Selective Localization and Rotational Immobilization of Univalent Cations on Quadruplex DNA†

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ABSTRACT: The quadruplex structure of the oligomer $d(T_2G_4T)$ is more stable in the presence of K^+ than in the presence of Na^+. This enhanced stability correlates with the preferential binding of K^+ to a small number of specific sites on the quadruplex. In contrast, Na^+ and K^+ compete on an equal footing for atmospheric binding. Both $^{39}K^+$ and $^{23}Na^+$ are, when specifically bound, significantly inhibited in their rotational mobility, so that the quadrupolar relaxation reflects the molecular tumbling of the oligomer, which occurs on the time scale of nanoseconds. This rotational immobilization is in distinct contrast to the high rotational mobility of atmospherically bound cations. On the other hand, all NMR-visible $^{39}K^+$ in solution is in rapid exchange among all environments (free, specifically bound, and atmospherically bound) implying that the lifetime of specifically coordinated $^{39}K^+$ must be significantly shorter than a millisecond. A similar conclusion holds for $^{23}Na^+$. The oligomer $d(T_2G_4T)$ forms two distinct Hoogsteen base-paired structures in NaCl solution, separated by a large kinetic barrier. Neither of these structures is as stable with respect to base pair opening as is the quadruplex structure formed in KCl solution. Only one of these two structures is associated with rotational immobilization of bound $^{23}Na^+$.

In the G-quartet structural motif, four guanine bases associate via Hoogsteen base pairing in a nearly planar array [reviewed by Guschlbauer et al. (1990) and Sen and Gilbert, (1991)]. In general, such a G-quartet structure does not occur in isolation but rather is stabilized by neighboring quartets. Although such structures have been known for some time (Arnott et al., 1974; Zimmerman et al., 1975), interest has been rekindled in recent years by the possibility that they may occur at the ends of chromosomes (Henderson et al., 1987) and may play a role in recombination events during immunoglobulin gene rearrangements (Sen & Gilbert, 1988). One striking aspect of G-quartet structures is a remarkable selectivity for univalent cations. In particular, K^+ stabilizes G-quartets much more strongly than does Na^+. Moreover, it seems clear that different quadruplex structures can be formed in the presence of K^+ and Na^+ from oligomers such as $d(T_2G_4)_4$ (Hardin et al., 1991) and $d(G_4T_4G_4)$ (Kang et al., 1992; Smith & Feigon, 1992). The picture that has arisen is of a cation cavity, much like those found in cation-specific ionophore complexes (Dobler, 1981). Selectivity and structural variability can be simply rationalized based on cation size.

Although the ionophore model is compelling, there has been little direct evidence to support it. In the crystal structure of $d(G_4T_4G_4)$, a suggestive region of electron density was found between planes of stacked G-quartets and was assigned to bound K^+ (Kang et al., 1992). In solution, preferential binding of K^+ compared to Na^+ was demonstrated in the pioneering cation NMR studies of Laszlo and colleagues on the formation of guanine nucleotide gels (Borzo et al., 1980; Detellier & Laszlo, 1980).

Cation NMR has contributed greatly to our understanding of how simple cations interact with double-helical DNA molecules (Padmanabhan et al., 1990). Early ^{23}Na NMR relaxation studies provided a picture of a loosely ("atmospherically") bound, largely salt-independent layer of rotationally mobile counterions. Similar results were obtained for $^{39}K^+$, and $^{87}Rb^+$, in support of the notion of nonspecific, electrostatic binding (Braunlin & Nordenskiöld, 1984; Padmanabhan et al., 1990). Studies with the multivalent cations $^{25}Mg^{2+}$, $^{43}Ca^{2+}$, and $^{59}Co(NH_3)_6^{3+}$ indicated a somewhat more complex situation involving an interplay between sequence-specific localized binding and nonspecific electrostatic association (Braunlin et al., 1991; Braunlin et al., 1987; 1989; Braunlin & Xu, 1992). Localized binding has been shown to correlate with dramatic structural transitions of duplex DNA (Xu et al., 1993a,b). Combining cation NMR with structural studies provides a powerful means to delineate the roles of simple cations in stabilizing DNA structures.

Oligonucleotides such as $d(T_4G_4)$ can form higher order aggregates in the presence of K^+, but not Na^+ (Sen & Gilbert, 1992; Lu et al., 1992). In contrast, oligomers such as the one studied here, $d(T_2G_4T)$, with 3′ and 5′ terminal thymines, form four-stranded structures in solution but do not polymerize to form higher order aggregates (Sen & Gilbert, 1992; Lu et al., 1992). Here we investigate the involvement of univalent cations in stabilizing G-quartet structures of the oligonucleotide $d(T_2G_4T)$. The oligonucleotide $d(T_2G_4T)$ was chosen because it does not aggregate, even in the presence of K^+, and because it can form a unique structure involving four parallel strands (Wang & Patel, 1992). In agreement with these predictions, *the narrow nonexchangeable proton NMR line widths that we observe under all conditions demonstrate that no higher order aggregates are formed with this oligomer.* The exchangeable imino and amino protons and the nonexchangeable base and thymine methyl protons of this oligonucleotide provide a convenient monitor of the DNA structure (Wang & Patel, 1992).

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MATERIALS AND METHODS

The oligonucleotide $d(T_2G_4T)$ was synthesized on an Applied Biosystems PCR-Mate DNA synthesizer on a 10 μM scale. Following synthesis, the oligonucleotide was dissolved in buffer, filtered to remove organic salts, and dialyzed using Spectrapore 1000 MW cutoff dialysis tubing. Dialysis changes were performed after at least 4 h. For the NaCl sample, the first change was against 0.5 M NaCl, followed by two changes of 0.1 M NaCl and then three changes of 0.001 M NaCl. The KCl sample was treated similarly but with KCl substituted for NaCl. The oligonucleotide concentrations were determined from UV absorbance measurements, which were performed under denaturing conditions. Extinction coefficients were calculated by the nearest-neighbor method (Cantor et al., 1970). The initial Na^+ concentrations of the DNA samples were determined by comparison of the integrated ^{23}Na signal intensities from a standard curve of NaCl in D_2O. Initial K^+ concentrations were determined by atomic absorption. D_2O was added to a final concentration of 10%.

