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^{1}H NMR Study of the Base-Pairing Reactions of d(GGAATTCC): Salt Effects on the Equilibria and Kinetics of Strand Association†

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Received May 7, 1990; Revised Manuscript Received September 28, 1990

ABSTRACT: Previously, we examined the imino proton relaxation of d(GGAATTCC) in order to characterize salt and polyamine effects on the base-pair opening kinetics of this oligonucleotide [Braunlin, W. H., & Bloomfield, V. A. (1988) *Biochemistry 27*, 1184-1191]. Here, we report salt-dependent measurements of the NMR behavior of the nonexchangeable base proton resonances of d(GGAATTCC). From chemical shift measurements, we find an unexpectedly large salt dependence of K_a, the equilibrium constant for helix association. A total of 1.8 ± 0.3 sodium ions are thermodynamically released upon dissociation of the octamer duplex. Most of the salt dependence of the equilibrium constant can be traced to a large salt dependence of the association rate. Thus, 1.4 ± 0.2 sodium ions associate during the rate-limiting step of helix association. In agreement with our previous imino proton results, we also find a significant salt dependence of the duplex dissociation rate. Activation energies for helix association are very small, and possibly negative; most of the temperature dependence of the association equilibrium can be traced to a large activation energy (~50 kcal/mol) for duplex dissociation.

Convenient access to milligram quantities of highly pure, synthetic oligonucleotides now offers unprecedented opportunities for the biophysical chemist concerned with understanding the details of sequence-dependent effects on DNA structural transitions and ligand-binding interactions. However, some caution must be exercised; oligomeric DNAs do not appear to be ideal models for some aspects of the behavior of polymeric DNA. For example, a large difference has been predicted in the ion-binding behavior of oligomeric and

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0006-2960/91/0430-754$02.50/0

polymeric DNAs (Olmsted et al., 1989). Such a dissimilarity in ion binding might be expected to manifest itself in substantial differences in the salt dependences of the helix–coil equilibria and kinetics of polymeric vs oligomeric DNA. Our objective in the current work is to examine the equilibria and kinetics of the helix–coil transition of the self-complementary deoxyoctanucleotide d(GGAATTCC) for comparison with theory, as well as with results obtained for polymeric DNAs (Record et al., 1981) and other DNA oligomers (Elson et al., 1970; Record & Lohman, 1978; Pörschke et al., 1973; Breslauer & Bina-Stein, 1977; Erie et al., 1987; Williams et al., 1989).

The proton NMR of d(GGAATTCC) has been well characterized (Patel & Canuel, 1979; Broido et al., 1984, 1985). The salt-dependent helix–coil thermodynamics of this molecule have also been reported (Patel et al., 1982b; Erie et al., 1987). In previous work from this laboratory, the exchangeable imino proton NMR relaxation of this octamer was examined and interpreted to obtain kinetic rate constants for (a) the "opening" of individual base pairs and (b) the salt-dependent dissociation of the octamer duplex (Braunlin & Bloomfield, 1987). Here are reported the results of NMR line width and chemical shift measurements for the nonexchangeable resonances of d(GGAATTCC). On the basis of these measurements, a fairly detailed picture of the salt-dependent duplex association and dissociation kinetics of d(GGAATTCC) has been obtained.

Materials and Methods

d(GGAATTCC) was prepared and purified as previously described (Braunlin & Bloomfield, 1988). Following the final dialysis step, the sample was lyophilized in D_2O to a total volume of 0.12 mL. The sodium ion concentration of this sample was determined to be 0.02 M by inductively coupled plasma spectroscopy, performed by the Analytical Services Laboratory of the University of Minnesota. The strand concentration was determined by using $\epsilon_{260} = 5.4 \times 10^4\ M^{-1}\ cm^{-1}$ (Patel & Canuel, 1979). NaCl titrations were performed by adding microliter amounts of 4.0 M NaCl in D_2O directly to the sample in 5-mm microtubes (Wilmad No. 508CP). NMR measurements were performed in D_2O solution on a Nicolet 300-MHz spectrometer. Suppression of the residual HDO peak was obtained by using the $1-\underline{1}$ pulse sequence (Hore, 1983). Chemical shifts were determined relative to DSS, $(CH_3)_3Si(CH_2)_3SO_3Na_2$, which was added to the samples as an internal standard.

