Binding Sites and Dynamics of Ammonium Ions in a Telomere Repeat DNA Quadruplex

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Guanine quartets are readily formed by guanine nucleotides and guanine-rich oligonucleotides in the presence of certain monovalent and divalent cations. The quadruplexes composed of these quartets are of interest for their potential roles in vivo, their relatively frequent appearance in oligonucleotides derived from in vitro selection, and their inhibition of template directed RNA polymerization under proposed prebiotic conditions. The requirement of cation coordination for the stabilization of G quartets makes understanding cation-quadruplex interactions an essential step towards a complete understanding of G quadruplex formation. We have used 15NH4‡ as a probe of cation coordination by the four G quartets of the DNA bimolecular quadruplex [d(G4T4G4)]2, formed from oligonucleotides with the repeat sequence found in Oxytricha nova telomeres. 1H and 15N heteronuclear NMR spectroscopy has allowed the direct localization of monovalent cation binding sites in the solution state and the analysis of cation movement between the binding sites. These experiments show that [d(G4T4G4)]2 coordinates three ammonium ions, one in each of two symmetry related sites and one on the axis of symmetry of the dimeric molecule. The NH4‡ move along the central axis of the quadruplex between these sites and the solution, reminiscent of an ion channel. The residence time of the central ion is determined to be 250 ms. The 15NH4‡ is shown to be a valuable probe of monovalent cation binding sites and dynamics.

Keywords: Oxytricha nova; G quartet; NMR; monovalent cation coordination

Introduction

The guanine quartet (G quartet) is a nucleic acid base-pairing motif formed by the cyclic hydrogen-bonding of four guanine bases in a coplanar arrangement (Figure 1(a)). The formation of such structures by GMP and polyguanine in vitro has been known for decades (Gellert et al., 1962; Guschlbauer et al., 1990). However, possible biological roles for G quartets have only been given serious consideration with the more recent discovery that quadruplexes composed of G quartets are formed by DNA oligonucleotides with repeat sequences from chromosome telomeres and other biologically relevant sources (Sen & Gilbert, 1988; Sundquist & Klug, 1989; Williamson et al., 1989). While direct evidence of G quadruplex formation and function in vivo is still lacking, the discovery that proteins associated with chromosome telomeres can act as molecular chaperones for the formation of G quadruplexes is highly suggestive of the presence of G quadruplexes in vivo (Fang & Cech, 1993a,b).

The exploration of nucleic acid molecules for possible diagnostic and therapeutic applications has also produced a number of DNA sequences which have been shown or implicated to contain G quartets. These include a thrombin-binding DNA aptamer (Macaya et al., 1993; Wang, K.Y. et al., 1993), the phosphorothioate oligonucleotide TTGGGGTT which inhibits human immunodeficiency virus (HIV) transmission in vitro (Wyatt et al., 1994), and a catalytic porphyrin metalation DNA (Li & Sen, 1996). The relatively frequent occurrence of putative G quartets in oligonucleotides isolated by in vitro selection from pools of
DNA polymers with random sequences suggests that this motif is one of the most versatile and stable structural motifs available to DNA. The remarkable stability of G quartets has also thwarted efforts to use G-rich single stranded RNA templates for the polymerization of nucleotide monomers under plausible prebiotic conditions (Joyce & Orgel, 1993; Roberts et al., 1997). Thus, even in the absence of definitive proof for their natural occurrence in vivo, G quartets are of considerable interest as a structural feature of nucleic acids.

At the center of each G quartet are four closely spaced O6 carbonyl oxygen groups of the guanine residues (Figure 1(a)). From the earliest structural studies of G quartets, it was proposed that the O6 free electron pairs are involved in cation coordination (reviewed by Guschlbauer et al., 1990). This idea was later given support by 23Na NMR spectroscopic studies which revealed that GMP assemblies into higher order structures with bound Na+ and K+ (Borzo et al., 1980; Detellier & Laszlo, 1980). More recently, X-ray crystallography has provided definitive evidence of cation coordination by G quartets with the high resolution (0.95 Å) structure of the tetramolecular quadruplex d(TG2T)4, where dehydrated cations are observed along the central axis of coaxially stacked G quartets (Laughlan et al., 1994; Phillips et al., 1997).