^{23}Na NMR measurements were performed at 132.3 MHz, and ^{39}K NMR measurements were performed at 23.3 MHz, on an Omega 500 NMR spectrometer. Proton NMR measurements were performed at 500 MHz on the same instrument. Single Lorentzian line shapes were fitted using the Omega NMR software. Longitudinal relaxation rates were obtained from three-parameter fits of the inversion recovery data, also using the Omega software. Non-Lorentzian line shapes were fitted to two Lorentzian components using the Marquardt algorithm. In performing this fitting, the two components were initially assumed to resonate at the same frequency and to have relative intensities in the ratio of 3:2 for the broad/narrow components. These fittings allowed us to determine quadrupole coupling constants and correlation times (Bull, 1972). Using these parameters, theoretical spectra were then simulated to include complex spectral density contributions (Einarsson & Westlund, 1988). Inclusion of these contributions resulted in a small frequency shift of the broad component compared to the narrow (Werbelow & Marshall, 1981). The introduction of this shift did not signficantly affect the fitting parameters of the two Lorentzian components; however, such simulations could successfully reproduce the observed asymmetry of the resonance spectrum. For spectra that were fitted to Lorentzian or bi-Lorentzian curves, free induction decays were zero-filled one time prior to Fourier transformation. Exponential weighting factors were *never* applied in the processing of these spectra.

It is worth pointing out that in all of our calculations and spectral simulations we assumed a single exponential decay of quadrupolar correlation. This corresponds to an isotropic rotational model, which gives a single Lorentzian spectral density function, characterized by a single correlation time (τ_c) for rotational motion.

THEORETICAL CONSIDERATIONS

Though the only *direct* evidence to support the hypothesis is a suggestive smear of electron density (Kang et al., 1992), it is generally assumed that G-quartet structures are stabilized by the binding of unhydrated univalent cations between the planes of guanine residues (Sundquist & Klug, 1989; Williamson et al., 1989; Hardin et al., 1991). This hypothesis is compelling in that it allows a simple rationalization of cation selectivity on the basis of ion size. Moreover, a great deal of cation NMR work has demonstrated that the surface binding of univalent cations to duplex DNA is diffuse, nonspecific, and accompanied by no significant DNA structural changes (Padmanabhan et al., 1990). Since the local surface features do not differ greatly between quadruplex and duplex structures, it is reasonable to assume that surface binding of univalent cations to quadruplex structure should be similarly diffuse and nonselective for one univalent cation over the other.

If univalent cations are coordinated between planes of guanines in such a manner, then a four-stranded quadruplex of $d(T_2G_4T)$ has the potential for three specific univalent cation binding sites. In addition to these putative specific sites, any DNA molecule in univalent salt solution will have associated with it a condensed layer of rotationally mobile, nonspecifically bound cations. The total fraction, r°, of associated cation bound per phosphate to polymeric duplex DNA is not well defined by experiment but appears to be between 0.5 and 0.8 (Anderson et al., 1976; Bleam et al., 1983; Braunlin et al., 1986). The fraction bound to oligomeric duplex has been predicted to be significantly less and dependent on the DNA length (Satoh et al., 1985; Olmsted et al., 1989; 1990; Fenley et al., 1990; Dewey, 1990). A dependence on the ionic conditions and on the DNA concentration is also expected (Satoh et al., 1985; Fenley et al, 1990). For quadruple-stranded DNA, no estimated of r° have been made; however, due to its higher charge density, r° should be greater than than for duplex DNA of comparable length. We can place an upper boundary on the total fraction of bound counterion by assuming that the high charge density of a tetrameric complex could result in nearly complete neutralization of phosphate charges by (specifically and nonspecifically) bound cation. Since a single strand of a heptamer oligonucleotide has six phosphate groups, this implies that a *maximum* of 24 univalent cations could be bound (specifically and nonspecifically) per quadruplex. Hence, if there are only three specific binding sites per quadruplex, a *minimum* of one of every eight bound cations will be specifically complexed. We will argue, based on our data, that the much enhanced relaxation rates of the specifically bound $^{23}Na^+$ and $^{39}K^+$ will cause these sites to dominate the relaxation even if only one of eight bound univalent cations is indeed specifically complexed. If fewer univalent cations are specifically bound, then the approximation improves.

It is clear that there are three potential types of binding environments for univalent cations in $d(T_2G_4T)$ solution. Such cations can be site-specifically bound, nonspecifically associated, or free in solution. *Moreover, since in general our measurements show decreases in relaxation rates with increasing temperature, the exchange among sites is rapid on the time scale of NMR relaxation.* Hence, we will assume a three-site, fast-exchange model:

$$R_i - R_{f,i} = p_a(R_{a,i} - R_{f,i}) + p_b(R_{b,i} - R_{f,i}) \qquad (1)$$

In this equation, under extreme narrowing ($\omega_o\tau_c \ll 1$) or near-extreme narrowing ($\omega_o\tau_c \approx 1$) conditions, R_i could be the single exponential rate constant describing the transverse ($i = 2$) or longitudinal ($i = 1$) relaxation. Here ω_o is the Larmor frequency and τ_c is the (rotational) correlation time for the motions that result in decorrelation of the quadrupolar interaction. More generally, R_i could be one of the two exponential rates describing the decay of transverse (or longitudinal) magnetization or it could represent a decay constant of a multiple quantum coherence. A comparison of different R_i's for the same sample allows one to calculate correlation times and, if the p_j's are known, quadrupole coupling constants. p_j is the fraction of cation in the jth environment. $R_{i,j}$ is the R_i relaxation rate of the cation in the

*j*th environment. Here $j = f$ refers to free cation, $j = a$ refers to atmospherically bound cation, and $j = b$ refers to specifically complexed cation.

The NMR behaviors of $^{23}Na^+$ and $^{39}K^+$ in polymeric duplex DNA solutions are quite similar (Braunlin & Nordenskiöld, 1984) and indicate loose, nonspecific binding. If the relaxation data are interpreted by an isotropic rotational diffusion model, then correlation times are calculated to be on the order of nanoseconds, a much shorter timescale than the end over end tumbling motions of a DNA polymer. In polymeric DNA solution, the primary differences between the NMR behaviors of $^{23}Na^+$ and $^{39}K^+$ reflect the higher NMR frequency of $^{23}Na^+$. The higher resonance frequency of $^{23}Na^+$ implies that deviations from extreme narrowing occur at a shorter value of the correlation time for $^{23}Na^+$, compared to $^{39}K^+$. The most noticeable manifestation of this effect is that although correlation times of DNA-bound cation are in the nanosecond range for *both* $^{23}Na^+$ and $^{39}K^+$, the $^{23}Na^+$ line shapes are bi-Lorentzian at higher magnetic fields, whereas those of $^{39}K^+$ are single Lorentzian at all currently available magnetic fields.