Data Analysis. (A) The Two-State Model for the Helix–Coil Transition. The chemical shifts of the base protons of d(GGAATTCC) provide a convenient means for monitoring the helix–coil transition (Patel & Canuel, 1979). According to the two-state model, the observed chemical shift is the population-weighted average of the proton in the duplex and coil forms. It follows that p_D, the fraction of oligomer in the duplex form, is given by

$$p_D = \frac{\delta_{obs} - \delta_C}{\delta_D - \delta_C} \tag{1}$$

where δ_{obs} is the observed chemical shift, δ_C is the chemical shift in the coil form, and δ_D is the chemical shift in the duplex form.

With p_D calculated from this equation, the equilibrium association constant K_a can be calculated from

$$K_a = \frac{p_D}{2(1 - p_D)^2 C_t} \tag{2}$$

where C_t is the total concentration of oligomer strand, in duplex and coil forms.

For the low-salt samples examined, δ_{obs} shows a significant temperature dependence in the "premelting" region. Since, for the resonances that we will focus on this work, these effects are not pronounced at higher salt concentrations, we have chosen the observed low-temperature, high-salt shifts to define the δ_D used to determine p_D and p_C. Our calculations are not very sensitive to the choice of δ_D. The consequences of applying a multistate model to analyze the data are discussed in the following section.

If the two-state model applies, and if n is the number of ions thermodynamically released per DNA duplex upon dissociation, then it can be shown that

$$\frac{dT_m}{d \log [Na^+]} = \left(\frac{2.3RT_m^2}{\Delta H^\circ}\right)n \tag{3}$$

where T_m is the transition midpoint, R is the gas constant, and ΔH° is the standard state association enthalpy (Krakauer & Sturtevant, 1968; Crothers, 1971). n can thus be calculated either from the slope of log K_a vs $-\log [Na^+]$ or, if ΔH° is known, from the salt dependence of T_m.

(B) Line Broadening in the Rapid-Exchange Regime. Exchange between helical and coil forms may result in line broadening in the near rapid exchange regime (Piette & Anderson, 1959). In this situation, the kinetic rate constants, k_d for the helix dissociation and k_a for association, are given by

$$k_d = \frac{4\pi^2 p_C^2 p_D [\Delta\delta]^2}{\Delta\nu_{1/2ex}} \tag{4}$$

$$k_a = K_a k_d \tag{5}$$

where $\Delta\delta$ is the chemical shift difference $\delta_D - \delta_C$ and $\Delta\nu_{1/2ex} = \Delta\nu_{1/2obs} - \Delta\nu_{1/2mag}$, where $\Delta\nu_{1/2obs}$ is the observed line width and $\Delta\nu_{1/2mag}$ is the line width expected in the absence of exchange. $\Delta\nu_{1/2mag}$ is determined by letting $\Delta\nu_{1/2obs} = \Delta\nu_{1/2mag}$ at low temperature, assuming that $\Delta\nu_{1/2mag}$ decreases as the viscosity divided by temperature, and by using the known dependence of the viscosity of water on temperature (Weast, 1983).

Results

Temperature Dependence of the NMR Spectrum. Broido et al. (1984, 1985) have completely assigned the low-temperature, nonexchangeable proton spectrum of d(GGAATTCC). In the discussion that follows, we shall use the conventional numbering scheme of these and other workers; i.e., the nucleotides are numbered sequentially from the 5′ end of the duplex. In Figure 1 we show the temperature dependence of the base proton region of the NMR spectrum of d(GGAATTCC). Note that, as the temperature is raised, the resonances shift, broaden in the intermediate range, and narrow again at higher temperatures.

For several of the resonances (AH4, AH2, GH8, and CH6 in particular) it is difficult to extract the exact peak positions in the intermediate range due to problems with spectral overlap. For the AH8 resonances, the chemical shift differences between coil and duplex forms are fairly small; hence these resonances are not very useful as probes of the helix–coil transition. Severe premelting behavior complicates the analysis of the terminal d(GG)·d(CC) units. As a consequence, in this work we have concentrated primarily on the analysis of the TH6 resonances. These resonances are well resolved over most

Table I: Salt Dependence of Helix–Coil Equilibria and Kinetics[a]

	association equilibrium			association kinetics		dissociation kinetics	
[Na^+] (M)	T_m[b] (°C)	$-\Delta H°$ (kcal mol^{-1})	K_a (M^{-1}) at 40 °C	E_a (kcal mol^{-1})	$k_a \times 10^{-6}$ (M^{-1} s^{-1}) at 40 °C	E_a (kcal mol^{-1})	$k_d \times 10^{-6}$ (s^{-1}) at 40 °C
0.046	37	48	130	0	0.6	48	4600
0.092	42	46	400	−5	1.7	41	4200
0.137	44	46	860	−8	2.7	38	3200
0.203	49	52	3800	−9	6.3	43	2000
0.267	50	52	3800	−9	6.9	43	1800

[a] The estimated uncertainties are ±2 °C for T_m, ±2 kcal mol^{-1} for $\Delta H°$, and ±5 kcal mol^{-1} for the activation energies. [b] T_m is calculated for a concentration of 3.0 mM strands.

