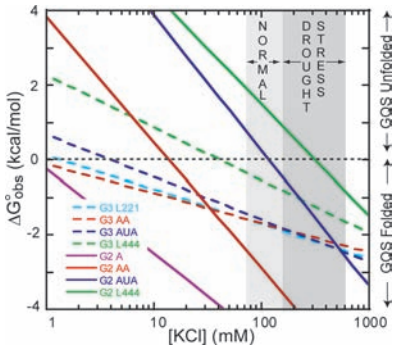

Figure 5. Observed free energies ΔG°_{obs} for RNA G3 (dashed lines) and G2 (solid lines) GQS oligonucleotides as functions of K^+ concentration, as calculated from K^+ CD titrations. The light-gray shading represents typical K^+ concentrations in plant cells under unstressed conditions and the dark-gray shading K^+ concentrations under water stress (~200−600 mM). The dashed line at $\Delta G^{\circ}_{obs} = 0$ separates strongly folding ($\Delta G^{\circ}_{obs} < 0$) and unfolding ($\Delta G^{\circ}_{obs} > 0$) conditions.This figure is color matched to Figure 1.

stress" range and even beyond (Figure 5). In addition, because of their larger Hill coefficients, the G2 sequences fold with a steeper K^+ dependence, causing the relative GQS stabilities to reorder at higher K^+ concentrations. Indeed, the G2 sequences become particularly stable at high K^+ concentrations: G2AA becomes more stable than any of the G3 sequences, and G2AUA and G2L444 begin to fold (Figure 5 "drought stress").

In conclusion, a switch in RNA structure due to changes in K^+ concentration could potentially affect gene expression in plants and other organisms where cellular ionic conditions change. Additionally, the more digital nature of the G2 sequences has general implications for computation with DNA and for engineering of genetic circuits, wherein a digital response is a desired element.[30]

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, CD titrations and fits, native PAGE, RNase T1 assays, and UV melts. This material is available free of charge via the Internet at http://pubs.acs.org.

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