For $^{23}Na^+$ in oligomeric DNA solution, extreme narrowing conditions are always obtained, and line widths are only modestly enhanced in the presence of DNA (Stein & Record, 1993). Hence, in this case, the rotational motions of the bound cations are much more rapid than the overall tumbling motions of the DNA. We find that, at 10 °C, for a solution containing 4.1 mM d(GGAATTCC) strand (28.7 mM DNA phosphate), and 30 mM Na^+, the natural ^{23}Na line width is 15 Hz, whereas the ^{23}Na line width obtained for a sample of 0.1 M NaCl is 10 Hz under the same conditions (unreported results).

For a quadruplex structure involving four heptameric strands, a reasonable estimate for the hydrodynamic radius of an equivalent sphere would be around 15 Å. The Stokes–Einstein equation predicts a rotational correlation time (τ_R) for such a sphere to be 4.6 ns at 10 °C. Since bound univalent cation cannot rotate more slowly than the DNA to which it is bound, this implies that the *maximum* correlation time for bound cation should be around 4.6 ns. For a tightly bound cation, for which the tumbling motions of the oligonucleotide dominate the cation quadrupolar relaxation, this would imply $\omega_0\tau_c \approx 3.8$ for $^{23}Na^+$ (observe frequency of 132.2 MHz), but only 0.7 for $^{39}K^+$ (observe frequency of 23.3 MHz). Hence, if bound cation is rotationally immobilized, clear bi-Lorentzian lineshapes are expected for $^{23}Na^+$, but not for $^{39}K^+$. *For neither cation are the motions of the DNA molecule sufficiently slow that dynamic quadrupolar splitting could conceivably result in a loss of signal intensity.*

It is not obvious what the proper baseline should be for the ^{23}Na and ^{39}K relaxation behavior of cations atmospherically bound to quadruplex DNA. In our view it is most likely that the shortness of the oligomer will give fairly slow relaxation rates for such atmosperically bound cations, comparable to the values obtained for ^{23}Na relaxation in oligonucleotide solution. On the other hand, it is conceivable that the high charge density of the quadruplex might facilitate increased cation localization, even for externally bound cations. Given the distribution of charges around the quadruplex exterior, we do view this latter possibility with some skepticism; however, it cannot be dismissed *a priori*. Rather, we initially considered two extreme possibilities for the atmospherically bound sodium and potassium ions. Either (1) these ions have similar relaxation properties to those of cations bound to polymeric DNA or (2) these ions show relaxation behavior similar to cations bound nonspecifically to short duplex oligonucleotides. We demonstrate below that only a small number of univalent cations are specifically and selectively bound to quadruplex DNA. When these cations are displaced by competing cation, the observed relaxation rates drop precipitously and obey extreme narrowing. Hence, our results are consistent with the second possibility, but not with the first.

Given that the relaxation behavior of atmospherically bound $^{23}Na^+/^{39}K^+$ is similar to that of cations nonspecifically bound to short oligonucleotides, then the second term on the right hand side of eq 1 will dominate. Since it is clear from the data presented below that $R_{b,i} \gg R_{f,i}$, then eq 1 can be approximated by

$$R_i - R_{f,i} \approx p_b R_{b,i} \tag{2}$$

Equation 2 is identical to the expression that would be obtained from a standard two-state model of free and bound ions. It is clear from the above analysis that this equation also holds for the less stringent case of a three-site model, provided that the relaxation rate of the atmospherically bound ions is sufficiently small compared to that of the site bound ions.

Pairs of such equations can be solved by standard methods to obtain the correlation time, τ_c, for the quadrupolar interaction and the quantity, $(p_b)^{1/2}X_b$, where X_b is the quadrupole coupling constant of the site bound nuclei. X_b is defined, e.g., in Forsén et al. (1987), and involves the product of the nuclear quadrupole moment and the square of the largest component (q_{zz}) of the electric field gradient at the nucleus. Since q_{zz} depends on the environment around the nucleus, it cannot be estimated with any accuracy, and hence a direct determination of p_b is not possible from these measurements.

Previous work has demonstrated that, once formed, G-quartet structures are quite stable (Smith & Feigon, 1992; Wang & Patel, 1992). This observation led us to the general experimental approach of first dialyzing against high salt, in order to form stable G-quartets. This is followed by low salt dialysis, which reduces the fraction of free cation in solution, and thereby enhances the cation NMR signal from the bound states. This procedure represents a balance between maintaining G-quartet structure and obtaining NMR signals with a dominant contribution from the bound states.

RESULTS AND DISCUSSION

Potassium Ion Preferentially Stabilizes Quadruplex Structure Compared to Sodium Ion. In Figure 1 we show the imino and base region (6.5–13.0 ppm) and the methyl region (1.4–2.0 ppm) of $d(T_2G_4T)$ at a concentration of 2.1 mM strand in the presence of 14 mM K^+, monitored as a function of temperature. Plots A–E show results obtained on raising the temperature from 10 to 70 °C. Plot F is the spectrum obtained at 10 °C after heating the sample to 90 °C and slowly cooling. Spectrum A, which we obtained at 10 °C, prior to heating, is nearly identical to the spectrum reported by Wang and Patel (1992). In the base proton region of this spectrum (7–9 ppm), we also find seven major peaks, indicating a single dominant low-temperature conformation. Low-intensity minor peaks are also discernible, in our spectra, as in those of Wang and Patel (1992). The thymine methyl group region shows three dominant, upfield shifted resonances between 1.5 and 1.7 ppm, which can be attributed to base-paired thymine methyl groups and small but perceptible peaks at 1.85 ppm, which can be assigned to two overlapping thymine methyls for the minor conformation. An additional methyl resonance at 1.75 ppm may also be discerned. As the temperature is raised, the minor peaks increase in intensity in both the base proton and the methyl proton regions. This

