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Effects of Spermidine and Hexaamminecobalt(III) on Thymine Imino Proton Exchange†

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ABSTRACT: We examine the influence of spermidine and hexaamminecobalt(III) binding on DNA base pair dynamics. Proton NMR line-width measurements are used to monitor the exchange of the thymine imino proton in poly[dA]·poly[dT] and in the alternating copolymer $[d(AT)]_{15}$·$[d(AT)]_{15}$. We employ the treatment by Benight et al. (1988) of diffusion effects in imino proton exchange to estimate catalytic rate constants and to extract apparent equilibrium constants for the interconversion between closed (nonexchanging) and solvent-accessible (exchangeable) states of the base pairs. The salt dependence of the spermidine-catalyzed imino proton exchange in poly[dA]·poly[dT] is qualitatively described by this theory. van't Hoff analysis of the temperature dependence of the apparent equilibrium constants suggests that the binding of spermidine and hexaamminecobalt(III) lowers the energy difference between the nonexchanging and exchanging states of the AT base pair in both duplexes.

Polyamines, as well as many other DNA binding molecules, stabilize the double helix against thermal denaturation (Mahler et al., 1961; Tabor, 1962). They may also promote alterations of the secondary structure of amenable sequences, the most profound being the transition between right-handed B-DNA and left-handed Z-DNA (Behe & Felsenfeld, 1981; Rich et al., 1984).

In addition to these major structural transitions, DNA undergoes a variety of subtle, small-scale alterations over the entire accessible temperature range (Palecek, 1976). These premelting transitions are observable by a number of techniques, particularly circular dichroism (Greve et al., 1977) and NMR (Patel, 1978).

Since polyamines and their analogues exhibit profound effects on the melting of double-stranded DNA, the question of their effects on other more subtle deformations of the DNA helix naturally follows. Well below the temperature where the DNA helix dissociates into two single-stranded polymers, transient disruptions of base pairing allow exchange with solvent of the imino protons of guanine and thymine (or uracil

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in RNA) and amino protons of adenine, guanine, and cytosine. The lifetime of the solvent-accessible state is on the order of milliseconds (Leroy et al., 1985). This transient breakage of base pairing is apparently not the same transition observed as base unstacking in DNA melting experiments (Benight et al., 1988). The exchange of the amino proton may not require disruption of the base pair (Leroy et al., 1985) so the imino protons serve as the best probe of the transient assumption of a solvent-accessible state. Observation of the rate of imino proton exchange as a function of the addition of polyamines and their analogues might give insight into the nature of their association with DNA and the nature of the solvent-accessible state of the DNA base pair.

The exchange of DNA protons with solvent may be observed by a number of techniques. Sephadex chromatography using tritium (Printz & von Hippel, 1968; Hanson, 1969; McConnell & von Hippel, 1970) is limited to conditions where exchange is slow and is no longer in wide use.

Exchange of a deuteron for a proton results in a very small shift in the ultraviolet absorption spectrum of the nucleic acid bases, which may be observed by stopped-flow techniques (Cross, 1975). Mixing of DNA in an H_2O solution with a D_2O solution results in a decay in absorbance, which is dependent on the rates of imino and amino proton exchange (Nakanishi & Tsuboi, 1978; Mandal et al., 1979). The proton exchange rate constants for each class of exchanging group may be extracted from these data. Deuterium exchange may also be observed by infrared spectroscopy (Pilet & Leng, 1982). NMR has been employed to study exchange of deuterons for protons in Z-DNA as a function of real time (Mirau & Kearns, 1985a). Exchange from Z-DNA is hundreds of times slower than from B-DNA. In general, observation of hydrogen exchange in nucleic acids by NMR requires relaxation techniques.

Proton exchange in calf thymus DNA (Bendel, 1987), a variety of synthetic nucleic acid polymers (Early et al., 1981; Mirau & Kearns, 1985a,b) and oligomers (Gueron et al., 1987; Benight et al., 1988; Braunlin & Bloomfield, 1988), and tRNA (Tropp & Redfield, 1983) has been observed by NMR techniques. NMR methods are particularly well suited to study imino proton exchange because imino proton resonances are found, well separated from other resonances, in the 12–14 ppm range. This facilitates the measurement of imino proton exchange rates uncomplicated by superimposed exchange of amino protons; deconvolution of multiple exponential decays is a difficulty in the analysis of UV stopped-flow data. In oligomers, it is possible to measure rates of independently exchanging imino protons of neighboring base pairs (Gueron et al., 1987; Benight et al., 1988).