Oligonucleotides with short, single runs of guanine bases (e.g. d(TGGGGT)) favor the formation of parallel-stranded intermolecular quadruplex structures containing G quartets (Aboul-ela et al., 1992; Cheong & Moore, 1992; Gupta et al., 1993; Laughlan et al., 1994; Wang & Patel, 1992, 1994). On the other hand, sequences with multiple runs of guanine bases (e.g. d(G4T4G4) and d(G4(T4G4)3)) have been shown to fold back on themselves and form bimolecular or unimolecular structures with antiparallel or mixed parallel and antiparallel backbones (Kang et al., 1992; Macaya et al., 1993; Schultz et al., 1994; Smith & Feigon, 1992; Smith et al., 1994, 1995; Wang, K.Y. et al., 1993; Wang & Patell, 1995). There is evidence that the topology of the quadruplex adopted by oligonucleotides with multiple runs of guanine bases (i.e. parallel versus foldback) depends upon the concentration and species of associated cation (Miura et al., 1995; Sen & Gilbert, 1990). This has led to speculation that cation driven structural transitions by DNA quadruplexes could be utilized in vivo as a molecular switch (Miura et al., 1995; Sen & Gilbert, 1990).

Studies of cation binding by G quartets in solution have thus far relied upon indirect methods to determine the number and location of cations bound within a quadruplex (Borzo et al., 1980; Deng & Braunlin, 1996; Detellier & Laszlo, 1980; Hardin et al., 1997; Hud et al., 1996; Scaria et al., 1992; Xu et al., 1993). It had been known that the ammonium ion (NH4+) stabilizes G quartets (Guschlbauer et al., 1990) to an extent comparable with that observed for Na+ (Laughlan et al., 1994). We have recently presented a preliminary account of the use of NH4+ as a probe for monovalent cation binding to nucleic acids in solution (Hud et al., 1998), using the dimeric G quadruplex formed by the repeat sequence found in Oxytricha nova telomeres (Kang et al., 1992; Smith & Feigon, 1992; Figure 1(b)). Here, we show that under slightly acidic conditions (pH 5.0), three distinct proton resonances are observed for NH4+ in the presence of this quadruplex. One of these three resonances corresponds with the bulk NH4+ of the solution, while the other two resonances correspond with NH4+ coordinated within the quadruplex. The coordinated NH4+ can be precisely localized between the planes of successive G quartets. Furthermore, the lifetimes of the bound NH4+ and their movement through the quadruplex can be directly monitored using two-dimensional (2D) heteronuclear NMR experiments.

**Results and Discussion**

**Three distinct 1H resonances are observed for ammonium ions in the presence of d(GT,G4)**

One-dimensional (1D) 1H NMR spectra of the aromatic/amino region of d(GT,G4), or Oxy-1.5, in solution with NH4+ as the principal counterion are shown in Figure 2. The chemical shifts of the DNA under these conditions are different from those observed for the same molecule in the presence of Na+ (Smith & Feigon, 1993). However, analysis of the 2D nuclear Overhauser effect (NOESY) spectra (see Materials and Methods) confirm that Oxy-1.5 adopts the same 2-fold symmetric dimeric diagonally looped quadruplex (Figure 1(b)) in the presence of NH4+ that has been reported for the Na+ form (Schultz et al., 1994;
Smith & Feigon, 1992, 1993) and the K⁺ form (Smith & Feigon, 1993; P.S., N.V.H., F.W.S. & J.F., unpublished data) of Oxy-1.5 and the related three quartet quadruplex formed by d(G₃T₄G₃) (Hud et al., 1996; Keniry et al., 1995; Smith et al., 1994). The spectra of Oxy-1.5 with the three different cations all show relatively large differences in many of the proton chemical shifts, but all give similar patterns of sequential and long-range NOE connectivities. Since the induced chemical shifts of the proton resonances are largely determined by ring current shifts from the G quartets, even small differences in quartet spacing or helical twist could result in significant chemical shift differences. Electronic effects from the bound cations could also contribute to chemical shift differences between the quadruplexes formed with different cations. There is some evidence for small structural differences between the quadruplexes formed with the three different cations, particularly in the loops (P.S., N.V.H., F.W.S. & J.F., unpublished results), but the overall helical structures and dimerization motif are the same.

The resonance for the bulk NH₄⁺ is observed at 7.10 ppm in the ¹H NMR spectrum of the NH₄⁺ form of Oxy-1.5 at pH 5.0, 283 K (Figure 2). The NH₄⁺ concentration is approximately 55 mM, producing a 44-fold more intense ¹H resonance compared with the 5.0 mM ¹H resonances of the DNA. Since the total cation concentration in this sample is equal to that of the DNA phosphates, the bulk of the NH₄⁺ in the sample is expected to be involved in electrostatic interactions with the phosphates on the surface of the quadruplex (Xu et al., 1993). The ¹H chemical shift of the bulk NH₄⁺ resonance is the same as the chemical shift of NH₄⁺ in the absence of DNA at the same pH, temperature and ionic strength. This is consistent with previous studies which have shown that monovalent cations associated with the phosphates of a DNA quadruplex are highly mobile and in fast exchange with solution (Xu et al., 1993).

