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Base Protonation Facilitates B–Z Interconversions of Poly(dG-dC)·Poly(dG-dC)†

Fu-Ming Chen

ABSTRACT: Comparative studies on the salt titration and the related kinetics for poly(dG-dC)·poly(dG-dC) in pH 7.0 and 3.8 solutions clearly suggest that base protonation facilitates the kinetics of B–Z interconversion although the midpoint for such a transition in acidic solution (2.0–2.1 M NaCl) is only slightly lower than that of neutral pH. The rates for the salt-induced B to Z and the reverse actinomycin D induced Z to B transitions in pH 3.8 solutions are at least 1 order of magnitude faster than the corresponding pH 7.0 counterparts. The lowering of the B–Z transition barrier is most likely the consequence of duplex destabilization due to protonation as indicated by a striking decrease (~40 °C) in melting temperature upon H^+ binding in low salt. The thermal denaturation curve for poly(dG-dC)·poly(dG-dC) in a pH 3.8, 2.6 M NaCl solution indicates an extremely cooperative melting at 60.5 °C for protonated Z DNA, which is immediately followed by aggregate formation and subsequent hydrolysis to nucleotides at higher temperatures. The corresponding protonated B-form poly(dG-dC)·poly(dG-dC) in 1 M NaCl solution exhibits a melting temperature about 15 °C higher, suggesting further duplex destabilization upon Z formation.

Continuous acidification of a DNA solution of medium salt concentration can result in acid denaturation at pH below 3 (Gulland et al., 1947; Cox & Peacocke, 1957; Sturtevant et al., 1958). However, in a moderately acidic solution (pH 3–5) the DNA exists in a partially base-protonated duplex state (Cavalieri & Rosenberg, 1957; Peacocke & Preston, 1958; Geiduschek, 1958) that exhibits a dramatic decrease (~40 °C) in melting temperature from the corresponding neutral counterpart (Zimmer et al., 1968; Courtois et al., 1968; Chen, 1983a). This protonated duplex greatly enhances binding to some polycyclic aromatic hydrocarbons (Chen, 1984) and causes a striking sign reversal for the induced circular dichroism (CD) of DNA-bound pyrene (Chen, 1983a). This interesting sign inversion has been shown to result from changes in base sequence specificity of pyrene from preference for dA-dT (and/or dT-dA) in neutral DNA (Chen, 1983b) to an affinity for all purine–pyrimidine sequences, especially dG-dC (and/or dC-dG), in protonated DNA (Chen, 1983a).

The exact nature of this protonated duplex state, however, is still unclear. There is controversy as to which base of the DNA is the first to be protonated. On the basis of the fact

† From the Department of Chemistry, Tennessee State University, Nashville, Tennessee 37203. *Received February 14, 1984.* Research supported by U.S. PHS Grant CA29817 and in part by MBRS Grant S06RR0892.

0006-2960/84/0423-6159$01.50/0

that of the four bases cytosine possesses the highest pK value (4.3 for dC) (Fasman, 1975), earlier studies suggested that cytosine is the site of the first protonation (Dove et al., 1959; Zimmer & Vernner, 1966). This view seems to be supported by a recent Raman study on the pH titration of DNA solutions (O'Conner et al., 1982). Extensive spectroscopic studies, however, led Guschulbauer to the opposite conclusion by suggesting that, since the N3 of cytosine is involved in base-pair hydrogen bonding, the protonation should occur at the N7 of guanine with a subsequent swinging around to a syn conformation to form a Hoogsten's base pair by sharing a proton with the N3 of cytosine (Guschulbauer & Courtois, 1968; Courtois et al., 1968). This conjecture is quite intriguing in view of the fact that guanosine in left-handed Z DNA exists in just such a syn conformation (Wang et al., 1979).