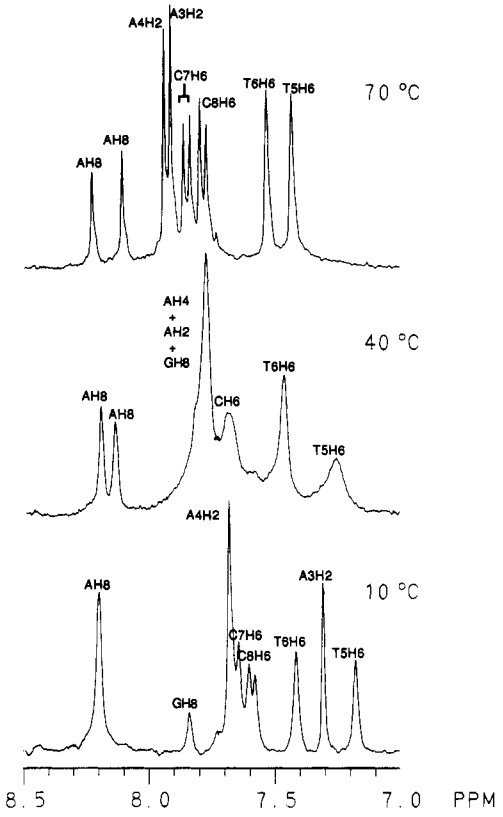

FIGURE 1: Temperature dependence of the base proton region of d(GGAATTCC) for a sample containing 0.12 M NaCl and 2.0 mM DNA. T6H6 resonates at 7.4 ppm in the 10 °C spectrum and at 7.5 ppm in the 70 °C spectrum. T5H6 resonates at 7.2 ppm at 10 °C and 7.4 ppm at 70 °C. These spectra agree with the temperature-dependent spectra first reported by Patel and Canuel (1979).

of the salt and temperature ranges examined and show relatively modest premelting behavior.

A minor point regarding Figure 1: The GH8 (7.85 ppm) resonances at low temperature already show reduced intensity owing to exchange with deuterium. In the 70 °C spectrum, the intensity remaining from these resonances has become indistinguishable from the noise (although the "blip" at 7.8 ppm on the upfield side of C8H8 may correspond to G2H8).

Premelting and Melting Transitions. Patel and Canuel (1979) have previously reported temperature-dependent chemical shift profiles for the nonexchangeable base protons of d(GGAATTCC). These workers found that significant changes occur for the chemical shifts of several of the base protons in the premelting temperature region of the spectrum. We find that low-salt samples show larger premelting chemical shift changes than do high-salt samples. Thus, as shown in Figure 2 for T6H6, significant premelting chemical shift changes are observed only for the low-salt samples ([Na^+] = 0.045, 0.096, and 0.15 M). In contrast, for the higher salt samples ([Na^+] = 0.20 and 0.27 M), the temperature dependences of the T6H6 shifts are consistent with a simple two-state model for helix dissociation. Similar behavior is seen for T5H6. Small temperature-dependent chemical shift changes are also observed at higher temperatures and can be attributed to partial stacking of the molecule in the coil form.