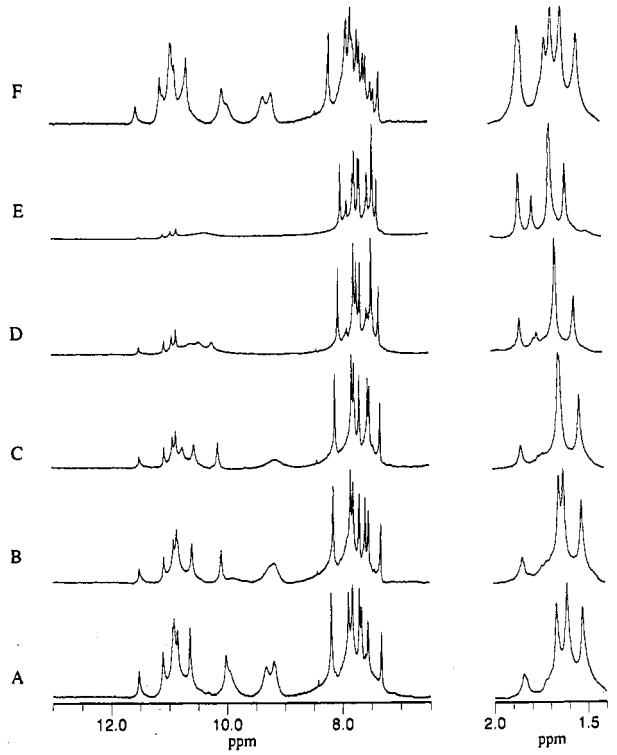

FIGURE 1: Base/exchangeable (6.5–13.0 ppm) and methyl (1.4–2.0 ppm) regions of the proton NMR spectrum of a sample containing 2.1 mM d(T_2G_4T) strands in KCl solution. The K^+ concentration of this sample was 14 mM. Spectra A–E were obtained following increasing the temperature to the indicated temperature: spectrum A (10 °C), spectrum B (20 °C), spectrum C (30 °C), spectrum D (50 °C), spectrum E (70 °C). Spectrum F was obtained at 10 °C following heating the sample to 90 °C and slowly reannealing.

Table I: ^{39}K NMR Parameters for a Potassium Sample of d(T_2G_4T)

temp (°C)	R_f (s^{-1})	R_1[a] (s^{-1})	R_2 (s^{-1})	τ_c (ns)	$(p_b)^{1/2} X_b$ (MHz)	f_q[b]
10	26	560	930	4.6 ± 0.3	0.26 ± 0.01	0.91
30	18	510	780	3.9 ± 0.5	0.25 ± 0.01	0.88
50	14	250	385	4.1 ± 0.4	0.17 ± 0.01	0.83
70	11	100	120	2.6 ± 0.6	0.11 ± 0.01	0.65
10[c]	26	450	640	3.6 ● 0.5	0.24 ± 0.01	0.61
10[d]	26	210	340	4.5 ± 0.7	0.16 ± 0.01	0.60
10[e]	26	85	130	4.8 ± 1.0	0.09 ± 0.01	0.75

[a] Also included are f_q (fraction quadruplex) determinations as determined from proton NMR intensity measurements of thymine methyl peaks. The solution contained 2.1 mM oligomer strand and 14 mM K^+. The uncertainties in the relaxation rates are ±5%. [b] The uncertainties in the values of f_q are ±0.05. [c] The sample was annealed by heating to 90 °C and then slowly cooled to 10 °C. [d] The sample was titrated with KCl to a concentration of 30 mM K^+, heated to 90 °C, and then slowly cooled to 10 °C. [e] The sample was titrated with KCl to a concentration of 100 mM K^+, heated to 90 °C, and then slowly cooled to 10 °C.

observation supports the assignment of these minor peaks to the single strand form (Wang & Patel, 1992). This assignment is also consistent with the downfield shift of the minor peak resonances, and we believe it to be correct. Nonetheless, some caution is in order since, as we discuss below, there is clear evidence for at least two types of Hoogsteen based-paired structures for d(T_2G_4T) in NaCl solution. Intensity determinations based on peak simulations in the methyl region allow estimates to be made of the fraction of the strands which are in the quadruplex form, f_q, and how this fraction varies with temperature. These determinations are given in Table I. It is notable that, even at 70 °C, over 60% of the oligonucleotide strands are still in the quadruplex (low temperataure) conformation. Upon raising the temperature to 90 °C and then lowering it back to 10 °C, significant peaks due to the minor form remain in the nonexchangeable spectrum, and the f_q remains at 0.6, even at this low temperature. Due to the apparent slowness of reforming quadruplex, it is not clear what f_q value would be obtained at thermodynamic equilibrium for this temperature.

The amino protons of the oligomer show two sets of resonances. The downfield peaks resonating between 9.0 and 10.0 ppm most likely correspond to Hoogsteen base-paired amino protons (Wang & Patel, 1992). Additional peaks are observed at around 6 ppm that correspond to the non-base-paired amino resonance. The Hoogsteen base-paired amino protons shift downfield, broaden sequentially, and disappear as the temperature is raised, reflecting exchange due to amino group rotation and/or proton exchange with water at higher temperature. There are six distinct peaks in the region between 10.5 and 12.0 ppm, in the spectra shown in Figure 1. Four of these peaks were assigned as guanine imino resonances. These guanine imino peaks decrease in intensity as the temperature is raised but do not broaden significantly, consistent with an unusually slow rate of base-pair opening (Wang & Patel, 1992; Smith & Feigon, 1992). The remaining two peaks in this region, and also the remarkably stable peak at 10.1 ppm, were not discussed by Wang and Patel (1992), though they are clearly evident in Figure 5 of their paper. All three peaks shift, broaden, and disappear as the temperature is raised. The most likely assignment of these three peaks is to thymine imino proton resonances. In this context it is worth noting that two uracil imino resonances were observed and assigned for RNA r(UGGGGU), providing evidence for hydrogen-bonded quartets of uracil bases (Cheong & Moore, 1992). In contrast to the base region, no new peaks appear in the exchangeable proton region between 9 and 12 ppm upon heating to 70 °C and then cooling back to 10 °C. This result provides the strongest evidence that a single quadruplex structure exists in KCl solution and that the minor peaks observed indeed correspond to single-stranded DNA.

