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The Selectivity for K^+ versus Na^+ in DNA Quadruplexes Is Dominated by Relative Free Energies of Hydration: A Thermodynamic Analysis by ^1H NMR†

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ABSTRACT: We have studied the competition between Na^+ and K^+ for coordination by G quartets using the oligonucleotide $d(G_3T_4G_3)$ as a model system. $d(G_3T_4G_3)$ forms a dimeric foldback structure containing three G quartets in the presence of either NaCl or KCl. Proton chemical shifts, which are particular to the species of coordinated ion, have been used to monitor the conversion between the sodium and potassium forms under equilibrium conditions. Analysis of titration experiments indicates that at least two K^+ are coordinated by the three quartets of the dimeric molecule, and perfect fits of the data are obtained for two Na^+ being displaced by two K^+. Our results also indicate that the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form is associated with a net free energy change ($\Delta G°$) of -1.7 ± 0.15 kcal/mol. It has long been suggested that the greater thermal stability of DNA quadruplex structures in the presence of K^+ is primarily a result of the optimal fit of this ion in the coordination sites formed by G quartets. However, a consideration of the relatively small change in free energy associated with the conversion from the sodium to the potassium form and the relatively large difference between the free energy of hydration for Na^+ and K^+ indicates that this cannot be correct. Rather, the preferred coordination of K^+ over Na^+ is actually driven by the greater energetic cost of Na^+ dehydration with respect to K^+ dehydration.

For over thirty years, it has been known that guanine nucleotides and polymers form quadruplex structures containing guanine quartets (G quartets) (Guschlbauer et al., 1990). G quartets are characterized by the cyclic hydrogen bonding of four guanine bases in a coplanar arrangement. These structures exhibit a remarkable ion-dependent stability which is, to a significant degree, the result of ion coordination by closely spaced guanine O6 atoms. The ion−G quartet interactions are analogous to those found in host−guest systems such as the crown ethers (Frensdorff, 1971; Weber & Vögtle, 1985). The discovery that G quartet structures are formed by guanine-rich oligonucleotides similar to those in immunoglobulin switch regions as well as the G-rich repeat sequences found in eukaryotic telomeres has led to the proposal of functional roles for G quartets *in vivo* and a renewed interest in their study [reviewed by Williamson (1993, 1994)].

The strong correlation observed between the melting temperature of guanosine gels and the ionic radii of coordinated cations was an early indication of site specific ion binding by G quartets (Chantot & Guschlbauer, 1969). The coordination of dehydrated cations between G quartets was first given experimental support by Laszlo and coworkers, who observed the broadening and upfield shift of the ^{23}Na resonance in solutions of 5′-GMP (Borzo et al., 1980; Detellier & Laszlo, 1980). Recently, X-ray diffraction studies have provided direct evidence of K^+ coordination between the two central G quartets of the dimeric quadruplex $[d(G_4T_4G_4)]_2$ (Kang et al., 1992) and a similar binding of Na^+ by the G quartets of the tetrameric quadruplex $[d(TG_4T)]_4$ (Laughlan et al., 1994). In the solution state, cation NMR studies have demonstrated directly that K^+ is preferentially bound by G quartets with respect to the smaller Na^+ and the larger Rb^+ (Detellier & Laszlo, 1980; Xu et al., 1993; Deng & Braunlin, 1996). On the basis of these observations and the intuitive nature of the "goodness of fit" model, it has been proposed that preferential binding of K^+ versus Na^+ or Li^+ in G quadruplexes is determined by an optimal fit of this cation between two G quartets, coordinated to the guanine carbonyls (Sundquist & Klug, 1989; Williamson et al., 1989). While this proposal has been widely accepted, questions still remain concerning the origin of the ion selectivity exhibited by G quartets (Williamson, 1993, 1994; Ross & Hardin, 1994) as well as the number of ion coordination sites in dimeric or monomeric foldback quadruplexes. A quantitative determination under equilibrium conditions of the difference in the free energy of ion binding ($\Delta G°$) by G quartets for various ions has never been reported, yet this would be a valuable step toward revealing the nature of ion selectivity.

G quadruplexes have been proposed to form *in vivo* in immunoglobulin switch regions (Sen & Gilbert, 1988), mutation hot spots associated with human disease (Smith et al., 1989; Murchie & Lilley, 1992), and G-rich repeat sequences found at the ends of telomeres (Williamson et al., 1989). Additionally, a variety of proteins which bind to and/or facilitate formation of G quadruplexes have now been

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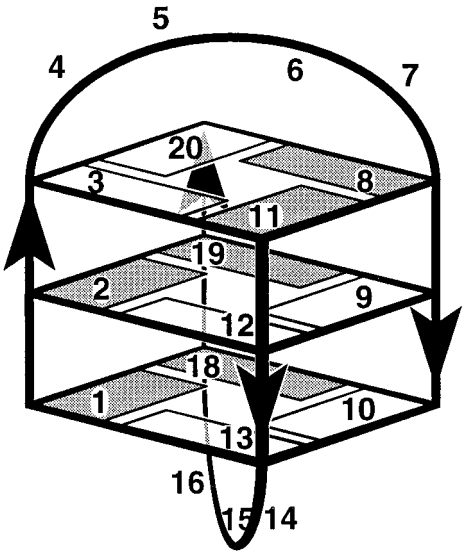

FIGURE 1: Dimeric foldback structural motif adopted by $d(G_3T_4G_3)$ in both NaCl and KCl (Smith et al., 1994; Keniry et al., 1995). Numbers indicate the 20 residues (1−10 for strand 1 and 11−20 for strand 2; strands are arbitrary) of the asymmetric structure. For illustrative purposes, guanine bases are represented as rectangles and thymine bases of the loops are omitted. Note that this structure is an asymmetric dimer due to the handedness and odd number of G quartets.

found (Walsh & Gualberto, 1992; Fang & Cech, 1993a,b; Weisman-Shomer & Fry, 1993; Giraldo & Rhodes, 1994; Giraldo et al., 1994). For both protein-catalyzed G quadruplex formation and spontaneous formation of G quadruplexes *in vitro*, it has been reported that more than one type of quadruplex can form, including both parallel tetrameric quadruplexes and foldback dimeric or monomeric quadruplexes, depending on the ionic conditions (Williamson, 1993, 1994). Thus, understanding ion selectivity by G quartet structures is of biological importance, for it may elucidate the potential for G quartet structures to participate in ion-driven regulatory mechanisms *in vivo* (Sen & Gilbert, 1988).