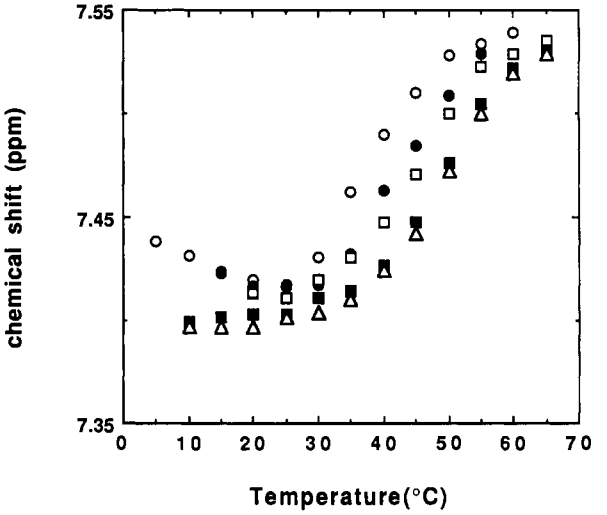

FIGURE 2: Temperature dependence of the ^1H chemical shift of T6H6. The sample contained 3.0 mM DNA single strand. (○) [Na^+] = 0.046 M, (●) [Na^+] = 0.092 M, (□) [Na^+] = 0.137 M, (■) [Na^+] = 0.203 M, and (△) [Na^+] = 0.267 M.

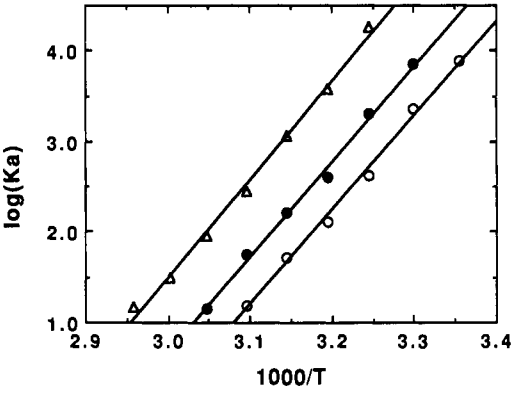

FIGURE 3: van't Hoff plots of K_a vs the inverse temperature determined from the data of Figure 2. (○) [Na^+] = 0.046 M, (●) [Na^+] = 0.092 M, and (△) [Na^+] = 0.267 M.

When, as outlined above, the chemical shift data of the T6H6 resonance are analyzed according to the two-state model, the results shown in Figure 3 are obtained, plotted as log K_a vs inverse temperature. From the slopes of these plots, the thermodynamic parameters listed in Table I were obtained. When the chemical shift data for T5H6 are analyzed in a similar fashion, plots are obtained that are superimposable within experimental uncertainty on those shown in Figure 3. In agreement with previous spectroscopic and calorimetric measurements (Patel et al., 1982; Erie et al., 1987), $\Delta H°$ for

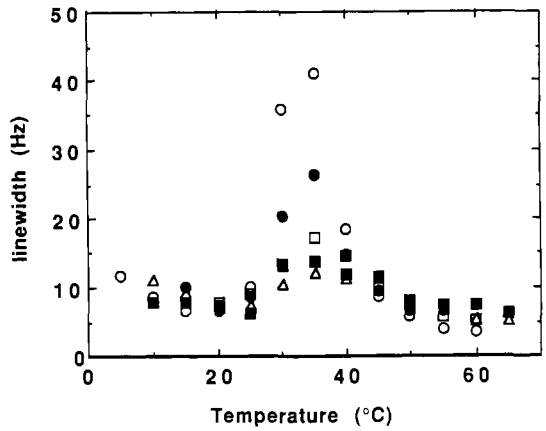

FIGURE 4: Temperature dependence of the line widths of T5H6. The sample is the same as in Figure 2. (○) $[Na^+] = 0.046$ M, (●) $[Na^+] = 0.092$ M, (□) $[Na^+] = 0.137$ M, (■) $[Na^+] = 0.203$ M, and (△) $[Na^+] = 0.267$ M.

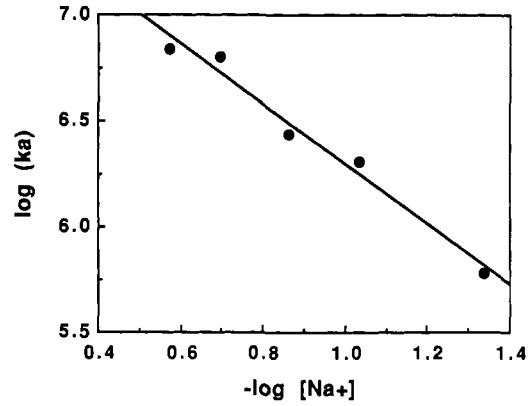

FIGURE 5: log k_a vs −log $[Na^+]$ at 40 °C, determined from the data of Figures 2 and 3, as outlined in the text. The points are fitted by log $k_a = 1.4$ log $[Na^+] + 7.7$.