In order to facilitate spectral analysis of the interactions of NH₄⁺ with the DNA, the samples were prepared with ¹⁵NH₄⁺. The ¹D ¹⁵N-filtered ¹H spectrum of the pH 5.0 sample shows resonances at 7.31 and 7.38 ppm, in addition to the bulk NH₄⁺ resonance at 7.10 ppm (Figure 2). The presence of these two smaller peaks indicates that approximately 7% of the total ammonium ions reside in two distinct environments, which are in turn distinct from the bulk solution. These peaks, as we have reported in a preliminary communication (Hud et al., 1998) and discuss in detail below, arise from NH₄⁺ coordinated within the Oxy-1.5 quadruplex.

The ammonium ions coordinated within Oxy-1.5 are protected from proton exchange with water

The linewidth of the bulk NH₄⁺ peak is dependent on pH and temperature. At pH 7.0 and 283 K, the bulk NH₄⁺ resonance in the sample containing 2.5 mM Oxy-1.5 (dimer quadruplex) is broadened to baseline, as a result of an increased rate of bulk NH₄⁺ proton exchange with water in going from pH 5.0 to 7.0. In contrast, the linewidths of the smaller lowfield shifted NH₄⁺ resonances are not appreciably altered by this change in pH (Figure 2). Based upon the linewidths of these resonances, the coordinated NH₄⁺ have bound lifetimes in excess of 50 ms. A similar differential line broadening is observed for the NH₄⁺ resonances as a function of temperature (Figure 3). ¹⁵N-filtered ¹H spectra of the pH 5.0 sample show that the exchange rate of the protons of bulk NH₄⁺ with water, as indicated by the broadening and reduced intensity of this resonance, increases monotonically with temperature. At 267 K the bulk NH₄⁺ resonance has a linewidth of 2.3 Hz, while at 333 K the linewidth increases to 40 Hz and the intensity is substantially reduced. In contrast, the linewidths of the two low-field shifted NH₄⁺ resonances of Oxy-1.5 are relatively insensitive to changes in temperature, and actually decrease slightly as the temperature increases from 267 K (9 Hz) to 288 K (6.5 Hz; Figure 3). The fact that these resonances do not
broaden with increasing temperature indicates that the protons on these cations are isolated from exchange with water for longer than 50 ms up to at least 333 K (extended time at this temperature or higher leads to melting of the quadruplex structure). The narrowing of these resonances with increasing temperature can be explained by a decrease in the correlation time of the entire DNA quadruplex within which the NH$_4^+$ are coordinated. This suggests that the coordinated NH$_4^+$ have restricted movement and tumble at a rate governed by the overall correlation time of Oxy-1.5.

All three NH$_4^+$ ¹H resonances (pH 5.0) exhibit lowfield (~0.017 ppm shifted) shoulders at 283 K or below (Figure 3). These shifted resonances are due to the presence of 17% singly deuterated ammonium ions (NH$_4^+$D$^+$), caused by the equilibrium distribution of deuterium from the 5% ²H$_2$O added for the magnetic field lock signal. At pH 5.0, the NH$_4^+$D$^+$ shoulder of the bulk ammonium peak is only clearly visible in spectra at temperatures below approximately 283 K. The NH$_4^+$D$^+$ shoulders of the ions coordinated within the quadruplex are visible over a much wider range of temperatures due to the protection of their protons and deuterons from chemical exchange with water. However, the linewidths of the coordinated NH$_4^+$ and NH$_4^+$D$^+$ resonances are too broad to be resolved from one another at any of the recorded temperatures, in contrast with the bulk NH$_4^+$ ¹H resonances at 267 K. This again appears to be a result of a longer correlation time for the coordinated NH$_4^+$ and resultant resonance line broadening due to restricted rotation within the quadruplex.

**Localization of the three ammonium ions coordinated within the four G quartets of Oxy-1.5**

The ratio of the integrated intensities of the two lowfield shifted NH$_4^+$ resonances at 7.31 and 7.38 ppm in the $^{15}$N-filtered ¹H spectrum acquired at 267 K is 1:2 (Figure 3). The ratio of the 7.31 ppm resonance to any of the non-overlapped DNA aromatic proton resonances in a corresponding ¹H spectrum is 2:1. Since each NH$_4^+$ has four protons and Oxy-1.5 forms a symmetric dimer, this means that two NH$_4^+$ resonate at 7.38 ppm and one resonates at 7.31 ppm per quadruplex (Hud et al., 1998). Thus, three NH$_4^+$ are coordinated in two distinct binding environments of Oxy-1.5, and these sites are fully occupied. The intensity of the NH$_4^+$ resonance at 7.38 ppm with respect to the DNA aromatic resonances decreases with increasing temperature (from 2:1 at 267 K to 1.5:1 at 333 K), while the relative intensity of the resonance at 7.31 ppm to the DNA aromatic resonances remains the same. This differential temperature dependence is the result of the NH$_4^+$ resonance at 7.38 ppm being less protected from exchange with solvent protons than the 7.31 ppm resonance. Support for this explanation is given by the following results.