Here, we report an analysis of the competition between K^+ and Na^+ for coordination by the oligonucleotide $d(G_3T_4G_3)$, a molecule which forms three G quartets in a dimeric foldback structure (Figure 1). These studies have been performed using proton NMR during NaCl and KCl titrations under equilibrium conditions. We find that, upon addition of KCl, two K^+ selectively replace the bound Na^+ in $[d(G_3T_4G_3)]_2$ with a net free energy change ($\Delta G°$) of −1.7 kcal/mol at 25 °C. However, this selectivity is determined not by an optimal fit of K^+ versus Na^+ but rather by preferential hydration of Na^+ versus K^+. These results explain the origin of K^+ versus Na^+ selectivity for G quadruplexes in general.

MATERIALS AND METHODS

Sample Preparation. The DNA decamer d(GGGTTTTGGG) [$d(G_3T_4G_3)$] was synthesized and purified as described previously (Smith & Feigon, 1993). Purified samples were passed over a cation exchange column (AG 50W-X8, BioRad) charged with the desired cation (Na^+ or K^+) and concentrated by lyophilization. NMR samples were dissolved in 90% H_2O/10% D_2O (pH 6.0) to a 1.5−2.0 mM oligonucleotide strand concentration and a final volume of 400 or 450 μL.

Oligonucleotide concentrations were determined by UV absorption using the extinction coefficient of 9900 cm^{-1} (mol of base)$^{-1}$ L^{-1} at 256 nm derived for $d(G_3T_4G_3)$ by Scaria et al. (1992). Na^+ concentrations for samples used in titrations were determined by comparing the integrated intensity of the ^{23}Na line at 132.28 MHz on a Bruker model AMX 500 spectrometer to the integrated intensities of a set of standard NaCl samples.

NMR Spectroscopy. One-dimensional NMR proton spectra for studies of ionic strength dependence (i.e., titrations of NaCl into NaCl and KCl into KCl) and NaCl into KCl titrations were collected at 500 MHz on a General Electric model GN500 spectrometer at 25 °C. Water suppression was accomplished with the $1\bar{1}$ spin echo pulse sequence (Sklenář & Bax, 1987). Spectra for titrations of KCl into NaCl were collected at 500 MHz on a Bruker model AMX 500 spectrometer at 5 °C increments between 15 and 35 °C. Water suppression was accomplished using the Watergate pulse sequence (Piotto et al., 1992). Spectra were acquired with a 10 kHz sweep width, 8K points, and 128−512 FIDs were accumulated for each one-dimensional spectrum.

Sample Titration. For each titration, 1−5 μL aliquots of concentrated salt solutions were added to 400 or 450 μL NMR samples and mixed thoroughly. NMR samples were concentrated as required under a stream of nitrogen gas so that the sample volumes did not increase more than 2% during a complete set of titrations. The NMR samples were thermally equilibrated in the magnet for 5−10 min before each data collection.

Data Analysis. Spectra were processed using the Bruker software package UXNMR or FELIX (Biosym Technologies, San Diego). Chemical shifts and line widths were extracted from the spectra by least-squares fits of a Lorentzian function with a linear baseline correction using the data analysis software IGOR Pro (WaveMectrics, Lake Oswego, OR). Chemical equilibrium models were fit to chemical shift data using a least-squares minimization program written and utilized within Mathematica (Wolfram Research, Inc., Champaign, IL).

RESULTS

^1H NMR Spectra of $d(G_3T_4G_3)$ in NaCl and KCl are Different. In 50 mM NaCl solution, the oligonucleotide $d(G_3T_4G_4)$ forms a dimeric quadruplex containing three G quartets with the thymines forming loops which go diagonally across the end G quartets (Smith et al., 1994; Keniry et al., 1995) (Figure 1). The structure is essentially the same as the symmetric quadruplex formed by $d(G_4T_4G_4)$ (Oxy-1.5; derived from the repeat sequence found in *Oxytricha* telomeres) except for the removal of one G quartet (Smith & Feigon, 1992; Schultze et al., 1994). However, due to the handedness of G quartets, $d(G_3T_4G_4)$ forms an asymmetric quadruplex, and thus, resonances for each of the two strands in the quadruplex are observed (Scaria et al., 1992; Smith et al., 1994) (Figure 2).

One-dimensional ^1H NMR spectra of the aromatic and methyl resonances of $[d(G_3T_4G_3)]_2$ in 50 and 300 mM NaCl and 50 and 300 mM KCl are shown in Figure 2. The ^1H chemical shifts of $[d(G_3T_4G_3)]_2$ in both NaCl (Smith et al., 1994; Keniry et al., 1995) and KCl (F. W. Smith and J. Feigon, unpublished results) have been completely assigned. While the proton chemical shifts of this molecule exhibit