Polyamine effects on the proton exchange behavior of nucleic acids have received some attention. The effects of spermidine on proton exchange in tRNA are complex (Tropp & Redfield, 1983); it increases the exchange rate for some protons and prevents others from exchanging. At low salt concentration, spermidine is observed to decrease the rate of helix dissociation of a DNA octamer (Braunlin & Bloomfield, 1988); spermidine is also an efficient catalyst of imino proton exchange. Basu et al. (1987) report that spermine reduces the imino proton exchange rates of calf thymus DNA, poly[d(GC)] and poly[d(G-me^{5}C)]; the rate of exchange in poly[d(AT)] was, however, increased.

In this paper, we employ NMR line-width measurements to study the effects of spermidine and hexaamminecobalt(III) on thymine imino proton exchange in the nonalternating double-stranded homopolymer poly[dA]·poly[dT] and in the double-stranded alternating copolymer $[d(AT)]_{15}$·$[d(AT)]_{15}$. Since the binding affinities of spermidine (Braunlin et al., 1982) and hexaamminecobalt(III) (Plum & Bloomfield, 1988) are known to be similar, hexaamminecobalt(III), which will not catalyze imino proton exchange, will provide a comparison to spermidine to uncouple the binding and catalytic effects.

Materials and Methods

Poly[dA]·poly[dT] (Sigma Chemical Co.) of suitable length for NMR experiments, approximately 120 base pairs, was prepared by digestion with bovine pancreatic deoxyribonuclease (DNase I). DNase I was purchased from Miles Laboratories. A solution of DNase I (10 mg/mL) was prepared in 20 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM $MgCl_2$, and 2 mM $CaCl_2$ at pH 7.5. Poly[dA]·poly[dT] was dialyzed into the same buffer.

A trial digest was performed to determine the time required to result in an acceptable poly[dA]·poly[dT] size distribution. DNase I solution was added to a portion of the poly[dA]·poly[dT] solution to give a ratio of 1:5 (mg of DNase I/mg of poly[dA]·poly[dT]). The solution was incubated at 30 °C. Aliquots were removed at 10-min intervals for 70 min. The digestion reaction was stopped by addition of an equal volume of buffer-saturated phenol:0.1 M EDTA (1:1). Electrophoresis on an 8% polyacrylamide gel in tandem with a series of molecular weight markers, a *Hae*III digest of pBR322 (Sutcliffe, 1978), revealed the poly[dA]·poly[dT] size distributions of the aliquots.

The remaining poly[dA]·poly[dT] was digested for 40 min under identical conditions. The poly[dA]·poly[dT] distribution is centered at approximately 120 base pairs; the bulk of the DNA is less than 250 base pairs.

Once the digestion reaction was stopped, residual protein was removed. The solution was extracted twice with buffer-saturated phenol, followed by several extractions with diethyl ether. The solution was made 0.2 M in ammonium acetate; the DNA was precipitated by cold isopropyl alcohol over dry ice. The DNA was resuspended and dialyzed against 0.02 M NaCl, 2 mM sodium cacodylate, and 0.2 mM EDTA at pH 6.5.

The volume at this point was approximately 1 mL. The solution was concentrated 5-fold by evaporation and placed in a 5-mm NMR microtube (Wilmad Glass Co., no. 508CP). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was added to provide an internal reference for proton chemical shifts. Concentrated 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid (TAPS) was added to give a final TAPS concentration of 10 mM. The proton resonances of TAPS in conjunction with that of cacodylate provided an internal pH reference (Plum, 1989; Valcour & Woodworth, 1986).

A 30 base pair alternating AT copolymer, $[d(AT)]_{15}$·$[d(AT)]_{15}$, was prepared by the phosphoramidite method by Dr. Eric Eccleston. After removal of protecting groups, the $[d(AT)]_{15}$·$[d(AT)]_{15}$ was treated identically to the poly[dA]·poly[dT] sample.

Concentrations were determined by ultraviolet absorption using $\epsilon = 6000\ M^{-1}$ at 258 nm for poly[dA]·poly[dT] (Greve et al., 1977) and $\epsilon = 6650\ M^{-1}$ at 262 nm (Inman & Baldwin, 1962). Concentrations were approximately 40 mM DNA phosphate.

Tris·HCl was purchased from Sigma Chemical Co. and used without further purification. Spermidine trihydrochloride was purchased from Sigma and further purified by recrystallization from 90% ethanol. Hexaamminecobalt(III) trichloride was also purchased from Sigma, recrystallized from hot HCl so-

lution, and washed with ethanol and ethyl ether.