In Figure 2 we show the corresponding temperature-dependent spectra for a sample of d(T_2G_4T) dialyzed against NaCl. The final DNA strand concentration was 2.1 mM, and the Na^+ concentration was 18 mM. Plots A–E show results obtained on raising the temperature from 10 to 70 °C. Plot F is the spectrum obtained at 10 °C after heating the sample to 90 °C and slowly cooling to 10 °C. Those peaks which largely disappear from the spectrum following reannealing are assigned to the "quadruplex form". Those which remain will be referred to as peaks from the "open form". In contrast to the potassium salt case, the spectrum obtained at 10 °C prior to heating shows clear evidence of two distinct conformations. This is particularly clear in the thymine methyl region, where the intensities of the downfield peaks (open form) are comparable to those of the upfield peaks (quadruplex form). An intensity comparison in this region allows us to calculate that 55% of the strands are in the quadruplex form. On the basis of comparison of the shifts of these spectra and those obtained in the presence of potassium ion, it seems that the quadruplex structure is similar, but not identical, in both cases. It is interesting that f_q increases on going from 10 to 20 °C. Since the quadruplex is more stable at lower temprature, this demonstrates that there is a kinetic block to quadruplex formation. The nonexchangeable base and thymine methyl spectra of Figure 2 show that the structural transition from quadruplex to open form is essentially complete

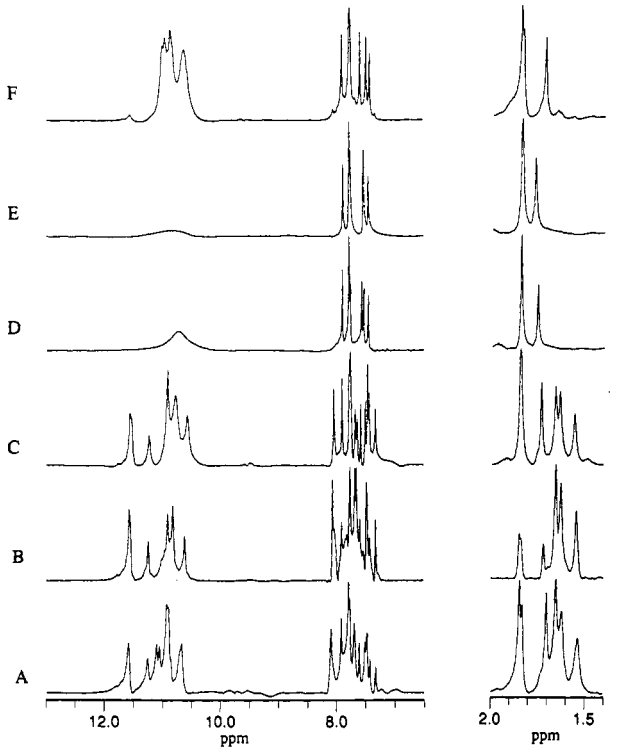

FIGURE 2: Base/exchangeable (6.5–13.0 ppm) and methyl (1.4–2.0 ppm) regions of the proton NMR spectrum of a sample containing 2.1 mM d(T_2G_4T) strands in NaCl solution. The Na^+ concentration of this sample was 18 mM. Spectra A-E were obtained following increasing the temperature to the indicated temperature: spectrum A (10 °C), spectrum B (20 °C), spectrum C (30 °C), spectrum D (50 °C), spectrum E (70 °C). Spectrum F was obtained at 20 °C following heating the sample to 90 °C and slowly reannealing.

at 50 °C. Hence, the quadruplex structure is significantly less stable in the presence of sodium ion than in the presence of potassium ion. Upon lowering the temperature back down to 20 °C, the open form dominates the spectrum, with the f_q calculated at 0.07.

In distinct contrast to the potassium salt sample, for the sodium sample no well-resolved amino proton peaks are apparent between 9.0 and 10.0 ppm. The behavior of the imino protons is also quite different for the two samples. Whereas for the potassium salt sample the imino peaks decrease in intensity without obvious line broadening, for the sodium case, these peaks progressively broaden and disappear as the temperature is raised. These results indicate a significantly more rapid rate of base-pair opening for the sodium sample compared to the potassium sample. The most remarkable aspect of Figure 2 is that, even though the f_q is only 5%, several rather broad imino peaks remain when the sample is reannealed at 20 °C (spectrum E). Since rapid proton exchange with solvent generally precludes the observation of imino peaks that are not base paired or otherwise trapped in a folded structure, this observation belies the interpretation of the open form as a structureless single strand. On the other hand, the fact that no distinct single strand resonances are ever observed suggests that the open form most likely constitutes a rapidly equilibrating mixture of a base-paired structure with single strands. We will return to a discussion of this point following our consideration of the cation NMR results. We do note however that a sodium sample that has been reannealed and left in the refrigerator for several days very slowly undergoes a transition to increased quadruplex form (for the sample in Figure 2, f_q increases from 0.07 to 0.15 over the period of 1 week at 4 °C). Hence, the open form constitutes a kinetic intermediate in going from single strand to quadruplex.

Both Sodium Ion and Potassium Ion Show Rotational Immobilization on the Time Scale of Oligomer Rotation. ^{39}K NMR line widths and longitudinal relaxation rates were also measured under the conditions of Figure 1. At all temperatures, the spectra are well fit by single Lorentzian line shapes. The most notable feature of these spectra is that the measured linewidths are significantly greater than has been observed previously for $^{23}Na^+$ or $^{39}K^+$ binding to duplex DNA. For example, the ^{39}K spectrum of this sample at 10 °C has a line width of 300 Hz. In addition to verifying the condition of extreme narrowing (or near extreme narrowing), the fact that a single Lorentzian line shape is observed (with no evidence of multiple components for the inversion recovery experiments) indicates that no NMR-visible sites are in slow exchange. Intermediate exchange can also be precluded since, as tabulated in Table I, both transverse and longitudinal relaxation rates decrease with increasing temperature. Hence, the NMR relaxation is in the rapid exchange regime. The transverse relaxation rate, as estimated from the line width, differs significantly from the longitudinal relaxation rate, measured using the inversion recovery experiment. Hence, the so-called near-extreme narrowing conditon applies, and the approximate equations suggested by Bull (1972) for the transverse and longitudinal relaxation were used to estimate the effective correlation time modulating the quadrupolar interaction [see also Halle and Wennerström (1981)]. These values are also listed in Table I. The value obtained for τ_c at 10 °C is equal to that given by the Stokes–Einstein equation for a particle in water of radius 15 Å, which is a reasonable estimate for the hydrated G-quartet structure. Hence, in contrast to previous results for duplex DNA, a class of bound potassium ion exists for which the decay of quadrupolar correlation reflects the time scale of oligomer rotation. The quantity $(p_b)^{1/2}X_b$ is initially constant and decreases at higher temperature as the f_q decreases. The relative constancy of τ_c on going from 30 to 50 °C may reflect a real expansion of the complex. Alternatively, the observed relative constancy of τ_c could reflect a modest contribution to the relaxation from chemical exchange among environments on the quadruplex, and bound to the high temperature form. Such exchange effects would not be observable at lower temperature due to the near-absence of the high temperature form.