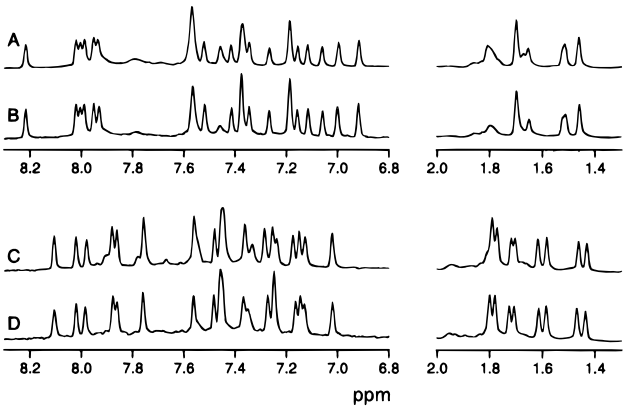

FIGURE 2: One-dimensional ^{1}H NMR spectra of $[d(G_3T_4G_3)]_2$ at 25 °C and pH 6.0: in 50 mM NaCl (A), after titration of sample A to 300 mM NaCl (B), in 50 mM KCl (C), and after titration of sample C to 300 mM KCl (D). Sample concentrations were 2.0 mM (A and B) and 1.5 mM (C and D) in oligonucleotide strand. The double set of resonances results from the asymmetric structure adopted by $[d(G_3T_4G_3)]_2$.

minimal changes as a function of ionic strength in both NaCl and KCl, the NaCl proton spectrum differs significantly from the KCl spectrum. This suggests that the sodium and potassium forms of $[d(G_3T_4G_3)]_2$ contain some structural differences. However, two-dimensional ^{1}H NMR spectroscopy studies performed in our laboratory (data not shown) reveal that the dimeric diagonally looped foldback structure is adopted in both NaCl and KCl at 50 mM ionic strength and is retained over the course of these ionic strength titrations for both cationic species. Similar cation-dependent spectra are observed for Oxy-1.5 (Smith & Feigon, 1993) for which NMR-derived structures have been solved in both NaCl solution (Schultze et al., 1994) and KCl solution (F. W. Smith et al., in preparation). While some local details of the structures vary in NaCl and KCl, the overall fold is the same in both cases. It is perhaps not surprising that apparently small structural changes can produce such distinct ^{1}H spectra when one considers the hyperpositional sensitivity of proton chemical shifts in proximity to aromatic rings (Wüthrich, 1986), the high density of closely packed aromatic rings in the quadruplex structures whose exact positions are likely to be directly affected by a change in the size of the coordinated ion, and the electronic effects of the coordination of different ions.

Titration of KCl into an NaCl Sample. Figure 3 shows the ^{1}H NMR spectra of the aromatic and methyl regions of $[d(G_3T_4G_3)]_2$ acquired at 25 °C during a KCl titration of a sample originally in 65 mM NaCl at pH 6.0. As KCl is titrated in, the resonance lines begin to shift. Spectral changes are detectable at KCl/NaCl ratios of less than 1/50. At the upper end of the titration, where the KCl/NaCl ratio is approximately 8/1, the spectrum is essentially identical to that exhibited in pure KCl. ^{1}H NMR spectra have also been collected for an analogous titration series in which NaCl was added to a solution of $[d(G_3T_4G_3)]_2$ originally containing only KCl (data not shown). In this case, the changes in the spectra are indicative of a gradual progression from the potassium form to the sodium form. The changes observed in the heterogeneous salt titration spectra (Figures 3) occur without the appearance of additional peaks or significant line broadening. This indicates that an exchange between NMR distinct species, i.e., $[d(G_3T_4G_3)]_2$ molecules coordinating

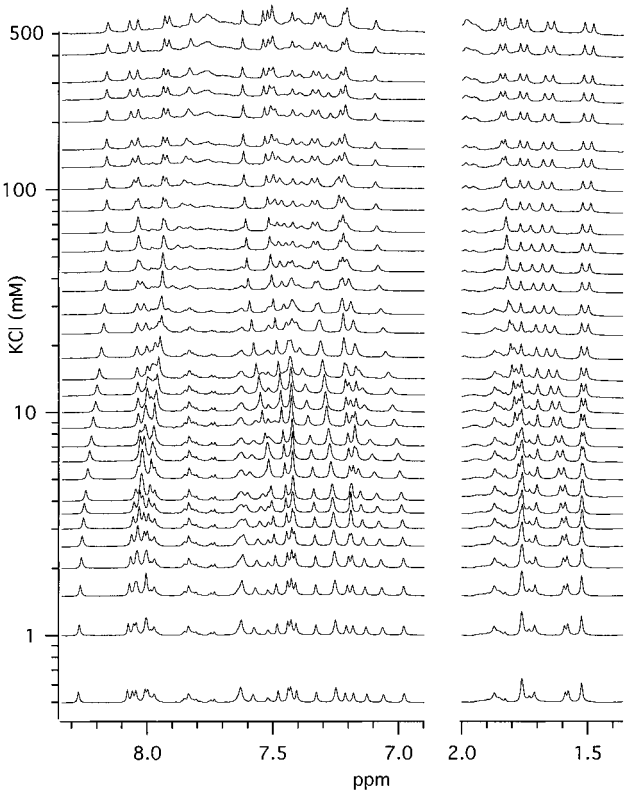

FIGURE 3: One-dimensional ^{1}H NMR spectra of $[d(G_3T_4G_3)]_2$ taken during a KCl titration at 25 °C. Sample is 1.68 mM in oligonucleotide strand at pH 6.0 and initially in 65 mM NaCl. Vertical placement of spectra corresponds to the logarithmic concentration of added KCl. Note the progression of the spectra from the pure NaCl form to essentially that of the pure KCl form shown in Figure 2.

different species of ions, is taking place at a rate which is fast on the NMR time scale. Similar results were obtained for spectra collected at 15, 20, 30, and 35 °C.

The spectral changes observed can be attributed to the displacement of Na^+ by K^+ from G quartet ion coordination sites. Phosphate−ion interactions (i.e., atmospheric binding) are not likely to be the source of any of the observed spectral alterations since it has been shown that DNA quadruplexes do not show selectivity in the atmospheric binding of K^+ with respect to Na^+ (Xu et al., 1993; Deng & Braunlin, 1996).