Imino proton exchange rates were determined by NMR line-width measurements. Line-width measurements are the preferred technique, since in large DNAs nonexchange contributions may dominate the relaxation (Early et al., 1980) and obscure the exchange contributions to inversion recovery experiments. In the absence of exchange, the NMR line width at half the peak height, W_0, is dependent on the effective transverse relaxation time, T_2, which contains contributions from the natural line width and instrumental factors, field inhomogeneities, etc. (Sandstrom, 1982).

$$W_0 = (\pi T_2)^{-1} \tag{1}$$

In the slow-exchange regime, the line width, W, is

$$W = \frac{1}{\pi}(k_{ex} + T_2^{-1}) \tag{2}$$

where k_{ex} is the proton exchange rate. Therefore, the catalyst-dependent exchange rate is easily extracted from the difference of line widths in the presence and absence of catalyst.

$$k_{ex} = \pi(W - W_0) \tag{3}$$

Proton NMR spectra were acquired on a Nicolet 300-MHz spectrometer. Samples were prepared with 10% D_2O, which provided a signal for frequency locking. Temperature was controlled by passing heated air through the probe. Suppression of the strong water signal was achieved by application of a $1\text{–}2\tau\text{–}\overline{5.4}\text{–}\tau\text{–}5.4\text{–}2\tau\text{–}\bar{1}$ pulse sequence (Starcuk & Sklenar, 1986), where τ is the delay between pulses and overbars indicate a 180° phase shift. This sequence results in suppression comparable to that with the standard binomial sequences (Hore, 1983) but has the advantage of a wider bandwidth (Hall & Hore, 1986).

Line widths were determined by fitting the imino resonance signals to a single Lorentzian with software packaged with the spectrometer. The interference of paramagnetic ions, which would cause broadening of NMR line widths, is guarded against by inclusion of EDTA.

Imino Proton Exchange

The DNA double helix is not a static structure in solution. At temperatures well below those where the melting transition occurs, there is evidence that the DNA base may transiently be exposed to solvent. This solvent exposure is sufficient to allow exchange of imino protons of thymine and reaction with formaldehyde (Frank-Kamenetskii, 1985). The standard model for the exchange of imino protons of double-helical DNA (Teitelbaum & Englander, 1975a,b) asserts that the exchange event is dependent upon the assumption of an "open" state by the DNA base pair. This "open" state allows access of solvent to the imino proton. The nature of this solvent-accessible state has widely, and incorrectly (Benight et al., 1988), been assumed to correspond to the unstacked state of the melted base pair. The true nature of the solvent-accessible state is not fully understood.

The general kinetic scheme supposes that a base pair is normally in a closed state, $[N\text{–}H]_{cl}$, which precludes proton exchange. Transient fluctuation of the base pair into the open state, $[N\text{–}H]_{op}$, may result in exchange with the solvent. This exchange is dependent on catalysis by hydroxide ion and added base catalysts (Leroy et al., 1985), which is governed by the proton transfer rate constant, k_{tr}.

$$[N\text{–}H]_{cl} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} [N\text{–}H]_{op} \xrightarrow{k_{tr}} [N\text{–}H]_{op} \tag{4}$$

The transfer of the proton is believed to occur via an hydrogen-bonded intermediate complex (Eigen, 1964). The rate of transfer is dependent on the collision rate, k_c, and the probability that a collision will result in transfer of a proton

$$k_{tr} = \alpha k_C/(1 + 10^{-\Delta pK}) \tag{5}$$

where $\Delta pK = pK_B - pK_{N\text{–}H}$; pK_B refers to the base catalyst and pK_{NH} to the imino group. The rate of diffusive collision is denoted k_C and is the product of the diffusion rate, k_D, and the base catalyst concentration, [B]. While the formation of the intermediate complex is assumed subject to diffusion control, an accessibility correction factor, α, is included to account for imperfect access of the catalyst to the imino proton in the open state. The proton transfer rate is, therefore

$$k_{tr} = \alpha k_D[B]/(1 + 10^{-\Delta pK}) = k_B[B] \tag{6}$$

where k_B is the rate constant for transfer of a proton from the open state to the catalyst B. Since hydroxide ion and added base are generally present, $k_{tr} = k_{OH^-}[OH^-] + k_B[B]$.

Since the electrostatic potential of DNA is considerable and most base catalysts are charged, a more sophisticated formulation of k_B would explicitly include electrostatic effects. Recently, a theory for diffusion-controlled catalytic rate constants that includes effects on the imino proton dissociation constant and diffusive collision rates from the electrostatic potential of the DNA has been developed (Benight et al., 1988). We follow this treatment of electrostatic and diffusive contributions to the catalytic rate constants, a short summary of which is included here.