Following heating to 90 °C, the sample was slowly cooled to 10 °C. As discussed above, the proton NMR of this sample (Figure 1E) showed a significant decrease in f_q. This decrease in the concentration of quadruplex is reflected in the ^{39}K line width of this sample, which decreased to 204 Hz, compared to 297 Hz for the 10 °C sample prior to heating. Titration with additional KCl followed by annealing (Table I) resulted in monotonic decreases in the observed ^{39}K line widths and in effective correlation times that remained nearly unchanged at around 4 ns. We were somewhat surprised to find that on the time scale of these experiments f_q showed only a modest increase from 0.60 to 0.75 upon increasing the K^+ concentration from 30 to 100 mM, again suggesting a kinetic block to reannealing.

^{23}Na NMR line shapes, obtained at temperatures below 50 °C, under the conditions of Figure 2, are distinctly non-Lorentzian. This is illustrated in Figure 3, where we show the ^{23}Na NMR spectrum for this sample at 20 °C, along with a spectral simulation of the curve to two Lorentzian lineshapes. It is also clear from the asymmetry of the spectrum that the broad Lorentzian component is shifted downfield with respect

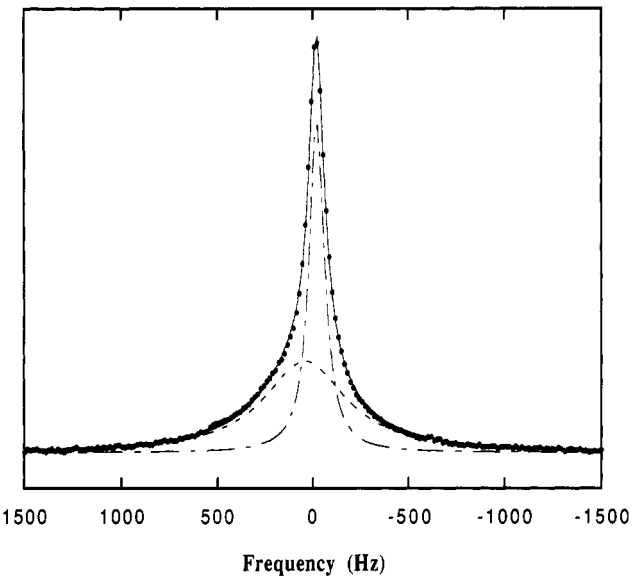

FIGURE 3: 132.3-MHz ^{23}Na spectrum obtained at 20 °C for a sample containing 2.1 mM d(T_2G_4T) strands in NaCL solution. The Na^+ concentration of this sample was 18 mM. Also shown is the best fit to the spectrum to two Lorentzian components with an area ratio of 3:2 for the broad to the narrow components. The fitted line widths are 530 Hz for the broad component and 97 Hz for the narrow component. The chemical shift difference between the two peaks was fitted at 62 Hz. A total of 4000 acquisitions were signal averaged. The spectral width was 4000 Hz, and the acquisition time was 64 ms. The FID was zero filled one time prior to Fourier transformation. No exponential weighting was applied.

to the narrow component. The non-Lorentzian behavior observed for ^{23}Na but not for ^{39}K reflects the higher Larmor frequency of the former. We have fit all non-Lorentzian ^{23}Na spectra to two Lorentzians, with intensity ratios of 2:3 for the narrow to broad components, and thereby obtained estimates of τ_c and $(p_b)^{1/2}X_b$ (Bull, 1972; Braunlin & Nordenskiöd, 1984). For all non-Lorentzian spectra that we have obtained, there is an inherent asymmetry that is impossible to remove completely by any phase adjustments. In the simulation shown in Figure 3 we have fitted as well for the chemical shift difference between the two Lorentzian components. The value that we obtain for the chemical shift difference by such fitting is 62 Hz, which is about half the theoretically expected value of 116 Hz (Einarsson & Westlund, 1988). Since asymmetric signals are notoriously difficult to phase, this relatively small difference between the theoretical prediction and our fitting results could reflect an improper phasing of an inherently asymmetric signal. In any case, although including a small shift did improve the agreement between simulation and experiment, the values obtained for τ_c and $(p_b)^{1/2}X_b$ were hardly affected by this procedure. At higher temperatures, ^{23}Na line shapes appear Lorentzian, and as for the ^{39}K case, the ratio of the transverse and longitudinal relaxation rates is used to estimate these two parameters. The results of these calculations are tabulated in Table II, along with the fitted relaxation rates (line width times π) of the two components. In general, both line widths decrease with increasing temperature, indicative of fast exchange. The one exception to this rule is that the line widths of both components increase slightly on raising the temperature from 10 to 20 °C. This line width increase can be correlated with an increase in f_q from 0.55 to 0.77, which, as discussed above, probably reflects a kinetic block to quadruplex formation at the lower temperature. From a comparison of Tables I and II, it seems that the effective correlation times for ^{23}Na are in general around a factor of 2 shorter than the corresponding ^{39}K correlation times. This difference may reflect greater overall rotational mobility of bound Na^+ compared to K^+.

Table II: ^{23}Na NMR Parameters for a Sodium Sample of d(T_2G_4T)

temp (°C)	R_f (s^{-1})	R_A[a] (s^{-1})	R_B[a] (s^{-1})	τ_c (ns)	$(p_b)^{1/2}X_b$ (MHz)	f_q[b]
10	25	300	1300	2.4 ± 0.5	0.47 ± 0.02	0.55
20	20	300	1580	2.7 ± 0.5	0.50 ± 0.02	0.77
30	17	230	1030	2.4 ± 0.7	0.42 ± 0.02	0.51
50	13	26	85	2.8 ± 1.0	0.13 ± 0.01	0.0
70	11	11	11			0.0
20[c]	20	35	95	2.5 ± 1.0	0.14 ± 0.01	0.07
20[d]	20	45	130	2.3 ± 0.4	0.17 ± 0.01	0.1
20[e]	20	60	190	2.2 ± 0.3	0.21 ± 0.01	0.14

[a] Also included are f_q (fraction quadruplex) determinations as determined from proton NMR intensity measurements of thymine methyl peaks. The solution contained 2.1 mM oligomer strand and 18 mM Na^+. For the first three entries in the table, R_A is the relaxation rate of the slow component (narrow line) of the transverse relaxation and R_B is the rate for the fast component. For the remaining entries, the signals are Lorentzian. For these entries, R_A is the longitudinal and R_B is the transverse relaxation rate (π times the line width). The uncertainties in these relaxation rates is estimated at ±5%. [b] The uncertainties in the values of f_q are ±0.05. [c] The sample was annealed by heating to 90 °C and then slowly cooled to 20 °C. [d] Following annealing, the sample was stored at 4 °C for 2 days prior to performing the measurements. [e] Following annealing, the sample was stored at 4 °C for 1 week prior to performing the measurements.

