Oligonucleotides Hot Paper

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A New Pathway of DNA G-Quadruplex Formation**

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Abstract: *A new folding intermediate of Oxytricha nova telomeric Oxy-1.5 G-quadruplex was characterized in aqueous solution using NMR spectroscopy, native gel electrophoresis, thermal differential spectra (TDS), CD spectroscopy, and differential scanning calorimetry (DSC). NMR experiments have revealed that this intermediate (i-Oxy-1.5) exists in two symmetric bimolecular forms in which all guanine bases are involved in GG N1-carbonyl symmetric base pairs. Kinetic analysis of K^+-induced structural transitions shows that folding of Oxy-1.5 G-quadruplex from i-Oxy-1.5 is much faster and proceeds through less intermediates than folding from single strands. Therefore, a new folding pathway of Oxy-1.5 G-quadruplex is proposed. This study provides evidence that G-rich DNA sequences can self-assemble into specific pre-organized DNA structures that are predisposed to fold into G-quadruplex when interacting with cations such as potassium ions.*

A great number of guanine-rich DNA sequences are predisposed to fold into four-stranded structures known as G-quadruplexes. These highly polymorphic compact DNA secondary structures consist of two or more stacked G-quartets, a co-planar arrangement of four guanine bases held together by eight Hoogsteen hydrogen bonds. G-quartet formation requires the presence of cations to reduce repulsion between carbonyl oxygen atoms.[1] Our recent NMR study has shown that hydrodynamic dimensions of model G-rich oligonucleotides without added salt do not correspond to an extended cylindrical shape of a linearized oligomer.[2] The inferred pre-organized structure is characterized by transient hydrogen bonds with distinctive fingerprint features and relatively poor signal dispersion in NMR spectra. Desalted G-rich oligonucleotides including those derived from the one and a half repeats of the Oxytricha nova telomere known as Oxy-1.5, d($G_4T_4G_4$), as well as d[$AG_3(T_2AG_3)_3$] derived from human telomeric sequence exhibit broad signal(s) at δ 11 ppm in ^{1}H NMR spectra (Figure 1a; Figure S1). Partial protection of guanine imino protons from fast exchange with bulk water raised two fundamental questions. First, what is the nature of hydrogen bonding between guanine residues in pre-organized structure(s) adopted by desalted G-rich oligonucleotides? Second, could these structure(s) of globular shape be on pathway to G-quadruplex formation and thus represent folding intermediate(s)? To address the above questions, a detailed study using NMR spectroscopy, native gel electrophoresis, thermal differential spectra (TDS), CD spectroscopy, and differential scattering calorimetry (DSC) were performed with Oxy-1.5 oligonucleotide solutions in the absence and in the presence of K^+ ions.

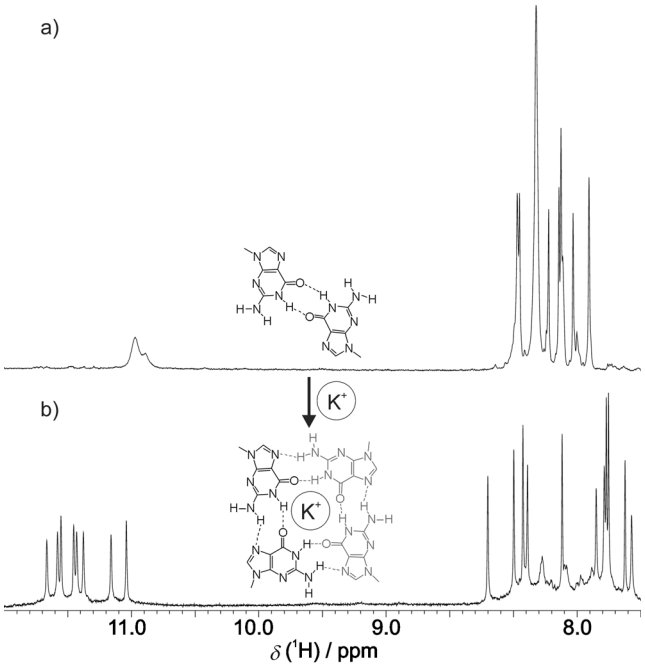

Figure 1. The imino and aromatic regions of 1D ^{1}H 600 MHz NMR spectra of desalted Oxy-1.5 a) in the presence of 30 mM KCl and b) at 25 °C in 100% H_2O and pH 5.5. The oligonucleotide concentration was 1.0 mM per strand. Guanine base pairs in GG N1-carbonyl symmetry and within a G-quartet are shown in panels (a and b), respectively.