Schurr's (1970a,b) treatment of diffusion effects in enzyme reactions was modified to describe the catalysis of imino hydrogen exchange in DNA (Benight et al., 1988). The catalytic rate constant, k_B, of base B is estimated by

$$k_B = \frac{k_D/f(z_I)}{1 + \dfrac{f(z_{II})}{f(z_I)}10^{-\Delta pK}} \tag{7}$$

where k_D is the diffusion-controlled association rate of the reactants and products in the absence of long-range interactions. The charge of the reaction B is z_I; that of product BH is z_{II}. $f(z)$ corrects for long-range interactions. Also, $\Delta pK \equiv pK_{BH^+} - pK_{NH}$ where pK_{BH^+} is for the catalyst and pK_{NH} is the pK for the imino proton corrected for the electrostatic potential at the DNA surface. The estimates of the association rate constants, k_D, include a steric factor to account for constraints not alleviated by rotation. The function $f(z)$, where z is the charge on the species interacting with the DNA, is

$$f(z) = \kappa R \int_{\kappa R}^{\infty} \exp[ze_0\zeta(x' + x_M)/kT]\, dx'/x'^2 \tag{8}$$

where κ is the Debye screening parameter, R the reaction radius, e_0 the elementary charge, x_M the product of κ and the distance from the cylinder axis to the exposed imino nitrogen, and $x' = \kappa r'$ where r' is the distance from the imino nitrogen. When spermidine is the catalyst, the reaction is protonated spermidine(2+) and the product, spermidine(3+). Thus, eq 7 becomes

$$k_B^{spd} = \frac{k_D^{spd}/f(+2)}{1 + \dfrac{f(+3)}{f(+2)}10^{-\Delta pK}} \tag{9}$$

The functional form of the electrostatic potential of DNA is a matter of considerable uncertainty (Anderson & Record, 1982); the potential $\zeta(x)$ calculated by Stigter (1975) is used.

While there are several parameters whose values are uncertain, this treatment of the catalytic rate constants successfully describes a large body of proton exchange experiments (Benight et al., 1988).

When the equilibrium constant for formation of the solvent-accessible state is small, that is, $K_{eq} = k_{op}/k_{cl} \ll 1$, the overall rate for proton exchange, k_{ex}, may be written as

$$k_{ex} = \frac{k_{op}k_{tr}}{k_{cl} + k_{tr}} \quad (10)$$

Two limiting cases of eq 10 are of interest. If $k_{tr} \gg k_{cl}$, then the exchange process is opening limited; exchange will occur at each opening event and $k_{ex} = k_{op}$. The opening-limited exchange mechanism is characterized by a lack of catalyst concentration dependence. Until recently, imino proton exchange was uniformly described as opening limited. These observations were due to insufficient base catalysis as shown by Leroy et al. (1985). In the nonopening limited case, the rate of proton transfer is slow compared to the closing rate and the exchange rate depends linearly on the catalyst concentration

$$k_{ex} = K_{eq}k_{tr} \quad (11)$$

Equation 10 is conveniently rearranged to

$$\frac{1}{k_{ex}} = \frac{1}{k_{op}} + \frac{1}{K_{eq}k_{tr}} \quad (12)$$

Since the proton transfer rate, k_{tr}, is linearly dependent on the base catalyst concentration, a plot of $1/k_{ex}$ versus $1/[B]$ will intersect the ordinate at $1/k_{op}$, the lifetime of the intact base pair, with a slope that depends on the base pair opening equilibrium constant, K_{eq} (Leroy et al., 1985). Accurate determination of k_{ex} (equation 3) is difficult at low rates of exchange due to small changes in the imino proton line width. When the exchange rates are high, accurate determination of line widths is complicated by excessive broadening. Due to these problems with data quality, the intercept of eq 12 is small and sometimes negative. Therefore, eq 11 is preferred for determination of K_{eq}.

Proton exchange observed in the absence of added catalyst is presumed to result from self-catalysis (Gueron et al., 1987), which is characterized by a rate constant k_{aac}. The rate of imino proton exchange is written $k_{tr} = k_{OH^-}[OH^-] + k_B[B] + k_{aac}$. Under conditions where the contributions of added catalyst and hydroxide ion to k_{tr} are small, the rate of exchange in the absence of added catalyst, k_{aac}, may dominate the observed imino proton exchange. This could result from either low concentrations of catalysts or small rate constants. Alternating A-T polymers are, apparently, particularly prone to exchange in the absence of added catalyst (Benight et al., 1988); that is, k_{aac} is unusually large for these polymers. Even in the presence of large amounts of base catalyst, poly[d(AT)]·poly[d(TA)] imino proton exchange is not dependent on catalyst concentration (Hartmann et al., 1986).

Results

Poly[dA]·Poly[dT]. In a solution containing 0.1 M Na^+, poly[dA]·poly[dT] melts at 68 °C (Riley et al., 1966); the transition breadth is 0.9 °C. In all of our experiments the temperature is well below the onset of the melting transition. Therefore, by monitoring imino proton exchange kinetics we are observing transient fluctuations in the intact DNA helix and not melting.

Four sets of spectra were collected as a function of temperature between 25 and 55 °C. Spectra were collected as a function of the concentrations of the base forms of Tris and spermidine at pH 7.0. Base concentrations were calculated by [base] = [total catalyst]/(1 + 10^{pK-pH}). Corrections for the temperature dependence of the pK of Tris (Good & Izawa, 1972) and the spermidine(3+) ⇄ spermidine(2+) equilibrium (Gold & Powell, 1976) were made. Two sets of spectra were collected as a function of $[OH^-]$, in the absence and presence of hexaamminecobalt(III).