Table III: ^{23}Na and ^{39}K NMR Parameters for the Cross-Dialysis Experiments with d(T_2G_4T)

sample	nucleus	R_1 (s^{-1})	R_2 (s^{-1})	τ_c (ns)	$(p_b)^{1/2}X_b$ (MHz)
A	^{39}K	900	1350	3.8 ± 0.4	0.34 ± 0.01
A	^{23}Na	45	80	1.6 ± 0.6	0.13 ± 0.01
B	^{39}K	580	845	3.6 ± 0.4	0.27 ± 0.01
B	^{23}Na	35	60	1.9 ± 0.7	0.11 ± 0.01

[a] Samples A and B were prepared as described in the text. f_q was 0.89 ± 0.05 for both samples.

Following heating to 90 °C, the sample was slowly cooled to 20 °C. As discussed above, the proton NMR of this sample (Figure 2E) showed nearly complete conversion to the high-temperature form (f_q = 0.07). Under these conditions, the ^{23}Na signal was found to be Lorentzian, with a line width of only 30 Hz. On the basis of line widths and longitudinal relaxation rates, this dramatic change in the NMR behavior could be correlated with decreases in both τ_c and in $(p_b)^{1/2}X_b$. As summarized in Table II, as the f_q increased with time upon storage in the refigerator, the ^{23}Na line widths and longitudinal relaxation rates also increased, as did the calculated values of τ_c and of $(p_b)^{1/2}X_b$.

Potassium Ion is Preferentially Bound to d(T_2G_4T) Compared to Sodium Ion but Only to a Small Fraction of the Total Number of Sites. Competitive dialysis experiments were performed, followed by ^{23}Na and ^{39}K NMR, in order to study the competition between sodium ion and potassium ion for atmospheric and site binding to d(T_2G_4T). The results of these experiments are summarized in Table III. In these experiments, the sodium and potassium concentrations were determined by ^{23}Na NMR and by atomic absorbance. The first sample (sample A) was prepared by dialyzing d(T_2G_4T) first against 0.5 M KCl, followed by successive changes (at 8-h intervals) against 0.1 M KCl, 10 mM NaCl, and finally againt 1 mM NaCl. The sample (roughtly 5 mL) was then lyophylized and dissolved in 2 mL of water containing 10% D_2O. The final DNA concentration of this sample was 1.8 mM strand, and the final concentrations of Na^+ and K^+ were, respectively, 5.4 and 5.7 mM. The second sample (sample B) was prepared in an identical manner, except that KCl was

substituted for NaCl and vice versa. That is to say, the sample was dialyzed against 0.5 M NaCl, 0.1 M NaCl, 10 mM KCl, and 1 mM KCl. The final DNA concentration for sample B was 1.6 mM strand, and the final concentrations of Na^+ and K^+ were, respectively, 7.1 and 7.2 mM. On the basis of the nearly equal concentrations of sodium and potassium ions in the two samples, there seems little or no selectivity for one ion over the other for atmospheric binding to DNA. The f_q for both samples was calculated to be nearly 90%. For both samples, the ^1H NMR spectra were very similar to the spectra obtained in the presence of potassium salt alone.

The ^{39}K line width at 10 °C was 430 and 270 Hz for samples A and B, respectively. This difference between the two samples could be attributed essentially entirely to a change in $(p_b)^{1/2}X_b$, since the ^{39}K correlation time is nearly identical for the two samples and is not surprising given the lower cation to DNA ratio of sample A compared to sample B. In contrast, the ^{23}Na line width is quite narrow for both samples (26 Hz and 20 Hz, respectively, for samples A and B) and well described by a single Lorentzian. Hence, the potassium ion competes much more effectively for sites associated with large relaxation effects, i.e., for specific sites. This conclusion is dramatically illustrated by the observation that when sample A is titrated with NaCl to give a total Na^+ concentration of 0.1 M, the ^{39}K line width decreases by less than a factor of 2. In contrast, under these conditions the sodium ion relaxation parameters are indistinguishable from those of the free ion.

The above cross dialysis experiment demonstrates K^+ selectivity over Na^+ for binding to a small number of specific sites. $^{39}K^+$ or $^{23}Na^+$ bound to these sites is in rapid exchange with much more slowly relaxing atmospherically bound cation. The condition of rapid exchange is in this context somewhat surprising, given the extremely slow imino proton exchange that has been found for quadruplex DNA (Wang & Patel, 1992; Smith & Feigon, 1992). On the basis of the above measurements we cannot, however, entirely preclude the possibility that there might exist an additional class of bound $^{39}K^+$ that might, for example, be bound to the central G-quartet and in slow exchange on the NMR time scale. Our inability to detect this class could reflect (a) the relatively small concentration of ions bound to such sites compared to the total cation concentration and (b) a very broad line width for such coordinated cations. Experiments designed to address this issue will be reported elsewhere (work in progress).

CONCLUDING REMARKS

Many questions remain regarding the peculiar nucleic acid structures which involve G-quartets and the synergistic and antagonistic roles of various cations in stabilizing them. On the basis of present study we have been able to answer a few of these questions and raise a few more. Our major conclusions can be summarized as follows. (1) Our temperature-dependent proton NMR experiments clearly demonstrate preferential stabilization of quadruplex DNA by K^+ compared to Na^+. (2) We have demonstrated that selectivity exists for K^+ compared to Na^+ for binding to a small number of sites on the quadruplex. (3) Both $^{39}K^+$ and $^{23}Na^+$ are, when bound to these sites, significantly inhibited in their rotational mobility. For atmospheric binding, no comparable binding selectivity can be discerned. (4) Though specifically bound cations are rotationally immobilized, all NMR-visible $^{39}K^+$ in solution is in rapid exchange among all environments. A similar conclusion holds for $^{23}Na^+$. (5) There are at least two distinct Hoogsteen base-paired structures in NaCl solution, separated by a very large kinetic barrier.