Chemical Shifts as a Function of KCl Concentration. Chemical shifts in the spectra as a function of KCl concentration can be followed for the majority of proton resonances. The methyl proton resonances are better-resolved than the aromatic and the imino proton resonances (data not shown) throughout the range of titrations, and their chemical shifts can therefore be determined with greater precision and are thus the most useful for analysis. The chemical shifts at 25 °C for the five best-resolved methyl protons of $[d(G_3T_4G_3)]_2$ as a function of KCl concentration for a sample of fixed NaCl concentration are plotted in Figure 4. The majority of the resonances shift monotonically from the initial (sodium form) to a final (potassium form) chemical shift. However, this is not the case for all of the resonances. The chemical shift of methyl 17, for example, decreases from an initial value of 1.71 to 1.70 ppm at 9.5 mM added KCl and then increases to a final value of 1.75 ppm. This change in chemical shift cannot be attributed to increased ionic strength, since no significant spectral changes occur upon

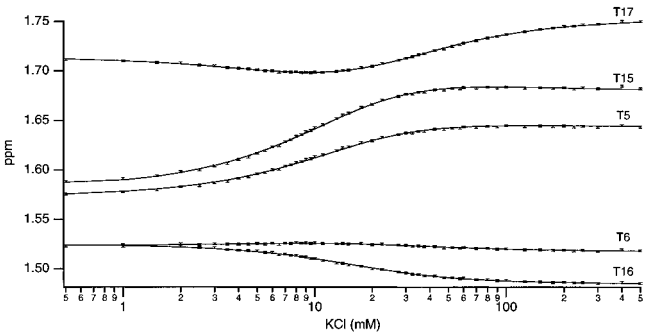

FIGURE 4: ^{1}H chemical shifts of methyls 17, 15, 5, 6, and 16 in 65 mM NaCl as a function of added KCl. Error bars represent $\pm 1/2$ spectral resolution of the data. Solid lines represent best fits of the two-site model (eq 1). For these fits, δ_a and δ_c have been restrained to be within the experimental error of the proton chemical shifts at 0 mM KCl and 500 mM KCl, respectively. The unobserved intermediate chemical shift (δ_b) is the only independent minimization parameter for each curve. K_1 and K_2 (3.15 and 5.4, respectively, for all curves) were derived from simultaneous fits of the ^{1}H chemical shifts curves of methyls 17 and 5. The concentration of free sodium was approximated as constant.

the titration of NaCl into NaCl samples or KCl into KCl samples up to at least 300 mM (Figure 2). Proton chemical shifts in the less-resolved aromatic region have also been found which exhibit features similar to those exhibited by methyl 17.

For a system undergoing chemical exchange which is fast on the NMR time scale, the observed chemical shift of a particular proton is equal to the weighted average of its chemical shifts in each species which participates in the exchange (Sandström, 1982). If titration from the sodium to the potassium form of $[d(G_3T_4G_3)]_2$ involves exchange between only two NMR distinct species, then all proton chemical shift changes would exhibit curves which progress monotonically from an initial to a final chemical shift. Such would be the case if the conversion from the sodium to the potassium form involved no intermediate forms. However, the chemical shift changes observed for methyl 17, as well as some of the aromatic resonances, are consistent only with an additional NMR distinct intermediate. This intermediate(s) is apparently in fast exchange with the pure sodium and the pure potassium form of $[d(G_3T_4G_3)]_2$ and is indicative of the presence of a mixed ionic species, i.e., $[d(G_3T_4G_3)]_2$ with simultaneously coordinated Na^+ and K^+. *We therefore conclude that at least two K^+ ion binding events occur during the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form.*

Determination of the Number of Ion Coordination Sites in $[d(G_3T_4G_3)]_2$. The chemical shift data reported above were analyzed in terms of possible ion binding models. $[d(G_3T_4G_3)]_2$, with three successive G quartets, would be expected to contain one to three ion coordination sites. The single-ion binding model has already been ruled out above on the basis of the shape of the chemical shift titration curves.

A two-ion binding model for the conversion from the sodium to the potassium form involves two equilibrium constants and three molecular species. Explicitly,

$$NaNaG_3 + K^+ \overset{K_1}{\longleftrightarrow} KNaG_3 + Na^+$$

$$KNaG_3 + K^+ \overset{K_2}{\longleftrightarrow} KKG_3 + Na^+$$

where $NaNaG_3$, $KNaG_3$, and KKG_3 represent $[d(G_3T_4G_3)]_2$ coordinating two Na^+, one K^+ and one Na^+, and two K^+, respectively. For this equilibrium model, the relative populations are determined by the following simultaneous equations:

$$[KCl] = [K^+] + [KNaG_3] + 2[KKG_3]$$

$$[G_3] = [NaNaG_3] + [KNaG_3] + [KKG_3]$$

$$[KNaG_3] = K_1[K^+][NaNaG_3]/[Na^+]$$

$$[KKG_3] = K_2[K^+][KNaG_3]/[Na^+]$$

where [KCl] is the concentration of added KCl, which is equal to the total concentration of all potassium species, bound and free, and $[G_3]$ is the total concentration of $[d(G_3T_4G_3)]_2$ in all three forms. For the spectra presented in Figure 3, the total sodium concentration was approximately 65 mM and $[G_3]$ was less than 1.7 mM in strand, so that the change in free sodium concentration with the displacement of all Na^+ by K^+ over the entire course of the titration would result in a change in the free sodium concentration ($[Na^+]$) of less than 3%. Thus, we make the approximation that the free sodium concentration is equal to the total sodium concentration and constant over the course of the KCl titration.

For the two-binding site model, with three species in fast exchange, the observed chemical shift (δ_o) for a particular proton will be

$$\delta_o = [NaNaG_3]\delta_a + [KNaG_3]\delta_b + [KKG_3]\delta_c \quad (1)$$

where δ_a, δ_b, and δ_c are the chemical shifts of the proton in $NaNaG_3$, $KNaG_3$, and KKG_3, respectively.