the helix–coil association equilibrium is salt-independent and is equal to about −50 kcal/mol. Salt-dependent transition midpoints, T_m, are also listed in Table I. If T_m is plotted versus −log $[Na^+]$, a straight line is obtained of slope 17.4 ± 1.0 and intercept 59.9 ± 2.0 °C at 1 M Na^+. Use of eq of 3, with T_m taken to be 45 °C, implies that, under the conditions of these experiments, 1.8 ± 0.3 sodium ions are thermodynamically released upon the dissociation of this octamer duplex. This conclusion is also in agreement with constant temperature plots of log K_a vs log $[Na^+]$. Thus, such a plot of 45 °C is well fitted to a straight line, with slope of 1.8 ± 0.2 and intercept of 4.1 at 1 M Na^+. A plot of T_m vs −log $[Na^+]$ obtained from an analysis of the T5H6 resonances gives a slope of 15.8 ± 0.8 and an intercept of 59.4 ± 2.0 at 1 M Na^+.

Kinetics of the Helix–Coil Transition. Patel and Canuel (1979) were the first to report temperature-dependent line broadening of the nonexchangeable proton resonances of d(GGAATTCC) and to analyze this line broadening to obtain kinetic parameters for the duplex dissociation event (Piette & Anderson, 1959). Here we report, as shown in Figure 4, a dramatic salt dependence of the observed line broadening. From an analysis of this line broadening, the salt-dependent kinetic parameters given in Table I are obtained. From these results it is apparent that the salt dependence of the helix–coil equilibrium mainly reflects the large salt dependence of the association kinetics. As is shown in Figure 5, a constant temperature plot of log k_a vs −log $[Na^+]$ is linear with a slope of 1.4 ± 0.2 and intercept of 7.7 U 0.4 at 1 M Na^+, indicating that 1.4 sodium ions associate with the DNA during the rate-limiting step of helix association. As is evident from Table I, only very small temperature dependences are found for k_a.

From the slopes of plots of log k_d vs inverse temperature, E_a's of around 50 kcal/mol are obtained for the dissociation rate constant. Evidently, the temperature dependence of the helix–coil equilibrium can be traced largely to the temperature dependence of the dissociation kinetics. Also, as was previously reported (Braunlin & Bloomfield, 1988), a small but significant salt dependence is found for k_d. The results shown in Table I are in good agreement with these previous measurements and indicate that 0.4 ± 0.2 sodium ion is released during the kinetic process of dissociation.

Discussion

Ionic Effects on Helix–Coil Transition. As illustrated in Figure 2, for both the T5H6 and T6H6 resonances of GGAATTCC, premelting chemical shift changes are observed under low-salt conditions. In contrast, under high-salt conditions, the chemical shift behaviors of these two interior resonances are well described by a two-state model. Premelting transitions of double-helical DNA are well documented and have been observed by circular dichroism (Gennis & Cantor, 1972) as well as by NMR (Patel & Canuel, 1979; Patel et al., 1982a,b). To our knowledge, there has not been a previous report documenting a significant salt dependence of such transitions.

The data of Figure 1 are not sufficient to define a reasonable multistate model. Nonetheless, it is of interest to consider how the limitations of the two-state model might influence our conclusions. If a multistate model is applied, it is apparent that the effective δ_D in eq 1 will decrease as a function of temperature for the low-salt samples. If, for example, a simple linear dependence of δ_D on temperature is assumed (the linear model), then the low-salt curves will be shifted to lower T_m compared to the predictions of the two-state model. Consequently, a slightly larger salt dependence of T_m is expected for the linear model than for a simple two-state model. We find that the linear model gives a plot of T_m vs −log $[Na^+]$ which can be fitted to a slope of −20.8 ± 2.0 and an intercept of 61.9 ± 4.0. The linear model thus predicts that 2.1 ± 0.3 sodium ions are thermodynamically released upon duplex dissociation, a prediction that is indistinguishable, within experimental uncertainty, from that of the two-state model. Similar small differences are found between the two-state and linear models with respect to the kinetic parameters of helix dissociation.