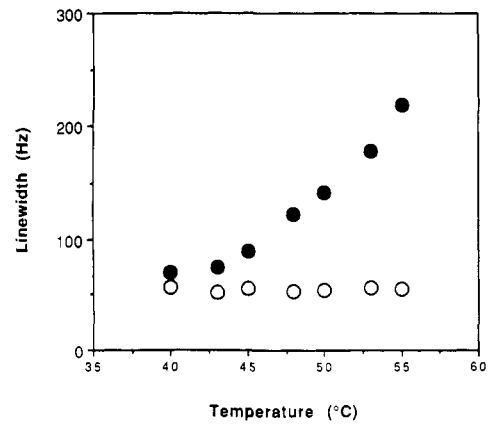

FIGURE 1: Dependence of poly[dA]·poly[dT] imino proton line width on temperature. Each point represents the average of two measurements. Open circles, pH 6.7; closed circles, pH 8.9.

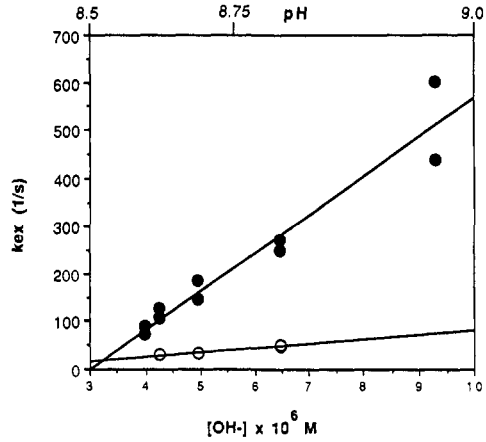

FIGURE 2: Dependence of poly[dA]·poly[dT] imino proton exchange rate, k_{ex}, on hydroxide ion concentration. Open circles, 40 °C; closed circles, 55 °C. The slopes of the linear least-squares best-fit lines that are shown are used to estimate K_{eq}, since $dk_{ex}/d[OH^-] = k_{OH}K_{eq}$ when exchange is limited by hydroxide ion catalysis. The open circles are duplicate pairs of points.

Figure 1 shows the dependence of imino proton line width on temperature at low (pH 6.7) and high (pH 8.9) hydroxide ion concentration. Similar behavior is observed when the other catalysts are present.

When the data are plotted in the fashion of Leroy et al. (1985) [$1/k_{ex}$ versus $1/[base]$), we find that at temperatures above 40 °C the intercepts ($1/k_{op}$) are indistinguishable from zero or negative values. This has also been observed by Benight et al. (1988). Therefore, to extract the equilibrium constant, K_{eq}, from the data we plot k_{ex} versus [base] (Figure 2). When the exchange is limited by base catalyst, the curve is linear (see eq 11); the slope of this line is k_BK_{eq}. The base catalyst rate constant, k_B, is estimated independently (eq 7), allowing estimation of K_{eq}.

Rate constants estimated for spermidine catalysis range from 4.4×10^7 M^{-1} s^{-1} at 25 °C to 8.7×10^7 M^{-1} s^{-1} at 55 °C. These estimates yielded equilibrium constants ranging from 5×10^{-3} to 2×10^{-2}. For Tris catalysis, k_{Tris} ranges from 9.7×10^6 M^{-1} s^{-1} to 7.6×10^6 M^{-1} s^{-1}; the reduction of k_{Tris} with

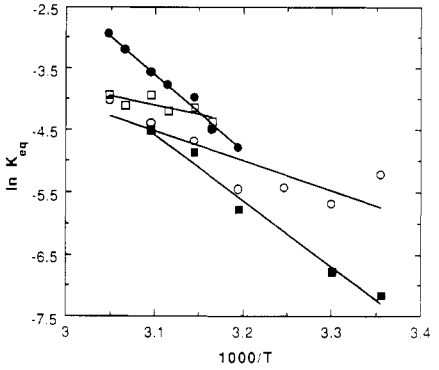

FIGURE 3: van't Hoff plots of poly[dA]·poly[dT] base pair dissociation constant. Closed circles: OH^- catalysis; ΔH = 24.9 ± 1.3 kcal/mol. Closed squares: Tris catalysis; ΔH = 21.1 ± 1.6 kcal/mol. Open circles: spermidine(2+) catalysis; ΔH = 9.5 ± 2.2 kcal/mol. Open squares: OH^- catalysis with hexaamminecobalt(III); ΔH = 11.6 ± 3.5 kcal/mol.

temperature is due to the strong temperature dependence of the Tris pK_a. Equilibrium constants are estimated to range from 8×10^{-4} to 8×10^{-3}. Rate constants estimated for hydroxide catalysis range from 1.1×10^9 M^{-1} s^{-1} at 40 °C to 1.5×10^9 M^{-1} s^{-1} at 55 °C. Equilibrium constants range from 8×10^{-3} to 5×10^{-2}. For estimation of k_{OH} in the presence of hexaamminecobalt(III), the effective charge of the DNA is reduced by 45% to account for screening by the trivalent cation. This alters the magnitude but not the temperature dependence of k_{OH}. At 40 °C the rate constant is estimated to be 3.5×10^9 M^{-1} s^{-1} and the equilibrium constant 7×10^{-3}. At 55 °C these become 3.5×10^9 M^{-1} s^{-1} and 2×10^{-2}.