If the two-binding site model accurately describes the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form, then eq 1 must be able to describe the chemical shift curves for all resonances as a function of relative KCl/NaCl concentrations. The parameters involved in fitting eq 1 to a particular proton chemical shift are K_1 and K_2, which determine the relative populations of the three species as a function of ion concentration, and the species specific chemical shifts δ_a, δ_b, and δ_c. The values of δ_a for all protons in the titration are given by the observed chemical shift in the initial NaCl spectrum before the addition of any KCl. Additionally, δ_c can be estimated by the value to which the observed chemical shifts asymptotically approach at high KCl concentrations. Thus, for each proton chemical shift curve, there are only three undefined parameters: the two chemical equilibrium constants and the chemical shift of the intermediate species (δ_b). Since the equilibrium constants must be the same for all proton chemical shift curves, δ_b is the only purely independent parameter for each curve.

Figure 4 shows the curves of the best least-squares fits for the two-ion binding site model to the chemical shifts of the five best-resolved methyl resonances as a function of added KCl. Perfect fits to the chemical shift data are obtained using this model. The quality of these fits supports the presence of two ion coordination sites in $[d(G_3T_4G_3)]_2$ and gives no indication of any additional site specific ion-binding events. Aromatic proton chemical shift curves are

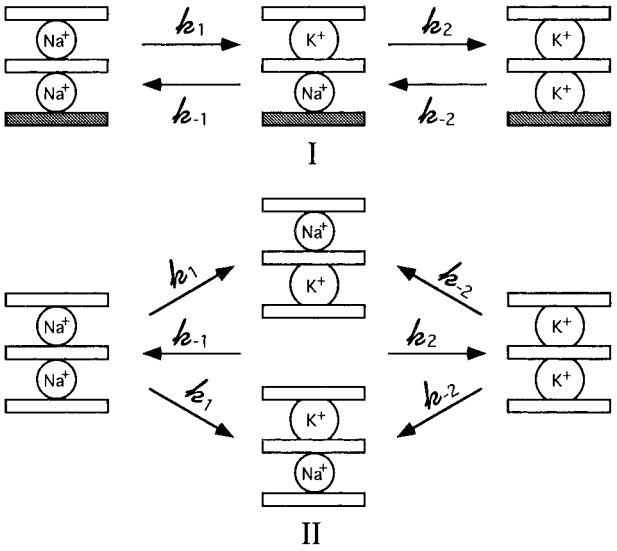

FIGURE 5: Two displacement pathways, I and II, represent the two extremes for the potential effect of unequivalent ion binding due to the two structurally distinct interplanar ion coordination sites of $[d(G_3T_4G_3)]_2$. In pathway I, the two ion coordination sites have ion binding affinities which differ to such a degree that one site always participates in ion exchange before the second site and there is no exchange between the sites (shaded bar, representing a G quartet, indicates the asymmetry of the molecule). In pathway II, the coordination sites are identical with respect to ion exchange. These two models are indistinguishable on the basis of the fitting of eq 1 to the chemical shift titration data.

also found to be consistent with the equilibrium constants derived from fits of the methyl chemical shifts.

Relative Magnitude of K_1 and K_2. The close proximity of neighboring G quartet ion coordination sites presents the possibility that direct or allosteric interactions resulting from the first K^+ binding event affect the second such binding in a cooperative or anticooperative manner. Thus, it can not be assumed *a priori* that the equilibrium constants K_1 and K_2 of the two-binding site model are of equal magnitude. Furthermore, the asymmetric nature of $[d(G_3T_4G_3)]_2$ also presents the possibility that the two ion binding sites, which are structurally distinct, have inherently different binding affinities for Na^+ and K^+.

The two limiting cases for ion exchange arising from binding site heterogeneity are illustrated schematically in Figure 5. These are as follows: (pathway I) the two coordination sites of $[d(G_3T_4G_3)]_2$ differ to such a degree that one particular site is always the first to exchange Na^+ for K^+ and there is no exchange between the two sites, and (pathway II) ion binding is absolutely equal for the two sites. Our chemical shift titration data can be equivalently fit by eq 1 for either ion-loading pathway, with the only difference being that $K_1 = [NaKG_3][Na^+]/([NaNaG_3][K^+])$ and $K_2 = [KKG_3][Na^+]/([NaKG_3][K^+])$ for loading pathway I, whereas $K_1 = [NaKG_3][Na^+]/(2[NaNaG_3][K^+])$ and $K_2 = 2[KKG_3][Na^+]/([NaKG_3][K^+])$ for loading pathway II. Thus, the product of K_1 and K_2 is independent of the loading pathway, but the ratio K_1/K_2 for pathway I is 4 times the corresponding value for pathway II.

We are unable to determine the absolute values of K_1 and K_2 as a result of our lack of knowledge concerning the actual values of the intermediate chemical shifts (δ_b). However, regardless of the pathway by which Na^+ is displaced by K^+ from $[d(G_3T_4G_3)]_2$, the relative magnitudes of the K_1 and K_2

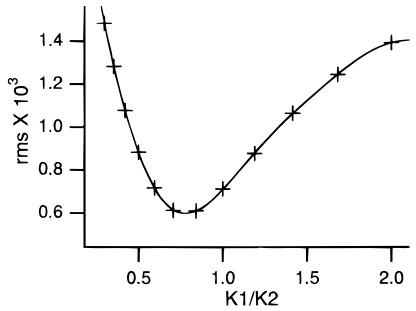

FIGURE 6: Root-mean-squared values from the best simultaneous fits of the chemical shift titration curves of methyls 17 and 5 as a function of the ratio K_1/K_2. The solid line is a polynomial fit of the calculated values which are designated by crosses. The particular values shown assume ion loading as represented by pathway I (Figure 5). The same results are obtained for pathway II, except K_1/K_2 values along the abscissa are divided by a factor of 4.