The imino region of the ^{1}H NMR spectrum of desalted Oxy-1.5 in aqueous solution and pH 5.5 revealed two broad signals with integral ratio of about 70:30 (Figure 1a). The signals at δ 10.97 and 10.89 ppm, albeit being broad, indicate that imino protons of guanine residues are involved in

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hydrogen bonds suggesting the existence of long-lived ordered structure(s), hereinafter referred to as i-Oxy-1.5. At higher pH of 6.8 in PIPES-LiOH buffer these signals were broadened to baseline presumably because of faster exchange of imino protons with bulk water molecules. In addition, simultaneous broadening of both imino signals in ^{1}H NMR spectra during temperature increase up to 50 °C was observed. Upon increase of the K^+ ion concentration to 30 mM a new set of eight resonances with chemical shifts characteristic of the Hoogsteen base pairing appeared in aqueous solution at pH 5.5 (Figure 1b), indicating formation of a well-known antiparallel fold-back G-quadruplex architecture consisting of four G-quartets and two diagonal T_4 loops.[3]

The bimolecular form of i-Oxy-1.5 was confirmed through its hydrodynamic properties that are comparable to those of Oxy-1.5 G-quadruplex. The translation diffusion coefficients of i-Oxy-1.5 ($1.53 \pm 0.04 \times 10^{-6}$ cm^{2}s^{-1}) and Oxy-1.5 G-quadruplex ($1.43 \pm 0.04 \times 10^{-6}$ cm^{2}s^{-1}) were determined by NMR diffusion experiments at 25 °C. Moreover, i-Oxy-1.5 exhibits a highly symmetric nature as evidenced by the similar (de)shielding of protons in the two oligonucleotide strands. Only one-half of the expected 24 aromatic resonances are observed in the ^{1}H NMR spectrum (Figure 1a). A nature of hydrogen bonds between guanine residues in i-Oxy-1.5 was evaluated by analyzing NMR spectra on seven ^{15}N residue-specific partially labeled oligonucleotides.[4] The observation of signals at δ 11 ppm in all seven acquired 1D ^{15}N-filtered ^{1}H spectra indicate that the imino proton of each guanine residue in i-Oxy-1.5 is involved in a hydrogen bond (Figure S2). In addition, the respective chemical shift values suggest formation of N1−H···O6 hydrogen bonds between guanines, which occur either in G-quartets or as a part of GG N1-carbonyl symmetric base pairs. However, the imino proton chemical shifts clearly demonstrate that i-Oxy-1.5 exhibits GG N1-carbonyl symmetric base pairs (Figure 1a). It has been suggested previously that the GG N1-carbonyl symmetric geometry is the most stable base pairing between two guanines based on the electrostatic potential energy and nonempirical ab initio calculations.[5] In addition, the examination of ^{15}N-filtered NMR spectra on ^{15}N residue-specific partially labeled i-Oxy-1.5 suggests the presence of two signals for each guanine imino proton. Characteristics of ^{1}H and ^{15}N-filtered NMR spectra, together with the proposed bimolecular symmetry suggest the coexistence of the two forms of i-Oxy-1.5 in different quantities and with the same thermal stabilities.