**Figure 3.** One-dimensional $^{15}$N-filtered ¹H spectra of $^{15}$NH$_4^+$ Oxy-1.5 as a function of temperature. The sample contains 2.5 mM Oxy-1.5 quadruplex (5 mM in single-strand), 55 mM $^{15}$NH$_4^+$ at pH 5.0. The $^{15}$NH$_4^+$ resonances are labeled B (bulk), I (inner NH$_4^+$) and O (outer NH$_4^+$). Spectra were acquired with $^{15}$N decoupled during collection of FID.

**Figure 4.** Portion of a ¹H ROESY spectrum of $^{15}$NH$_4^+$ Oxy-1.5 containing the imino/amino-$^{15}$NH$_4^+/H_2$O cross-peaks. The spectrum was acquired at 283 K with $^{15}$N decoupling in both dimensions. Sample is the same as described in the legend to Figure 3. Positive and negative cross-peaks are contoured with broken and continuous lines, respectively. Assignments of the NH$_4^+$ and G imino resonances are indicated on the sides of the spectra. The G amino resonances with exchange cross-peaks to water and bulk NH$_4^+$ are also labeled. These have been assigned to the outer G quartets based upon their linewidth, chemical shifts, and lack of cross-peaks with a non-hydrogen-bonded amino, which are observed for the more slowly exchanging aminos of the inner G quartets (Smith & Feigon, 1993). Due to the absence of NOE cross-peaks between the outer G aminos and any other DNA resonances, it was not possible to assign these resonances to specific bases.
The three bound $^{15}$NH$_4^+$ can be precisely localized in the quadruplex by analysis of cross-peaks observed in a 2D rotating frame Overhauser effect spectroscopy (ROESY; Bothner-By et al., 1984) spectrum (Figure 4). The ROESY spectrum was used to allow distinction between cross-peaks arising from NOE transfers and those from pure chemical exchange. NOE cross-peaks between the NH$_4^+$ resonances and the eight G imino resonances are observed in the spectrum; this unambiguously demonstrates that these ions must be bound inside the quadruplex. To simplify nomenclature, the eight G imino protons of Oxy-1.5 are divided into two sets: outer G imino resonances, G1, G4*, G9* and G12 (the asterisk designates bases of the symmetry related strand); and inner G imino resonances, G2, G3*, G10* and G11 (Figure 1(b)). The NH$_4^+$ resonance at 7.31 ppm has cross-peaks with all four imino proton resonances (eight imino protons total) of the inner G quartet, but no cross-peaks with the outer G imino resonances. This pattern of NOE cross-peaks in the ROESY spectrum, and the stochiometric ratio of one cation with this chemical shift per quadruplex, firmly establishes that this cation is situated at the center of the two inner G quartets on the 2-fold symmetry axis. This NH$_4^+$, with a 1H chemical shift of 7.31 ppm, is henceforth referred to as the inner NH$_4^+$. The NH$_4^+$ resonance at 7.38 ppm has cross-peaks in the ROESY spectrum with both the inner and the outer G imino resonances. Integrated volumes of the cross-peaks between the inner quartet imino protons of G3 and G11 and the inner NH$_4^+$ are equal in volume to the cross-peaks between these same G imino protons and the NH$_4^+$ with a chemical shift of 7.38 ppm. This localizes the other NH$_4^+$ coordination site to precisely the same distance above the plane of the inner G quartet as the single NH$_4^+$ is below, i.e. in between the planes of the inner and outer G quartets (outer NH$_4^+$). Since the quadruplex has 2-fold symmetry, there are two such binding sites per quadruplex, which is consistent with the area under the peak at 7.38 ppm being twice that of the peak at 7.31 ppm (Figure 2).

An energy minimized model of Oxy-1.5 with three coordinated NH$_4^+$ is shown in Figure 5. This model was calculated starting from the structure of the K$^+$ form of Oxy-1.5 (P.S., N.V.H., F.W.S. & J.F., unpublished results) with the ions placed symmetrically in the sites as determined by the NOEs observed between the NH$_4^+$ and G imino resonances (see Materials and Methods). The coordination of NH$_4^+$ within Oxy-1.5, midway along the axis between two consecutive stacked G quartets of the quadruplex, is different from that which has been observed for Na$^+$ coordination by G quartets. In the crystal structure of d(TG$_4$T), which was crystallized in the presence of Na$^+$, two tetramolecular quadruplexes are coaxially stacked such that eight G quartets appear in each unit cell. Seven ions are coordinated by the eight successive G quartets, with the location of Na$^+$ coordination varying along the central axis, from midway between the central two G quartets of the dimer of tetrads to in-plane coordination by the outer most G quartets (Laughlan et al., 1994).