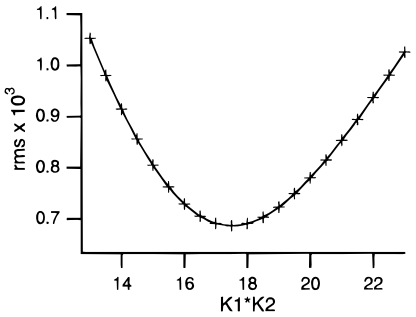

FIGURE 7: Plot of rms values for a series of fits to the ^1H chemical shift titration curves of methyls 17 and 5 as a function of the product K_1K_2. The solid line is a polynomial fit of the calculated values which are designated by crosses. While 17.5 provides an optimal fit of the two-site model to the data presented in Figure 4, values from 13.5 to 21.5 yield curves with rms values which are within experimental error.

values which satisfy the data are considerably constrained. Curve fits which have overall root mean square (rms) values within experimental error of our data (on the basis of simultaneous fits of the chemical shift curves of methyl 17 and methyl 5, the two curves which exhibit the greatest degree of change over the course of titrations and thus provide the most information) are found to be restricted to a range of K_1/K_2 values between 0.4 and 1.4 for ion exchange via pathway I or, alternately, to a range indicative of significant cooperativity of 0.1−0.35 for pathway II (Figure 6). The two ion-loading scenarios presented (Figure 5) only represent the two most extreme cases. It may very well be the case that the ion exchange kinetics of $[d(G_3T_4G_3)]_2$ follow a pathway intermediate between the two extremes represented by pathway I and II, which would also be indistinguishable by our analysis.

Free Energy Difference of Sodium and Potassium Binding by $[d(G_3T_4G_3)]_2$. The product K_1K_2, which depends primarily upon the curvature of the chemical shift curves and only weakly upon the actual values of the intermediate chemical shifts, is found to be essentially constant for the entire range of relative K_1 and K_2 values which give satisfactory fits of the data. In order to extract the net free energy from these data, we have performed a series of least-squares fits to the chemical shift data for methyl 17 and methyl 5 where the product K_1K_2 has been constrained to particular values during minimization. Shown in Figure 7 is a plot of rms values (as an indication of the quality of the fit) versus the product K_1K_2. A value of 17.5 provides an optimal fit of the data

(i.e., Figure 4). No pairs of values for K_1 and K_2 with products outside the range of approximately 13.5−21.5 can be found that give curves which are within experimental error of the data. These values reveal that the free energy change for the displacement of two Na^+ from $[d(G_3T_4G_3)]_2$ by two K^+ is -1.7 ± 0.15 kcal/mol at 25 °C.

Ion Selectivity as a Function of Temperature. In addition to the 25 °C data presented in Figure 3, we have acquired titration spectra at temperatures ranging from 15 to 35 °C with the goal of determining $\Delta H°$ and $\Delta S°$ for the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form. However, over this range of temperatures, $\Delta G°$ values are found to differ in magnitude less than our interval of confidence (±0.15 kcal/mol). While this does not permit a precise determination of $\Delta H°$ or $\Delta S°$, we are able to define a finite range of possible $\Delta H°$ and $\Delta S°$ values at 25 °C of -1.7 ± 4.5 kcal/mol and 0 ± 15 cal mol^{-1} K^{-1}, respectively. It should be noted that the rather low level of precision in these values is not as much a reflection of an imprecise determination of $\Delta G°$ as it is of the error inherent to what is essentially an extrapolation to absolute zero from a 20 K temperature range centered around 298 K.

DISCUSSION

The Coordinated Ions of $[d(G_3T_4G_3)]_2$ Are in Fast Exchange. The proton NMR spectra of $[d(G_3T_4G_3)]_2$ in NaCl are distinct from those in KCl (Scaria et al., 1992). Since two-dimensional ^{1}H NOESY spectra indicate that the same general structure is adopted by this molecule in the presence of both cations, the differences in ^{1}H chemical shifts are apparently the result of minor differences in local structural features which depend upon the type and perhaps simply the size of the coordinated ion. In a solution containing both Na^+ and K^+, the ^{1}H chemical shifts of $[d(G_3T_4G_3)]_2$ differ from those exhibited in the corresponding homogeneous salt solutions. The absence of additional peaks and significant line broadening in heterogeneous salt solutions (Figure 3) indicates that the sodium and potassium forms of $[d(G_3T_4G_3)]_2$ are in fast exchange on the NMR time scale over the temperature range in the present study (15−35 °C). That is, cations bound by G quartets are exchanging with ions in the bulk solution at a rate greater than 10^3 s^{-1}. This is consistent with the results of Deng and Braunlin (1996), who have shown that the bound lifetime of Na^+ in the closely related molecule $[d(G_4T_4G_4)]_2$ is approximately 180 μs at 20 °C. In contrast, the imino protons within the quartets of the same molecule are protected from exchange for on the order of weeks to months (Smith & Feigon, 1992). The difference in these time scales suggests that ions enter and exit through the ends of the DNA quadruplex without disruption of G quartet hydrogen bonding (Deng & Braunlin, 1996).

Two Ions Are Bound by $[d(G_3T_4G_3)]_2$. Analysis of changes in the one-dimensional ^{1}H NMR spectra of $[d(G_3T_4G_3)]_2$ at different Na^+ and K^+ concentrations reveals that the binding of at least two K^+ is required for the complete conversion of this molecule from the sodium to the potassium form. Furthermore, a chemical equilibrium model in which two Na^+ are displaced by two K^+ is able to fit the titration data within experimental error. While these results support the two-ion binding site model, they alone do not rule out the possibility of more than two Na^+ or K^+ ion binding sites. Three or more ion binding sites would, however, be difficult if at all possible to reconcile with features of G quartets reported in the literature.