There is no significant difference between TDS spectra of i-Oxy-1.5 and Oxy-1.5 G-quadruplex (Figure S3). A maximum at 273 nm and a minimum at 295 nm in the TDS spectra of both samples clearly suggest that intrinsic base-stacking interactions of neighboring guanine residues in the two forms are similar.[6]

To obtain additional conformation on the bimolecular nature and overall molecular shape of i-Oxy-1.5 suggested by NMR diffusion experiments, several other 12-mer oligonucleotides (see Table S1 in the Supporting Information) that fold into well-defined DNA structures in PIPES-LiOH buffer were analyzed together with i-Oxy-1.5 using native gel without added salt (Figure 2a).

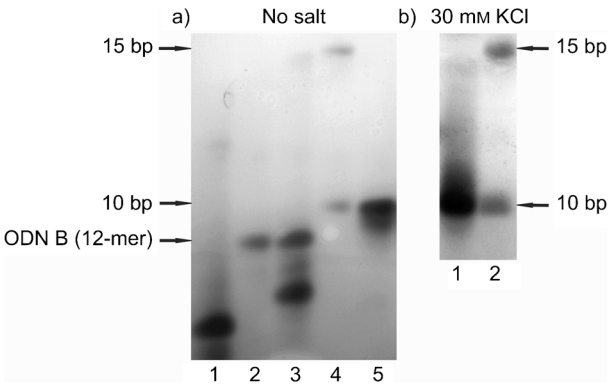

Figure 2. Gel mobility of i-Oxy-1.5 monitored in comparison to the hairpin, dimeric DNA with internal loop and Oxy-1.5 G-quadruplex forms. a) Native gel electrophoresis in the absence of salt. Lane 1: 12-mer hairpin (ODN A). Lane 2: 12-mer unstructured MW marker (ODN B), marked also with an arrow. Lane 3: Mixture of ODN B (1.2 equiv) and ODN C (MW = 7257 g mol^{-1}). Lane 4: 10-base-pair (10 bp) and 15-base-pair (15 bp) fragments in the DNA ladder. Lane 5: i-Oxy-1.5. b) Native gel electrophoresis in the presence of 30 mM KCl. Lane 1: Oxy-1.5 G-quadruplex. Lane 2: 10 bp and 15 bp fragments in DNA ladder.

Oligonucleotide A (ODN A) was designed to fold into a 12-mer hairpin and migrated fastest. ODN B was designed to be an unstructured 12-mer molecular weight marker when used alone, or to hybridize with ODN C into a dimeric DNA with internal loop. The lowest mobility was observed for i-Oxy-1.5. Its migration is the same as for the 10-base-pair (bp) fragment in the DNA ladder, indicating a slightly looser intermolecular DNA structure when compared to the ODN B/ODN C dimeric construct. The intermolecular nature of the ODN B/ODN C structure was confirmed by concentration-dependent melting temperature (T_m) values (Figure S4). As a control, the unfolding transition temperature T_m of ODN A is concentration-independent (Figure S5). Interestingly, the Oxy-1.5 G-quadruplex exhibits the same mobility as the 10 bp fragment on native gel in the presence of K^+ ions (Figure 2b). The same migration pattern observed for Oxy-1.5 G-quadruplex and i-Oxy-1.5 suggests a bimolecular nature of the latter with a similar compactness as the G-quadruplex structure.

The absence of G-quartets in the i-Oxy-1.5 structure suggested by NMR spectroscopy is in accordance with differences in the enthalpy of unfolding between Oxy-1.5 G-quadruplex and i-Oxy-1.5 [$\Delta\Delta H_u = \Delta H_u$(Oxy-1.5 G-quadruplex)$-\Delta H_u$(i-Oxy-1.5)] determined by DSC. ΔH_u(i-Oxy-1.5) of 90 kJ mol^{-1} estimated as the area under the DSC thermogram (Figure S6) is about three times lower than ΔH_u(Oxy-1.5 G-quadruplex)[7] resulting in $\Delta\Delta H_u$ of about 200 kJ mol^{-1}. Since thermally induced unfolding results in the same unfolded state for both i-Oxy-1.5 and Oxy-1.5 G-quadruplexes, the observed $\Delta\Delta H_u$ can be ascribed to a twice lower number of hydrogen bonds, less favorable stacking interactions, and the absence of metal ion coordination in i-Oxy-1.5 compared to Oxy-1.5 G-quadruplex. To estimate the extent of reversibility of i-Oxy-1.5 unfolding/folding transition another heating DSC scan was conducted after the first heating/

cooling cycle at 1 °C min^{-1} and 24 h equilibration at 5 °C (Figure S6). In contrast to the first heating scan, the second heating scan shows no detectable DSC peak, indicating that even after one day equilibration at low temperature the population of i-Oxy-1.5 or other ordered structures is negligibly small. This clearly shows that the formation of i-Oxy-1.5 is a slow kinetically governed process.