The K$^+$ with an ionic radius of 1.33 Å is too large to be coordinated by a single G quartet in a coplanar fashion (Detellier & Laszlo, 1980; Sundquist & Klug, 1989) and is likely to be coordinated in a very similar fashion to that demonstrated here for NH$_4^+$ (ionic radius 1.43 Å). It is well documented that G quartets favor the coordination of K$^+$ over Na$^+$ (Deng & Braunlin, 1996; Hud et al., 1996; Williamson, 1993, 1994). However, this observed preference is primarily due to the greater cost of Na$^+$ dehydration with respect to K$^+$, while the intrinsic free energy of Na$^+$ binding by G quartets is actually more favorable than that of K$^+$ (Hud et al., 1996; Ross & Hardin, 1994). The

Figure 5. Stereo pair of the calculated model structure of Oxy-1.5 with three coordinated NH$_4^+$. One strand is shown in red and the other (symmetry equivalent) strand in green. The ammonium ions are shown in CPK representation. For clarity, the T residues of the loops are drawn with thinner lines than the G residues.
locations of NH$_4^+$ coordinated by Oxy-1.5 illustrate that the more favorable free energy of Na$^+$ binding within G quartet molecules, compared to K$^+$, could primarily be the result of K$^+$ coordination being restricted to between the planes of successive G quartets. In contrast, the smaller ionic radius of Na$^+$ allows in-plane coordination. In addition, multiple Na$^+$ within a quadruplex are not restricted to the spacing of stacked G quartets, and they can move farther away from each other in order to reduce mutual electrostatic repulsions (Laughlan et al., 1994), whereas the larger K$^+$ and NH$_4^+$ cannot.

**Bulk ammonium ions produce exchange and exchange-relayed NOE cross-peaks with Oxy-1.5**

In addition to the cross-peaks between the bound NH$_4^+$ and Oxy-1.5, negative cross-peaks are observed between the bulk NH$_4^+$ resonance and the G imino resonances of the outer G quartets of Oxy-1.5 in the ROESY spectra shown in Figure 4. A virtually identical pattern of cross-peaks is also observed between the water resonance and the outer G imino resonances. These are most likely exchange-relayed NOEs which arise from protons of bulk NH$_4^+$ and water exchanging with the amino protons of the outer G quartets during the ROESY mixing time, followed by an NOE transfer to the G iminos of the same bases. This is supported by the fact that exchange cross-peaks are also observed between the outer G amino resonances and both the bulk NH$_4^+$ and water resonances (Figure 4). This explanation is consistent with the absence of cross-peaks from the inner G imino protons to water and bulk NH$_4^+$, since it is known that the amino protons of the Oxy-1.5 inner G quartets exchange much more slowly with solvent than the outer G aminos (Smith & Feigon, 1993).

**Ammonium ions move between the cation coordination sites of Oxy-1.5 on the millisecond time scale**

The linewidths of the lowfield shifted NH$_4^+$ resonances indicate that the coordinated NH$_4^+$ of the Oxy-1.5 quadruplex have lifetimes of at least 50 ms (Figure 2). However, exchange cross-peaks are observed in the ROESY spectrum between the proton resonances of the inner and outer NH$_4^+$ and with the bulk NH$_4^+$ (Figure 6). This indicates that NH$_4^+$ ions move from one Oxy-1.5 coordination site to another, as well as out into the bulk solution, during the mixing time of the ROESY experiment (50 ms). In principle, analysis of cross-peaks in the ROESY spectrum could be used to obtain rates of NH$_4^+$ exchange, but since these cross-peaks are near the diagonal their volumes are difficult to quantify. This analysis would also be complicated by the fact that the cross-peak intensities can be reduced by NOE transfer between neighboring NH$_4^+$.