Of particular relevance to the question of how many Na^+ ion binding sites are in a DNA quadruplex is the recent high-resolution (1.2 Å) crystal structure of the oligonucleotide $d(TG_4T)$ reported by Laughlan et al. (1994). In the crystal form studied, pairs of parallel-stranded tetramers stack together such that a continuous quadruplex with eight equally spaced G quartets is formed. Although Na^+ is small enough to fit in the plane of a G quartet, this is not the case for most of the bound Na^+ in the crystal structure. Within the eight quartets, seven Na^+ are located along the central axis and primarily bind between successive planes of G quartets. If this interplanar coordination of ions is generally favored by DNA quadruplexes, then N successive G quartets will generally contain $N - 1$ ion binding sites. The solution state investigations of Deng and Braunlin (1996) give additional support to this being the case, for they have shown that at most three Na^+ are coordinated by the four G quartets of the dimeric quadruplex of Oxy-1.5 which is structurally very similar to $[d(G_3T_4G_3)]_2$. K^+ is too large to fit in the plane of a G quartet. In the crystal structure of Oxy-1.5, diffuse electron density for only one K^+ was identified, located between the central two quartets (Kang et al., 1992). However, our data are consistent only with at least two K^+ displacing the Na^+ in $[d(G_3T_4G_3)]_2$. Combining our data with the literature data, we conclude that there are only two specific ion binding sites in $[d(G_3T_4G_3)]_2$ and these can be occupied by either Na^+ or K^+.

Relative Free Energy of Na^+ and K^+ Binding by $[d(G_3T_4G_3)]_2$. Our analyses indicate that the product of the equilibrium constants for the displacement of two Na^+ by two K^+ from $[d(G_3T_4G_3)]_2$ is between 13.5 and 21.5, which corresponds to a free energy difference ($\Delta G°$) of -1.7 ± 0.15 kcal/mol at 25 °C. This result has been determined under equilibrium conditions, for the exchange time between the ionic forms of $[d(G_3T_4G_3)]_2$ is short (less than 10 ms) with respect to the time given for thermal equilibration of the sample (5−10 min) prior to data collection.

Due to the extraordinarily slow kinetics of G quartet formation and dissociation, thermodynamic parameters for G quartet molecules determined on the basis of temperatures of denaturation are prone to considerable error (Williamson, 1994). For example, errors in T_m values for two different G quartet molecules have been reported to be as great as 15−50 °C when a heating rate of 1 °C/min, a rate commonly used in many laboratories, was used during the denaturation studies (Williamson, 1994; Wyatt et al., 1996). Furthermore, Wyatt et al. (1996) have recently demonstrated that even at a rate as slow as 0.025 °C/min the T_m for the tetrameric quadruplex $d(T_2G_4T_2)$ is overestimated by almost 40 °C. Under these circumstances, it is not surprising that the relative free energy of the potassium and sodium forms of $d(G_3T_4G_3)_2$ based upon the difference in the reported free energies of quadruplex formation for this molecule in 100 mM NaCl and 100 mM KCl is in disagreement with our results (Scaria et al., 1992). This T_m-based value, −4.2 kcal/mol at 25 °C, corresponds to an equilibrium constant in excess of 10^3. This large equilibrium constant is inconsistent with the ^{1}H chemical shift curves which are observed; i.e., the chemical shifts would approach their final position at much lower KCl concentrations than found here.

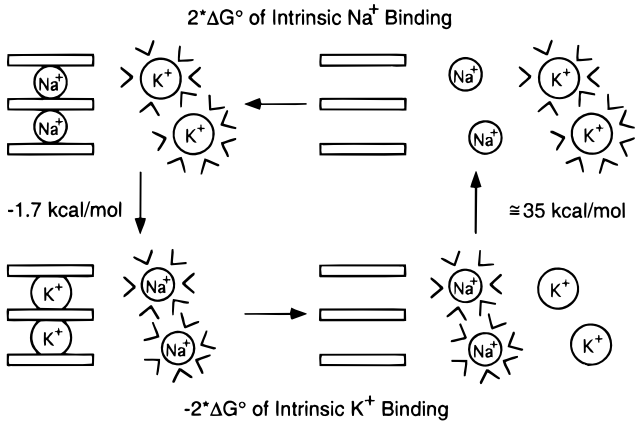

FIGURE 8: Free energy cycle for the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form in aqueous solution. Note that the relatively large difference between the free energy of dehydration for two Na^+ and that for two K^+, with respect to the free energy determined here for the conversion of $[d(G_3T_4G_3)]_2$ from the sodium to the potassium form, indicates that Na^+ is actually more favorably coordinated by G quartets than K^+. However, K^+ is selectively bound over Na^+ because of its less unfavorable free energy of dehydration.

Raghuraman and Cech (1990) have estimated the free energy of formation for $d(T_4G_4)_4$ on the basis of the kinetics of quadruplex formation to be −2.2 and −4.7 kcal/mol in 50 mM NaCl and 50 mM KCl, respectively. This corresponds to an average free energy difference of −0.8 kcal/mol for each coordinated ion, assuming three binding sites for their four-G quartet molecule, which is in agreement with our result.

Role of Ion Hydration in G Quadruplex Ion Selectivity. The free energy for two bound K^+ with respect to two bound Na^+ at 25 °C (−1.7 ± 0.15 kcal/mol) is surprisingly small compared to the estimated 17.6 kcal/mol difference between the free energies of Na^+ and K^+ hydration at 25 °C (Burgess, 1978). A free energy cycle which includes the free energies of hydration and the relative free energies we have determined here for ion replacement reveals that *Na^+ actually binds the coordination sites of $[d(G_3T_4G_3)]_2$ with a more favorable free energy than K^+* (Figure 8). However, Na^+ is displaced by K^+ because of the more negative hydration free energy of Na^+. As a further illustration of this point, we consider the hypothetical case in which Na^+ and K^+ bind the coordination sites of $[d(G_3T_4G_3)]_2$ with equal affinity. In that case, the difference between the stability of the potassium form and the sodium form would be equal to the difference of the free energy of hydration of two Na^+ ions versus two K^+ ions, i.e., approximately −35 kcal/mol. Such a free energy would correspond to an equilibrium constant between the two forms on the order of 10^{12}. The consequence of this would be proton chemical shift curves that approach their final values at an added KCl concentration which is equal to the concentration of G quartet ion binding sites, which is not observed experimentally. Furthermore, no spectral changes would occur upon the addition of NaCl to a sample initially in excess KCl, whereas we observe changes at and below equal molar ratios of added NaCl.