Structural features and kinetics of the i-Oxy-1.5 intermediate into Oxy-1.5 G-quadruplex were also investigated by CD spectroscopy. Figure 3a shows typical CD spectra measured at different times after addition of KCl to the solution of i-Oxy-1.5. The CD spectrum of i-Oxy-1.5 is characterized by two peaks—a minimum at 262 nm and a maximum at 294 nm. Although the overall shape of the spectra does not change significantly, a CD signal increase at 294 nm and a drop at 262 nm can be detected upon addition of KCl (Figure 3a). The corresponding CD profiles (Figure 3c) were used to analyze the kinetics of the i-Oxy-1.5 to Oxy-1.5 G-quadruplex transition. The simplest model mechanism consistent with the experimental CD profiles includes two consecutive elementary reactions (Mechanism S1) suggesting that formation of Oxy-1.5 G-quadruplex proceeds through one intermediate state. The details on global model analysis of the experimental CD data and the values of the corresponding kinetic parameters are presented in the Supporting Information.

In addition, Oxy-1.5 G-quadruplex formation from single-stranded DNA (ss-Oxy-1.5) was investigated to compare its rate and mechanism with the one suggested for i-Oxy-1.5 to Oxy-1.5 G-quadruplex transition. To obtain ss-Oxy-1.5, a sample of i-Oxy-1.5 was heated up to 85 °C and cooled to 5 °C. In contrast to the CD spectrum of i-Oxy-1.5 which shows characteristics of Oxy-1.5 G-quadruplex (Figure 3a; □), the CD spectrum of ss-Oxy-1.5 (Figure 3b; □) exhibits characteristics of a random coil with limited base-stacking interactions.[8] CD spectra were also measured at different times after addition of KCl to the solution of ss-Oxy-1.5 (Figure 3b) which enabled us to study kinetics of the ss-Oxy-1.5 to Oxy-1.5 G-quadruplex transition by monitoring the CD intensities at 294 and 262 nm (Figure 3c). In contrast to the i-Oxy-1.5 to Oxy-1.5 G-quadruplex transition, the simplest model able to describe these CD profiles is based on a mechanism consisting of three consecutive elementary reactions (Mechanism S2). Thus the kinetic analysis of Oxy-1.5 folding induced by binding of K^+ ions suggests the presence of one intermediate ($I_{2,A}$) upon i-Oxy-1.5 to Oxy-1.5 G-quadruplex transition and two intermediates ($I_{2,B}$, $I_{2,C}$) during ss-Oxy-1.5 to Oxy-1.5 G-quadruplex transition. In this context it should be emphasized that several kinetic models were tested for their ability to describe the time dependence of CD signals and that models involving one or two intermediate states are the simplest models able to describe the experimental data. Good agreement of these models with experimental data can be achieved only when different spectral (CD) characteristics are ascribed to intermediates $I_{2,A}$, $I_{2,B}$, $I_{2,C}$, and i-Oxy-1.5 ($I_{2,A} \neq I_{2,B}$ or $I_{2,C}$; $I_{2,B}$ or $I_{2,C} \neq$ i-Oxy-1.5). Mechanisms of i-Oxy-1.5 to Oxy-1.5 G-quadruplex and ss-Oxy-1.5 to Oxy-1.5 G-quadruplex transitions may well be more complex, involving more intermediates, however, such a complexity cannot be justified by model analysis of the experimental data presented here. On the other hand, kinetic analysis clearly shows (Figure 3d, Figure S8, and Table S2) that the formation of Oxy-1.5-G-quadruplex from ss-Oxy-1.5 is much slower and probably proceeds through more intermediates than the structural transition of i-Oxy-1.5 to Oxy-1.5 G-quadruplex.