Major questions raised by our work include the nature of the open and quadruplex structures that are formed in NaCl solution and on the roles of cations in stabilizing them. Neither structure is as stable as is the KCl quadruplex structure with respect to base pair opening, as judged by the exchangeable imino and amino protons. The quadruplex structure is formed at low temperatures during the dialysis and lyophilization procedure used to prepare the NaCl sample. The proton NMR of this structure is similar, but not identical to the quadruplex formed in KCl solution. As for the KCl structure, thymine methyl group resonances are shifted upfield, indicating stacking of thymines, and thymine imino peaks can be discerned. However, in contrast to the KCl structure, peaks in the imino region show significant exchange line broadening. Moreover, no amino peaks are apparent for the NaCl sample in the regions around 9.5 and 6 ppm. The NaCl quadruplex structure traps sodium ions in a rotationally immobilized state and is correspondingly associated with bi-Lorentzian ^{23}Na NMR signals. Following heat denaturation and reannealing, another base-paired structure, the "open structure" is observed, with even broader imino resonances and with thymine methyl resonances in the downfield shifted region normally associated with single-stranded thymines. ^{23}Na signals are Lorentzian, indicating an increase in sodium ion rotational mobility. Following reannealing, over a period of days at 4 °C, the fraction of strands in the quadruplex form gradually but perceptibly increases and is accompanied by an increase in the ^{23}Na linewidth.

A model suggested by Lee (1990) provides a nice rationale for this behavior. In this model, four strands of DNA can first form a Hoogsteen base-paired tetraplex, prior to ion complexation. This tetraplex structure melts cooperatively, as does our open complex, and we can regard the two as equivalent. Over time, the open structure can slowly complex univalent cations, provided they are of the proper size, to form what Lee terms G-DNA, and we refer to as quadruplex. This model is supported by our ^{23}Na line shape results, for which bi-Lorentzian line shapes are associated with the quadruplex but not the open structure.

REFERENCES

Anderson, C. F., Record, M. T., Jr., & Hart, P. A. (1982) *Biophys. Chem. 7*, 301–316.

Arnott, S., Chandrasekaran, R., & Martilla, C. M. (1974) *Biochem. J. 141*, 537–543.

Bleam, M. L., Anderson, C. F., & Record, M. T., Jr. (1983) *Biochemistry 22*, 5418–5425.

Borzo, M., Detellier, C., Laszlo, P., & Paris, A. (1980) *J. Am. Chem. Soc. 102*, 1124–1134.

Braunlin, W. H., & Nordenskiöld, L. (1984) *Eur. J. Biochem. 142*, 133–137.

Braunlin, W. H., Anderson, C. F., & Record, M. T., Jr. (1986) *Biopolymers 25*, 205–214.

Braunlin, W. H., Drakenberg, T., & Nordenskiöld, L. (1987) *Biopolymers 26*, 1047–1062.

Braunlin, W. H., Drakenberg, T., & Nordenskiöld, L. (1989) *Biopolymers 28*, 1339–1342.

Braunlin, W. H., Nordenskiöld, L., & Drakenberg, T. (1991) *Biopolymers 31*, 1343–1346.

Braunlin, W. H., & Xu, Q. (1992) *Biopolymers 32*, 1703–1711.

Bull, T. E. (1972) *J. Magn. Reson. 8*, 344–353 (1972).

Cantor, C., Warshaw, M. M., & Shapiro, H. (1970) *Biopolymers 9*, 1059–1077.

Cheong, C., & Moore, P. B. (1992) *Biochemistry 31*, 8406–8414.

Detellier, C., & Laszlo, P. (1980) *J. Am. Chem. Soc. 102*, 1135–1141.

Dewey, T. G. (1990) *Biopolymers 29*, 1793–1799.
Dobler, M. (1981) *Ionophores and Their Structure*, Wiley-Interscience, New York.
Einarsson, L., & Westlund, P.-O. (1988) *J. Magn. Reson. 79*, 54–65.
Fenley, M. O., Manning, G. S., & Olson, W. K. (1990) *Biopolymers 30*, 1191–1203.
Forsén, S., Drakenberg, T., & Wennerström, H. (1987) *Quart. Rev. Biophys. 19*, 83–114.
Guschlbauer, W., Chantot, J. F., & Thiele, D. (1990) *J. Biomol. Struct. Dyn. 8*, 491–511.
Halle, B., & Wennerström, H. (1981) *J. Magn. Reson. 44*, 89–100.
Halle, B., Wennerström, H., & Picullel, L. (1984) *J. Phys. Chem. 88*, 2482–2494.
Hardin, C. C., Henderson, E., Watson, T., & Prosser, J. K. (1991) *Biochemistry 30*, 4460–4472.
Henderson, E. R., Hardin, C. C., Wolk, S. K., Tinoco, I., Jr., & Blackburn, E. H. (1987) *Cell 51*, 899–908.
Kang, C., Zhang, X., Ratliff, R., Moyzis, R., & Rich, A. (1992) *Nature 356*, 126–131.
Lee, J. S. (1990) *Nucleic Acids Res. 18*, 6057–6060.
Lindman, B., & Forsén, S. (1978) in *NMR and the Periodic Table* (Harris, R. & Mann, B., Eds.) pp 129–181, Academic Press, New York.
Lu, M., Guo, Q., & Kallenbach, N. R. (1992) *Biochemistry 31*, 2455–2459.
Olmsted, M. C., Anderson, C. F., & Record, M. T., Jr. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7766–7770.
Olmsted, M. C., Anderson, C. F., & Record, M. T., Jr. (1991) *Bipolymers, 31*, 1593–1604.
Padmanabhan, P., Paulsen, M., Anderson, C. F., & Record, M. T., Jr. (1990) in *Cations in Biological Systems* (Pasternak, C., Ed.) pp 321–338, CRC Press, Boca Raton, FL.
Satoh, M., Komiyama, J., & Iijima, T. (1985) *Macromolecules 18*, 1195–1200.
Sen, D., & Gilbert, W. (1988) *Nature 334*, 364–366.
Sen, D., & Gilbert, W. (1991) *Curr. Opin. Struct. Biol. 1*, 435–438.
Sen, D., & Gilbert, W. (1992) *Biochemistry 31*, 364–366.
Smith, F. W., & Feigon, J. (1992) *Nature 356*, 164–168.
Stein, V., & Record, M. T., Jr. (1993) *Biophys. J. 64*, A267.
Werbelow, L. G., & Marshall, A. G. (1981) *J. Magn. Reson. 43*, 443–448.
Wang, Y., & Patel, D. J. (1992) *Biochemistry 31*, 8112–8119.
Xu, Q., Jampani, S., & Braunlin, W. H. (1993) *Biochemistry* (in press).
Xu, Q., Shoemaker, R. K., & Braunlin, W. H. (1993) *Biophys. J. 65*, 1039–1049.
Zimmerman, S. B., Cohen, G. H., & Davies, D. R. (1975) *J. Mol. Biol. 92*, 181–192.