At 27 °C, the equilibrium constant for base pair opening, K_{eq}, for the RNA analogue of poly[dA]·poly[dT], poly[rA]·poly[rU], is reported to be approximately 10^{-3} (Leroy et al., 1985) when hydroxide ion is the catalyst. We find, extrapolating our data, that K_{eq} for poly[dA]·poly[dT] is about 1.4×10^{-3}.

From the temperature dependence of K_{eq}, the dissociation enthalpy change of the base pair may be calculated from the van't Hoff equation

$$\left(\frac{\partial \ln K}{\partial (1/T)}\right)_P = -\frac{\Delta H}{R} \qquad (13)$$

Figure 3 shows van't Hoff plots when Tris base, spermidine(2+), and hydroxide ion, with and without hexaamminecobalt(III), are used as catalysts. When hydroxide ion is the catalyst, we find ΔH = 24.9 ± 1.3 kcal/mol, where the uncertainty is the estimated standard error of the slope. Similarly, the enthalpy change when Tris is the catalyst = 21.1 ± 1.6 kcal/mol. In contrast, ΔH = 9.5 ± 2.2 kcal/mol when spermidine(2+) is the catalyst. In the presence of 2.6 mM hexaamminecobalt(III) (1 hexaamminecobalt(III)/7 base pairs), we find ΔH = 11.6 ± 3.5 kcal/mol when hydroxide ion is the catalyst.

The addition of salt reduces the effectiveness of spermidine as a catalytic agent. The rate of spermidine-catalyzed (3.5 mM) imino proton exchange in poly[dA]·poly[dT] (25 mM DNA phosphate) was monitored as a function of sodium ion concentration at 40 °C and pH 7.0. At 0.1 M Na^+, k_{ex} is 120 s^{-1}. At 0.5 M Na^+, k_{ex} is reduced to 35 s^{-1}. If it is assumed that K_{eq} is invariant with sodium ion concentration, the calculated dependence of k_B on [Na^+] is in the correct direction but does not fully account for the reduction in k_{ex}. This may be due to a salt-dependent change in K_{eq}. There is negligible sodium ion dependence observed for Tris catalysis, because the catalytic species of Tris is uncharged.

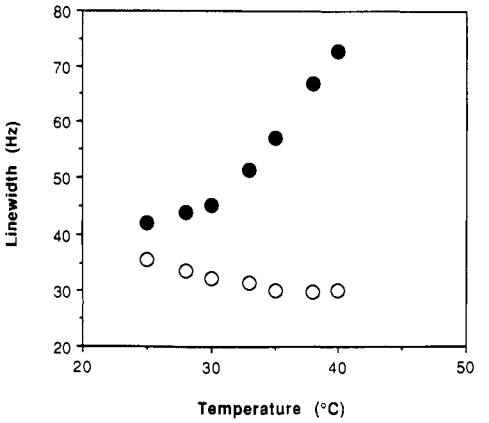

FIGURE 4: Dependence of $[d(AT)]_{15}$·$[d(AT)]_{15}$ imino proton line width on temperature. Each point represents the average of two measurements. Open circles, pH 6.6; closed circles, pH 9.0.

$[d(AT)]_{15}$·$[d(AT)]_{15}$. The $[d(AT)]_{15}$·$[d(AT)]_{15}$ polymer is long enough to possess three full turns of a B-form helix and is expected to melt at only slightly lower temperature than poly[d(AT)]·poly[d(AT)] (Elson et al., 1970). Also, the chemical shift of the nonexchangeable thymine CH_3-5 protons is very sensitive to melting (Patel, 1978). In intact helices the resonance is found at 1.3 ppm; upon melting an abrupt shift to 1.7 ppm is observed. We find no shift in this resonance over the entire experimental temperature range. Alternating sequences, such as $[d(AT)]_{15}$, may fold back on themselves and form hairpin structures (Baldwin, 1971). The imino protons of the bases in the loop region of a DNA hairpin resonate between 10 and 11 ppm (Haasnoot et al., 1983); no evidence of hairpin structures is observed in the NMR spectra of the sample. We cannot, however, conclude that there is no aggregation of multiple $[d(AT)]_{15}$ strands to form double helices longer than 30 base pairs. There is good evidence that imino proton exchange occurs with individual base pairs (Gueron et al., 1987; Benight et al., 1988); therefore, extended helices need not be considered deleterious.