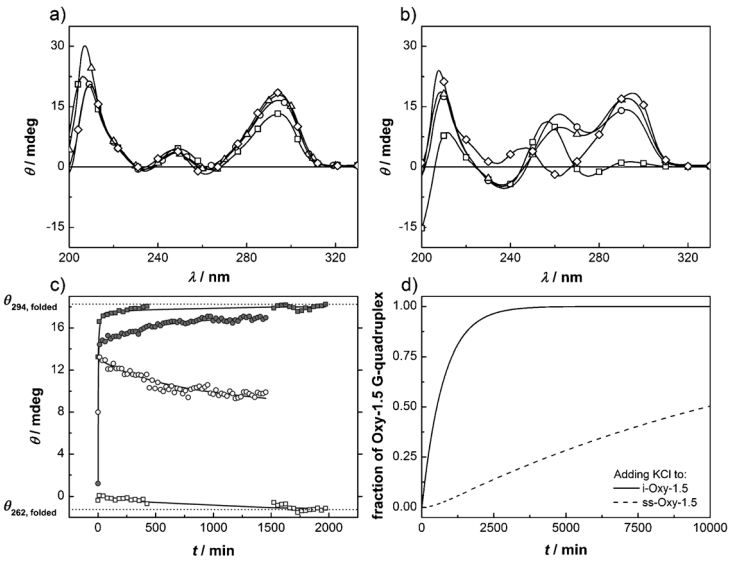

Figure 3. Following the structural rearrangement of i-Oxy-1.5 and ss-Oxy-1.5 in 20 mM PIPES-LiOH pH 6.8 buffer at 5 °C as a function of time after addition of KCl to a final concentration of 30 mM. a) CD spectra of i-Oxy-1.5 sample measured at $t = 0$ (□), $t = 0.03$ h (○), $t = 24$ h (△), and $t = 96$ h (◇). b) CD spectra of ss-Oxy-1.5 sample measured in the time range from hours to one month; $t = 0$ (□), $t = 0.03$ h (○), $t = 24$ h (△), and $t = 1$ month (◇). c) Changes in ellipticity of the i-Oxy-1.5 sample monitored at 294 (■) and 262 nm (□), and ss-Oxy-1.5 sample monitored at 294 (●) and 262 nm (○). Solid lines represent the best fit of the kinetic models (Mechanisms S1 and S2), whereas the two dashed lines represent the ellipticities of the folded Oxy-1.5 G-quadruplex. d) Model predicted fraction of Oxy-1.5 G-quadruplex formed from i-Oxy-1.5 (solid line) or ss-Oxy-1.5 (dashed line). Fractions of all model predicted species are presented in Figure S8.

Analysis of all the experimental data presented above suggests a new Oxy-1.5 G-quadruplex folding pathway that includes the intermediate i-Oxy-1.5 (Figure 4). According to the proposed mechanism Oxy-1.5 G-quadruplex can be formed from symmetric bimolecular forms of i-Oxy-1.5 where all guanine bases are involved in GG N1-carbonyl symmetric base pairs. The proposed fold-back topology of i-Oxy-1.5 is based on a detailed NMR analysis and the observed similarities in CD spectra, TDS profiles, migration patterns on native gel, and translation diffusion coefficients with Oxy-1.5 G-quadruplex, which exhibits a well-characterized fold-back topology.[3] According to the topology of i-Oxy-1.5 topology, for example, G1 can be base paired either with G4 or with G9. As a result, G-rich strands involved in base pairing can be in parallel or antiparallel orientations. The transition from i-Oxy-1.5 to Oxy-1.5 G-quadruplex has to be accompanied by breaking one of the N1–H···O6 hydrogen bonds