In order to quantify the rate of NH$_4^+$ exchange between coordination sites on Oxy-1.5 and the solution more precisely, we obtained a series of N$_2$ chemical exchange heteronuclear single-quantum coherence (N$_2$-Ex HSQC) spectra as a function of mixing time. This experiment is a modified version of the $^{15}$N-$^1$H correlated S$_2$-chemical exchange HMQC (Montelione & Wagner, 1989). Figure 7 shows regions from two N$_2$-Ex HSQC spectra acquired with mixing times of 13 and 63 ms, respectively. The N$_2$-Ex HSQC spectra contain
The weak cross-peak observed between the inner coordination sites to bulk are all equal (Figure 7). The cross-peaks in this type of experiment arise from exchange between sites during the mixing time, and quantification of their intensities as a function of mixing time gives the exchange rate. Specifically, the cross-peaks between the autocorrelation peaks of the two coordinated $^{15}$NH$_4^+$ indicate that cations move from the inner to the outer coordination site, and vice versa, during the mixing time. The cross-peak between the autocorrelation peaks of the outer NH$_4^+$ and the bulk NH$_4^+$ indicates that cations also move from the outer coordination site into solution. Qualitative analysis of the cross-peak intensities in the N$_2$-Ex HSQC spectrum with a 63 ms mixing time shows that the number of cations which have moved from the outer to the inner coordination sites, inner to outer coordination sites, and outer coordination sites to bulk are all equal (Figure 7).

The weak cross-peak observed between the inner NH$_4^+$ resonance and the bulk NH$_4^+$ resonance in Figure 6 can be explained by the two-step movement of an NH$_4^+$ from solution into the inner site. We see no evidence to support the direct movement of NH$_4^+$ from the bulk to the inner site. Thus, our results show that NH$_4^+$ cations move along the central axis of the Oxy-1.5 quadruplex as illustrated schematically in Figure 8. Vacant coordination sites are likely to exist, at least transiently, during NH$_4^+$ movement. However, the lifetime of these vacancies must be short compared with the lifetime of occupied sites, since we see no evidence for vacant coordination sites (e.g. an additional set of G imino chemical shifts).

A quantitative analysis of the autocorrelation and cross-peak intensities of the inner NH$_4^+$ has been used to determine the bound lifetime of this ion. Figure 9 shows a graph for the ratio of NH$_4^+$ which have moved from the inner coordination site to an outer coordination site as a function of time. A fit of this data with a first-order rate equation gives a bound lifetime of 250 ms for the inner NH$_4^+$. This value should be considered an upper limit on the residence time of an NH$_4^+$ coordinated in the inner site because, unlike in an ion channel with a mechanism to drive cation movement unidirectionally, ions within the Oxy-1.5 quadruplex could be moving back and forth between coordination sites at random.

**Figure 8.** Schematic of NH$_4^+$ movement through the Oxy-1.5 quadruplex. Red, yellow, and blue spheres represent inner, outer, and bulk NH$_4^+$, respectively, in the initial state (left). The movement of NH$_4^+$ ions depicted in step I is revealed by N$_2$-Ex HSQC spectroscopy to take place at a rate of approximately 4 s$^{-1}$. If this movement is repeated in the same direction (step II), an NH$_4^+$ originally in the bulk enters the inner site.

**Figure 9.** Plot of the ratio of cross-peak volumes with respect to autocorrelation peak volumes for the inner NH$_4^+$ from a series of N$_2$-Ex HSQC acquired at 283 K with various mixing times. The continuous line represents the best fit of the data to a first-order rate equation for movement of the inner ion to the outer coordination site.

**Are the cations really moving, or is it just the protons?**

Since the protons of NH$_4^+$ are exchangeable, the formal possibility exists that only protons of NH$_4^+$ are moving through Oxy-1.5 on the millisecond time scale, rather than the whole ion. The cross-peaks observed in the ROESY spectrum do not distinguish between ammonium ion movement and ammonium proton exchange. However, the N$_2$-Ex HSQC experiment proves that whole ammonium ions are, in fact, moving. This is because the magnetization transfer pathway of this experiment gives rise only to cross-peaks from the protons attached to the specific nitrogen which has moved during the mixing time, i.e. the magnetization coherence that will eventually be detected is solely on the $^{15}$N during the mixing time. No cross-peak intensity would be produced by protons that have moved to a different nitrogen during the mixing time. Since the relative cross-peak intensities observed in the ROESY and N$_2$-Ex HSQC experiments are the same, the possibility that both types of transfer take place can also be eliminated.

The fact that NH$_4^+$ ions are moving through Oxy-1.5 is consistent with results obtained from the titration of this quadruplex with different monovalent cations. For example, titration of a sample of Oxy-1.5 in 55 mM Na$^+$ with NH$_4^+$ results...
in displacement of the Na\(^+\) by NH\(_4\)^+ in less than five minutes, approximately the time needed to acquire a \(^1\)H spectrum (Hud et al., 1998). It has also been shown that Na\(^+\) and K\(^+\) exchange with each other from the coordination sites of the closely related quadruplex [d(G\(_3\)T\(_3\)G\(_3\))]\(_2\) at a rate which is fast on the NMR time scale, and similar results are observed with Oxy-1.5 (Hud et al., 1996).