There are some indications in the literature of a change in the dissociation mechanism of oligonucleotides as a function of salt concentration. Thus, on the basis of temperature-jump measurements, Williams et al. (1989) found k_d to be relatively salt-independent for the oligonucleotide GCATGC, except for a significant increase in k_d for the lowest salt examined. On the basis of a comparison of calorimetric and van't Hoff enthalpies, Patel et al. (1982a) found that the duplex–coil transition of the oligomer CGCGAATTCGCG approximates two-state behavior better at low salt than at high salt. This observation seems to conflict with the spectroscopic measurements reported here, which indicate that premelting behavior is more dramatic at low salt. However, it might, for example, be the case that the low-salt duplex form monitored spectroscopically undergoes the helix–coil transition more according to the calorimetric two-state model than does the high-salt form. Also, the size of the cooperative unit for the CGCGAATTCGCG dodecamer was determined to be 9 ± 1 base pairs under low-salt conditions. Hence, a salt-dependent *calorimetric* deviation from two-state behavior might not be

expected for the octamer GGAATTCC.

Thermodynamics of the Helix–Coil Transition. In excellent agreement with the results reported here, previous spectroscopic and calorimetric measurements indicate that the helix–coil thermodynamics of GGAATTCC are well described by a two-state model with $\Delta H^\circ = -50$ to -60 kcal/mol (Patel et al., 1982b; Erie et al., 1987). The salt dependence of the helix–coil thermodynamics reported here is also in reasonable agreement with that reported previously. Thus, we find that $\Delta i = 1.8/14 = 0.13 \pm 0.02$ sodium ion is released per DNA phosphate upon helix dissociation, whereas Erie et al. (1987) find $\Delta i = 0.096$. The small difference between these two numbers, if real, may reflect differences in the solution conditions of the two studies. In particular, the NMR work reported here was performed at considerably higher DNA strand concentrations than those used by Erie et al. (1987) in their salt-dependent UV melting studies. It is an interesting possibility that, in contrast to the case of polymeric DNA, Δi for the transition of oligomeric DNA may depend significantly on the total DNA phosphate concentration.

An empirical model of salt effects on oligonucleotide melting was proposed by Record and Lohman (1978) to describe the hairpin–melting data of Elson et al. (1970). According to this model, for an octamer duplex with 14 phosphate groups, 1.0 sodium ion should be released upon duplex dissociation, compared to the 1.8 sodium ions determined from the present study. For comparison, if $\Delta i = 0.17$ as determined from melting studies of polymeric DNA, then 2.4 sodium ions should be released upon octamer dissociation. Thus, as previously seen by several other groups (Williams et al., 1989; Erie et al., 1987), as far as salt effects on thermal denaturation are concerned, oligomers show behavior that is surprisingly similar to that found for polymers.

Recent Monte Carlo calculations predict that i_{helix}, the fraction of thermodynamically associated sodium ion per DNA phosphate, should equal about 0.28 for a double-helical oligonulceotide with 14 phosphate groups such as the one studied here (Olmsted et al., 1990). In contrast, i_{helix} is predicted to be about 0.8 for an infinitely long DNA molecule. Nonetheless, from the data reported here, we calculate that $\Delta i = i_{helix} - i_{coli}$ is equal to about 0.13, which is quite close to the value of $\Delta i = 0.17$ determined from the thermal denaturation of polymeric DNA. If the theoretical predictions of Olmsted et al. (1990) are correct, then i_{helix} and i_{coli} must decrease in a compensatory fashion so that Δi remains roughly constant, independent of DNA length. Clearly, more experimental work is required to test this prediction.

Kinetics of the Helix–Coil Transition. The salt dependence of k_a reported here is in good agreement with that reported for other oligomers of similar length (Pörschke et al., 1973; Williams et al., 1989). However, in contrast to results for other oligomers, we also find a significant salt dependence of k_d. This salt dependence is in agreement with our previous determinations based on selective T_1 relaxation measurements of the imino proton resonances of GGAATTCC (Braunlin & Bloomfield, 1987).

Pörschke et al. (1973) studied oligomers of the family A_nGCU_n, where $n = 2$–4. For such oligomers, the internal GC residues seem to provide the nucleus whose dissociation provides the rate-limiting step. Since the breaking of two base pairs should involve at most a minor release of bound counterions, it is not surprising that Pörschke found no salt dependence of k_d. Less readily explained are the data of Williams et al. (1989), who studied an oligomer with an AT-rich interior. These workers did find a 2-fold increase in k_D upon going from 0.012 to 0.042 M Na^+. However, k_d remained roughly constant above 0.042 M Na^+. We do not at present have a satisfactory explanation for this apparent discrepancy. It does, however, remain an intriguing possibility that both our data and those of Williams et al. (1989) are reflecting salt-dependent changes in the helix-dissociation mechanism.

Registry No. d(GGAATTCC), 70755-49-6; Na, 7440-23-5.

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