As expected for smaller molecules, the line width in the absence of catalyst is smaller for $d(AT]_{15}$·$[d(AT)]_{15}$ than for poly[dA]·poly[dT]. The peak is located about 1 ppm upfield of the poly[dA]·poly[dT] resonance; this is characteristic of alternating AT base pairs. The dependence of the imino proton line width on temperature and catalyst is analogous to that of poly[dA]·poly[dT]; in the absence of base catalysis the line width decreases with increasing temperature and with sufficient catalysis the line width increases with temperature. Figure 4 shows the $[d(AT)]_{15}$·$[d(AT)]_{15}$ imino proton line width as a function of temperature.

We find that, like the poly[dA]·poly[dT] data, the slopes of $1/k_{ex}$ versus 1/[base] curves are large and result in negative intercepts. Therefore, equilibrium constants are extracted from k_{ex} versus [base] curves in the catalysis-limited region (Figure 5). Hartmann et al. have reported that the exchange rate of imino proton of poly[d(AT)]·poly[d(AT)] is insensitive to hydroxide ion concentration up to about pH 8. Our data are consistent with this; we see no significant change in imino proton line width until the pH is 8.5 or higher. Suggestions have been made (Benight et al., 1988) that the insensitivity of poly[d(AT)]·poly[d(AT)] exchange to added catalyst is due to an abnormally important contribution from the mechanism involving exchange in the absence of added catalyst (Gueron et al., 1987).

Estimates of k_B and K_{eq} are made in the same fashion as for the poly[dA]·poly[dT] case. For spermidine catalysis k_{spd} is estimated to range from 4.4×10^7 M^{-1} s^{-1} at 25 °C to 7.4

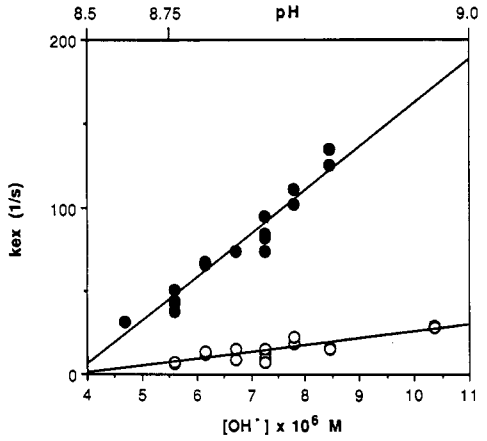

FIGURE 5: Dependence of $[d(AT)]_{15}$·$[d(AT)]_{15}$ imino proton exchange rate, k_{ex}, on hydroxide ion concentration. Open circles, 25 °C; closed circles, 40 °C. The slopes of the linear least-squares best-fit lines that are shown are used to estimate K_{eq}, since $dk_{ex}/d[OH^-] = k_{OH}K_{eq}$ when exchange is limited by hydroxide ion catalysis.

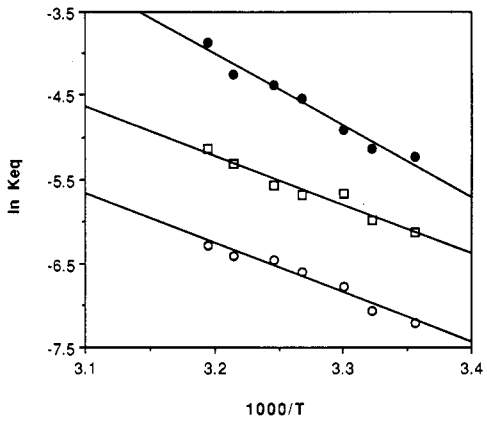

FIGURE 6: van't Hoff plots of the $[d(AT)]_{15}$·$[d(AT)]_{15}$ base pair dissociation constant. Closed circles: OH^- catalysis; $\Delta H = 16.9 \pm 1.3$ kcal/mol. Open circles: spermidine(2+) catalysis; $\Delta H = 11.8 \pm 1.1$ kcal/mol. Open squares: OH^- catalysis with hexaamminecobalt(III); $\Delta H = 11.6 \pm 1.1$ kcal/mol.

$\times 10^7$ M^{-1} s^{-1} at 40 °C, resulting in estimates of K_{eq} ranging from 7×10^{-4} to 2×10^{-3}. When hydroxide ion is the catalyst, k_{OH} ranges from 7.8×10^8 M^{-1} s^{-1} to 1.1×10^9 M^{-1} s^{-1} and K_{eq} from 2×10^{-3} to 6×10^{-3}.

van't Hoff plots (Figure 6) are used to extract ΔH for the base pair dissociation. We find that when exchange is catalyzed by hydroxide ion, $\Delta H = 16.9 \pm 1.3$ kcal/mol. As in the case of poly[dA]·poly[dT], this enthalpy is lowered by the presence of hexaamminecobalt(III), $\Delta H = 11.6 \pm 1.1$ kcal/mol. Catalysis by spermidine leads to $\Delta H = 11.8 \pm 1.1$ kcal/mol.