Previous analyses of the \(^{23}\)Na linewidth for sodium ions coordinated within the Oxy-1.5 quadruplex have estimated the lifetime of bound Na\(^+\) at 283 K to be 250 quartets, may permit Na\(^+\) to move freely along the central axis of a quadruplex. In contrast, the larger ionic radius of Na\(^+\), which allows it to be coordinated in-plane by G quartets (Laughlan et al., 1994; Phillips et al., 1997), solution state evidence has remained indirect. We have shown here that NH\(_4\)^+ provides a probe for the direct localization of the monovalent coordination sites of G quadruplexes using \(^1\)H NMR spectroscopy. This represents a novel method for the direct localization of monovalent cations on a macromolecule in solution. Using this technique, we have shown that three cations are coordinated within Oxy-1.5. We are further able to show that cations do not enter and exit the quadruplex through the grooves, but rather move between coordination sites along the central axis of the quadruplex and out through the ends. The relatively large size of the NH\(_4\)^+ is likely to hinder its movement through the quadruplex and provides a rationale for its longer residence time compared with Na\(^+\). The coordination of the K\(^+\) within G quadruplexes is expected to closely resemble the interplane coordination we have demonstrated here for NH\(_4\)^+. Our results are consistent with previous estimates of the stoichiometry of K\(^+\) bound to DNA quadruplexes (Hardin et al., 1997; Hud et al., 1996), but are inconsistent with the crystal structure of Oxy-1.5, for which diffuse electron density for a single K\(^+\) in the center of the quadruplex was reported (Kang et al., 1992).

Structurally significant monovalent cation (K\(^+\) and NH\(_4\)^+) binding sites have also been reported for specific RNA molecules, including the antico-

Conclusions

It was first suggested 25 years ago that the O6 carbonyl groups of G quartets coordinate cations (Arnott et al., 1974). While single crystal X-ray crystallography has recently provided definitive evidence of this mode of cation coordination by G quartets (Laughlan et al., 1994; Phillips et al., 1997), solution state evidence has remained indirect. We have shown here that NH\(_4\)^+ provides a probe for the direct localization of the monovalent coordination sites of G quadruplexes using \(^1\)H NMR spectroscopy. This represents a novel method for the direct localization of monovalent cations on a macromolecule in solution. Using this technique, we have shown that three cations are coordinated within Oxy-1.5. We are further able to show that cations do not enter and exit the quadruplex through the grooves, but rather move between coordination sites along the central axis of the quadruplex and out through the ends. The relatively large size of the NH\(_4\)^+ is likely to hinder its movement through the quadruplex and provides a rationale for its longer residence time compared with Na\(^+\). The coordination of the K\(^+\) within G quadruplexes is expected to closely resemble the interplane coordination we have demonstrated here for NH\(_4\)^+. Our results are consistent with previous estimates of the stoichiometry of K\(^+\) bound to DNA quadruplexes (Hardin et al., 1997; Hud et al., 1996), but are inconsistent with the crystal structure of Oxy-1.5, for which diffuse electron density for a single K\(^+\) in the center of the quadruplex was reported (Kang et al., 1992).

Materials and Methods

Preparation of DNA quadruplex samples

DNA was synthesized and purified as described (Smith & Feigon, 1993). Cations associated with purified DNA were replaced with \(^{15}\)NH\(_4\)\(^+\) by passage over a 1.2 m Sephadex G-25 size-exclusion column (Sigma, St. Louis) equilibrated and eluted with 1 mM \(^{15}\)NH\(_4\)Cl. Samples were then concentrated to 5.0 mM in oligonucleotide strand by freeze drying and resuspension in 5\% \(\text{H}_2\text{O}\). The pH of each sample was adjusted with LiOH to either pH 5.0 or 7.0. LiOH was chosen since Li\(^+\) is not coordinated by G quartets (Acevedo et al., 1991; Chantot & Guschlbauer, 1969). The \(^{1}\)H NMR concentration of a sample was verified at pH 5.0 by comparing the integrated intensity of the bulk \(^{15}\)NH\(_4\) resonance in an \(^{15}\)N-filtered \(^1\)H spectrum with that of the same sample following the addition of a known amount of \(^{15}\)NH\(_4\)Cl. The concentration of Na\(^+\) contamination in each sample was determined by the comparison of the integrated \(^{23}\)Na resonance with a set of NaCl standards. The Na\(^+\) contamination was found to be less than 100 \(\mu\)M in all samples.