Selectivity between two ionic species by an ionophore that is predominately driven by the more favorable hydration of the smaller ion is not a novel concept. Over thirty years ago, Eisenman (1962) showed that a simple electrostatic model of ion binding by cation selective glass electrodes versus hydration gave rise to eleven possible selectivity sequences for the five alkali metals (Li^+, Na^+, K^+, Rb^+, and Cs^+) out of the total of 120 possible sequences. These selectivity sequences are among those most commonly found in chemistry and biology. The eleven so-called Eisenman series begin with a large binding site where the electrostatic field is correspondingly weak. Here, ion selectivity is governed solely by hydration free energies yielding selectivity sequence I: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. At the other end of the spectrum is the small binding site with a correspondingly larger field strength. Here, the smaller ions are able to approach attractive groups within the site more closely than larger ions, yielding sequence XI: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. On the basis of the T_m of br^8G gels (Guschlbauer et al., 1990), G quartets exhibit Eisenman sequence V: $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$. This is also consistent with the stabilities reported for a variety of oligonucleotide quadruplexes as a function of coordinated ion (Williamson, 1994).

While Eisenman's theory of equilibrium selectivity of ions has undergone some revision from its more simple original form (Eisenman & Horn, 1983), the basic premise that selectivity sequences reflect the competition between hydration and ligand interactions which are both continuously decreasing functions of increasing ionic radius has remained a very informative model for understanding the ion selectivity exhibited by peptide ionophores and ion channels, for example (Masut & Kushick, 1985; Hille, 1992). Recently, Ross and Hardin (1994) used free energy perturbation calculations to investigate the origin of ion selectivity by G quartets. While the results of this theoretical study incorrectly predict the preferential binding of Na^+ with respect to K^+, the free energy of ion binding within a G quartet was predicted to be a monatomically increasing function of ionic radius, which is what would be expected by a system which follows an Eisenman selectivity series.

Ion-Induced Polymorphism. Several investigations have shown that oligonucleotides which form G quartet structures can exhibit a structural polymorphism between parallel- and antiparallel-stranded structures, with K^+ in some instances showing a preference for parallel structures (Williamson, 1994). A recent Raman spectroscopic study by Miura et al. (1995) has provided a detailed analysis of the ion-dependent conformational polymorphism of $d(T_4G_4)_4$. On the basis of analysis of the data, they conclude that $d(T_4G_4)_4$ (Oxy-4) is in the foldback monomeric structure in low-concentration NaCl and KCl solutions, similar to the monomeric quadruplex $d(G_4T_4G_4T_4G_4T_4G_4)$ (Oxy-3.5) (Smith & Feigon, 1992; Smith et al., 1995; Wang & Patel, 1995) and the dimeric quadruplexes $[d(G_4T_4G_4)]_2$ (Oxy-1.5) (Smith & Feigon, 1992) and $[d(G_3T_4G_3)]_2$ (Smith et al., 1994; Keniry et al., 1995). However, Miura et al. (1995) find that $d(G_4T_2)_4$ favors a parallel tetrameric structure in higher concentrations of either ion. This structural transition occurs at 65 mM KCl and at 225 mM NaCl for homogeneous salt solutions and at intermediate ionic strengths for heterogeneous salt solutions. A similar transition of $[d(G_3T_4G_3)]_2$ from a foldback to a parallel structure might also be expected. However, the minimal alterations observed in the proton spectra from our homogeneous salt titrations and two-dimensional ^1H NMR spectroscopy indicate that $[d(G_3T_4G_3)]_2$ remains in the dimeric foldback structure for all of the data presented in the present study. In the study presented by Miura et al.

(1995), data were obtained from solutions of $d(G_4T_2)_4$ which had been brought to thermodynamic equilibrium by annealing at 80 °C for 1 h. In the present study, samples were not heated above 35 °C once titrations had been initiated. Thus, the ^{1}H NMR spectra collected for $[d(G_3T_4G_3)]_2$ at high NaCl and KCl concentrations may represent structures which have been kinetically trapped at a lower ionic strength. Such behavior for a G quartet structure would not be surprising considering the slow kinetics of quadruplex dissociation. For example, $d(T_4G_4)_4$ has been shown to be kinetically trapped in quadruplex structures even in the presence of complementary strands which thermodynamically favor the formation of Watson−Crick duplex structures (Hardin et al., 1991, 1992).

Conclusions. Analysis of ^{1}H NMR spectra of $[d(G_3T_4G_3)]_2$ as a function of added NaCl and KCl has shown that the preferential binding of K^+ versus Na^+ to this quadruplex is dominated not by a better fit of K^+ compared to that of Na^+ in the cavity between the planes of two G quartets but rather by the preferential hydration of Na^+ compared to K^+, analogous to what has previously been observed for peptide ionophores and ion channels. We find that, upon addition of KCl to a sample of $[d(G_3T_4G_3)]_2$ originally in the sodium form, two K^+ selectively replace two bound Na^+. The free energy change ($\Delta G°$) for the conversion from the sodium to the potassium form is -1.7 ± 0.15 kcal/mol, with $\Delta H°$ and $\Delta S°$ having limits of -1.7 ± 4.5 kcal/mol and -15 cal mol^{-1} K^{-1} at 25 °C, respectively. On the basis of the results presented here and similar ion-dependent chemical shifts observed for the related quadruplex Oxy-1.5 (manuscript in preparation), we conclude that in general one K^+ ion is bound between each pair of G quartets in G quadruplexes.

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