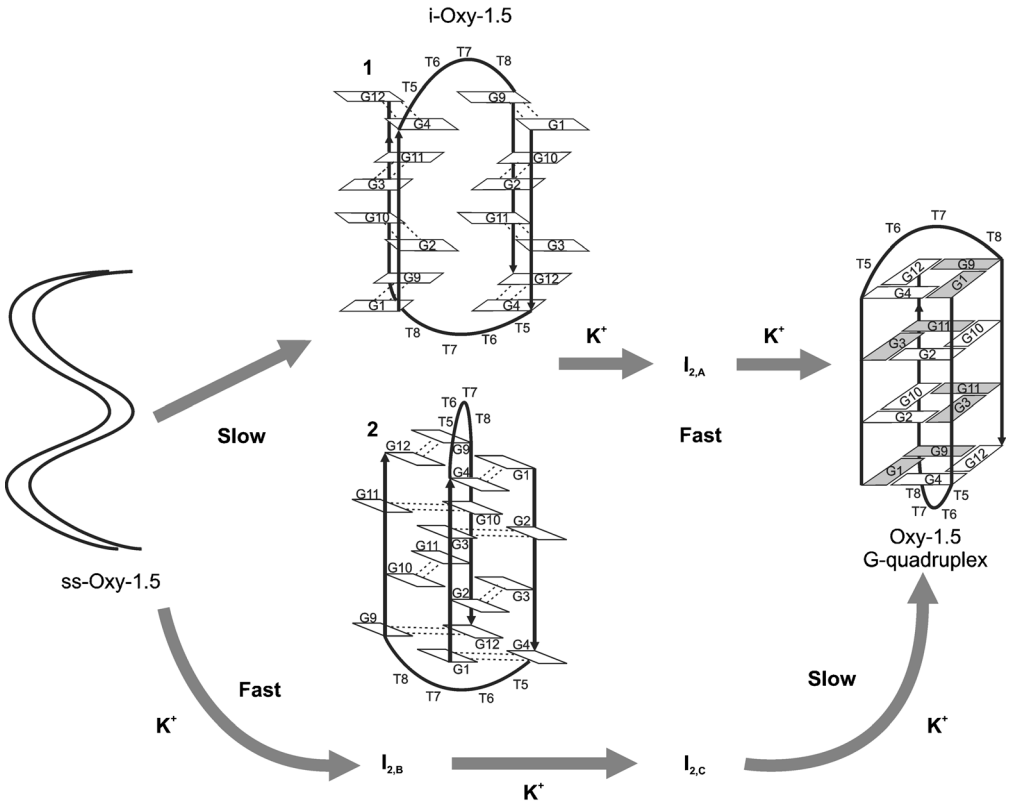

Figure 4. Oxy-1.5 G-quadruplex folding pathway involving long-lived i-Oxy-1.5 intermediate. Suggested topologies for i-Oxy-1.5 are denoted as 1 and 2. $I_{2,A}$, $I_{2,B}$, and $I_{2,C}$ denote bimolecular intermediates formed during structural transition either from i-Oxy-1.5 or ss-Oxy-1.5 to Oxy-1.5 G-quadruplex in the presence of K^+ ions.

between each GG N1-carbonyl symmetric base pair in i-Oxy-1.5 before amino···N7 hydrogen bond can be established (Figure 1). Consequently this leads to the reorientation of the strands enabling the formation of the G-quartets. The analysis of DSC data suggests that the majority of free-energy needed for this structural rearrangement comes from highly negative enthalpy of transition ($= -\Delta\Delta H_u \approx -200\ kJ\ mol^{-1}$).

In general, G-rich DNA sequences that are pre-organized in the way described here could fold very rapidly and not by chance into a large number of structurally diverse G-quadruplex structures with mechanisms of varying complexity. G-quadruplex formation by telomeric repeats and gene regulatory G-rich regions could simply go through populating various intermediate states, which are generally unstable and hard to detect. This kind of studies where intermediate states can be characterized structurally, thermodynamically, and kinetically provide an important step in the quest to elucidate general principles by which G-quadruplexes adopt their native folds.

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