NMR spectroscopy

All spectra were collected on a Bruker model DRX 500 spectrometer at 500 MHz. The 1D \(^1\)H and \(^{15}\)N-filtered spectra were acquired with 8192 complex points, a spectral width of 5000 Hz, 256 accumulated scans and \(^{15}\)N decoupling. Water suppression was accomplished using the WATERGATE pulse sequence (Piotto et al., 1992). A spin lock field of 4950 Hz was used with the maximum offset 2.5 ppm from the carrier frequency. The spectrum was acquired with 2048 complex points in F2, 512 blocks in F1 and a mixing time of 50 ms, 32 scans per block, and a spectral width of 7246 Hz in F2 and F1. Quadrature detection in F1 was achieved using the States-TPPI phase cycle (Marion et al., 1989). Spectra were \(^1\)H deconvoluted in both F1 and F2. Water suppression was accomplished with the gradient enhanced 11 spin echo pulse sequence (Sklenář & Bax, 1987) with the first maximum set to approximately 11 ppm.

The \(\text{N}_2\)-Ex HSQC pulse sequence is a modified version of the \(\text{S}_2\)-chemical exchange HMOC pulse sequence (Montelione & Wagner, 1989). The forward and reverse DEPT coherence transfer steps were replaced by INEPT elements with refocusing delays \(\sigma = 1/6\ J\), in order to suppress the interference of zero quantum coherences during the \(\text{N}_2\) mixing. The \(\text{N}_2\)-Ex HSQC spectra were acquired with 2048 complex points in F2 and 256 blocks...
in F1, eight scans for each block, and a spectral width of 5000 Hz in F2 and 2027 Hz in F1. Spectra were acquired with total mixing times of 13, 18, 23, 33, 43, 53, and 63 ms (including the inherent 13 ms delay).

Assignment of proton resonances of Oxy-1.5 in the presence of NH₄⁺

Resonance assignments for the guanine H1' and aromatic protons were obtained based on the analysis of the corresponding cross-peaks in 2D NOESY spectra. These spectra contain characteristically strong H1'-H8 cross-peaks showing that four of the G residues are in the syn conformation, as previously observed for the forms containing Na⁺ and K⁺ (Smith & Feigon, 1992, 1993). The spectra of the NH₂⁺ form of Oxy-1.5 show a high degree of similarity with the Na⁺ and especially the K⁺ form, differing only in chemical shift positions. This clearly shows that all three forms have the same topological fold. Assignments could, therefore, in part be based unambiguously as the only G in anti-conformation with sequential connectivities to a T. All four sequential syn-anti-steps show sequential H1'-H8 contacts in both directions. In the anti-syn-steps, no sequential H1'-H8 peak is present at all. The resulting ambiguities could be resolved by the presence of a non-sequential contact between 2H8-11H8 (Smith & Feigon, 1992), which occurs analogously in the other two forms. The remaining H8-H8 cross-peaks were found to be consistent with distances observed in the K⁺ form. Four of the eight G imino protons exchange so slowly with water that they are still observable several days after transfer of the sample into 2H₂O. These were assigned to the inner quartets formed by G2, 3, 10 and 11, by analogy with assignments obtained for the Na⁺ and K⁺ form of Oxy-1.5 (Smith & Feigon, 1993). Base-specific assignments for the four inner quartet imino protons were obtained by analysis of NOESY cross-peaks with aromatic protons in a 2H₂O spectrum. The remaining imino protons of the outer quartet were identified from imino-imino and imino-aromatic cross-peaks in H₂O NOESY.

Modeling of NH₂⁺ bound within Oxy-1.5

A model of the quadruplex containing three ammonium ions was constructed as follows. The conformation with lowest overall energy obtained by X-PLOR refinement for the K⁺ form of Oxy-1.5 (P.S., N.V.H., v.S. & J.F., unpublished results) served as starting structure. This starting structure was calculated exactly as described for the Na⁺ form of Oxy-1.5 (Schultze et al., 1994). The K⁺ form was used, since the ionic radius of K⁺ is closer to that of NH₂⁺ than Na⁺. The force field parameters for NH₂⁺ were derived by analogy with the corresponding values given for lysine. In the initial step of the calculations, the ammonium ions were placed at the geometric centers of the eight O₆ carbonyl oxygen atoms closest to each ion position. Steric clash was relieved by a short energy minimization run using all restraints and energy function parameters exactly as in the refinement procedure for the starting structure. At this point, the starting structure remained practically unchanged, with only slight movements of the ammonium ions. For the final step of simulated annealing, complete van der Waals and electrostatic potentials were used in addition to all restraints from the K⁺ form, using the “refine gentle” protocol (Brünger, 1992). Although no explicit distance restraints for the ammonium ions were used at any step of the model building, the ions remained near their starting positions between the quartets. During the simulated annealing they adopted slightly larger ion-ion distances (3.2 versus 2.5 Å), probably because of electrostatic repulsion. This is correlated with slightly greater outwards buckling of some of the bases in the outer quartets. Overall the model structure derived from refinement on the experimental data of the K⁺ form can accommodate the NH₂⁺ ions without significant changes.

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