Discussion

By application of NMR line-width measurements we have measured the rates of thymine imino proton exchange in two model DNA polymers: poly[dA]·poly[dT] and $[d(AT)]_{15}$·$[d(AT)]_{15}$. Clearly, from previous studies of these polymers and our observations of the NMR spectra, this exchange is occurring well below the onset temperature of the melting transition.

In the standard model for hydrogen exchange in double-stranded nucleic acids, the base pair must transiently adopt a configuration that allows solvent access to the exchangable protons. The probability of an exchange event occurring while the proton is accessible is determined by the presence of base catalyst. By measuring the rate of imino proton exchange under conditions where the exchange is limited by the concentration of base catalyst, we may extract estimates of the equilibrium constant K_{eq} for the assumption of the solvent-accessible state of the base pair.

The values of K_{eq} that we determine for poly[dA]·poly[dT] are similar to measurements of the equilibrium constant for poly[rA]·poly[rU] (Leroy et al., 1985). Also the temperature dependence of K_{eq} is similar for both polymers; $\Delta H = 20.6$ kcal/mol for the RNA polymer and for the DNA polymer it is 24.9 ± 1.3 kcal/mol and 21.1 ± 1.6 kcal/mol when hydroxide ion and Tris are catalysts, respectively.

When catalysis is provided by hydroxide ion, the enthalpy change for the base pair dissociation is larger for poly[dA]·poly[dT] than for $[d(AT)]_{15}$·$[d(AT)]_{15}$, 24.9 ± 1.4 kcal/mol and 16.9 ± 1.3 kcal/mol, respectively. This is consistent with the slightly lower stacking enthalpy for the alternating polymer, which has been measured calorimetrically (Marky & Breslauer, 1982); $\Delta H_{stack} = 7.1$ kcal/mol for poly[d(AT)]·poly[d(AT)] and $\Delta H_{stack} = 8.6$ kcal/mol for poly[dA]·poly[dT]. The coincidental similarity of the dissociation enthalpy change and twice the stacking enthalpy change suggests to some that the open state observed by imino proton exchange may be identical with that of the unstacked base pair of the melting transition. Recent evidence renders unlikely the conclusion that the two open states are identical. Benight et al. have shown that the melting and exchange properties of the two central A·T base pairs of a 16 base pair double-helical region of a closed DNA circle do not correspond.

We have shown that spermidine and hexaamminecobalt(III) lower the enthalpy change for the transition between closed and solvent-accessible states in both poly[dA]·poly[dT] and $[d(AT)]_{15}$·$[d(AT)]_{15}$. The effects of the trivalent cations on the magnitude of the equilibrium constant K_{eq} are less readily determined. Since the estimates of the catalyst-dependent proton exchange rate constants k_B are somewhat uncertain in magnitude, K_{eq} is also uncertain. The prediction of temperature dependence of k_B should, however, be better than the prediction of its magnitude. Electrostatic effects on the catalytic rate constant should be rather small; ϵT is nearly invariant with temperature over this range. Therefore, the major contribution to the temperature dependence of the rate constant, which would depend on the base catalyst, is the temperature dependence of the pK of the catalyst. The temperature dependence of the pKs for the bases of interest is well-known; consequently, estimations of the temperature dependence of K_{eq} are likely to be better than the estimates of their magnitude.

We find that the catalytic efficiency of spermidine is reduced by increasing monovalent salt. This effect is partially described by a reduction in the estimate for k_{spd}. The effect may be described simply by a reduction in the local spermidine concentration; binding of spermidine is known to decrease as a function of monovalent salt concentration (Braunlin et al., 1982).

The general conclusion we draw from our observations is that spermidine and hexaamminecobalt(III) reduce the difference in energy between the closed and solvent-accessible states of poly[dA]·poly[dT] and $[d(AT)]_{15}$·$[d(AT)]_{15}$. Our observations reveal nothing about the origins of this alteration. Whether interaction with the trivalent cations alters the closed state or the open state or both is not addressed by our analysis.

Computer simulations of binding spermine to alternating AT sequences (Feuerstein et al., 1986) suggest that spermine binding to the DNA major groove causes the minor groove to widen. Enlargement of the minor groove would increase

the accessibility of the thymine imino proton to solvent. Basu et al. (1987) speculate that this would result in an increase in the exchange rates of imino protons in poly[d(AT)]·poly-[d(AT)]. Perhaps a similar distortion of the DNA helix is responsible for the lowering of the enthalpy that we observe.

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