Pohl & Jovin (1972) first reported that poly(dG-dC)·poly(dG-dC) exhibits a cooperative conformational transition at high salt concentrations as evidenced by a near inversion of its CD spectrum. In 1979, a novel left-handed Z conformation was discovered by the X-ray crystal diffraction on hexanucleotide $(dC\text{-}dG)_3$ (Wang et al., 1979). Shortly after, a laser Raman study (Thamann et al., 1981) confirmed that the high-salt form of poly(dG-dC)·poly(dG-dC) is indeed that of the newly discovered Z type. Several reports have since appeared to indicate that some chemical modification of the bases can facilitate the B–Z interconversion (Behe & Felsenfeld, 1981; Moller et al., 1981; Sage & Leng, 1980; Malfoy et al., 1982). Of particular interest to us are the N7-methylating agents since it has been suggested (Moller et al., 1981) that methylation of the N7 of guanine facilitates the Z formation in poly(dG-dC)·poly(dG-dC) in part through the positive charge that is put on the guanosine residue, which shields the phosphate repulsions between the two polynucleotide chains. If indeed this is the case, it is reasonable to suspect that, if protonation does occur at the N7 position of guanine, it may also facilitate such interconversion. In order to investigate such a possibility, comparative studies on the salt titration and the related kinetics for poly(dG-dC)·poly(dG-dC) in pH 7.0 and 3.8 buffer solutions have been carried out, and the results appear to support that suspicion.

Materials and Methods

Poly(dG-dC)·poly(dG-dC) was purchased from P-L Biochemicals and dissolved in either a 10 mM sodium phosphate buffer of pH 7 or a 10 mM sodium citrate buffer of pH 3.8. Both buffer solutions contain 0.01 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Extinction coefficients of 8400 and 7300 M^{-1} cm^{-1} at 255 nm were used for the concentration determination at pH 7 and 3.8, respectively.

Salt-jump experiments were carried out either by adding concentrated NaCl solution (5 M) in the appropriate buffer or by direct solid addition. Mixing is accomplished with rigorous manual shaking in the CD measurements but with the use of the stirrer accessory in the absorbance measurements. All absorption measurements were made by a Cary 210-plus spectrophotometric system. Melting experiments were performed in stoppered quartz cuvettes by using the "chart" utility of the temperature-readout accessory with a 0.3 °C/min heating rate maintained by a Neslab RTE-8 refrigerated circulating bath and a EPT-4RC temperature programmer. Absorbance vs. temperature data were also acquired every 15 s with an Apple II microcomputer. Numerical differentiations were performed to obtain differential melting profiles from which melting temperatures and half-widths were deduced. CD spectra were measured with a JASCO J-500A recording spectropolarimeter in jacketed cylindrical cells. Base-line corrections are made unless stated otherwise.

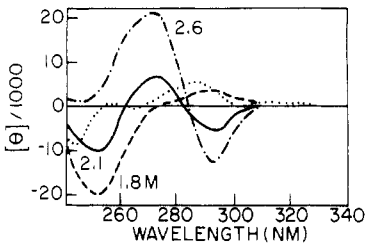

FIGURE 1: CD spectra at room temperature of 23 μM poly(dG-dC)·poly(dG-dC) in pH 3.8 solutions of some representative NaCl concentrations: (···) 0.01, (– –) 1.8, (—) 2.1, and (–·–) 2.6 M.

Results

Salt Titration of Protonated Poly(dG-dC)·Poly(dG-dC). Some representative CD spectra of poly(dG-dC)·poly(dG-dC) in pH 3.8 buffer solutions of varying salt content are presented in Figure 1. The spectrum in the acidic solution containing 0.01 M NaCl is much weaker and distinctly different from the corresponding pH 7 solution, which is characterized by a strong negative CD band at 250 nm (compare with Figure 4). The acidic solution spectrum at low salt is similar to that of Narasimhan & Bryan (1975). A moderate increase in the salt concentration alters the spectrum to resemble more the B-form DNA in neutral solutions as suggested by the prominent presence of the 250-nm band. This is most likely the result of a pK decrease in DNA bases due to the enhanced ionic strengths (Michelson, 1963). It is apparent that a B to Z conversion does indeed take place at high salt concentrations as indicated by the appearance of a characteristic negative CD band at 293 nm and a positive maximum near 272 nm. In contrast to the approximately equal intensities of the two oppositvely signed bands for the Z form in the neutral solution (Pohl & Jovin, 1972), the magnitude of the positive shorter wavelength band is about twice the intensity of the negative band with slight red shifts for both. It is interesting to note that similar spectral characteristics are also exhibited by the Z form of the N7-methylated poly(dG-dC)·poly(dG-dC) (Moller et al., 1981), thus lending some credence to Guschulbauer's suggestion that protonation occurs at the N7 position of guanine.

CD measurements extended down to 200 nm were also made by using a 1-mm cell. Instead of a negative CD maximum observed at 205 nm for poly(dG-dC)·poly(dG-dC) in a 0.01 M NaCl neutral solution, negative-going CD intensities are apparent at this same wavelength for the high salt solutions (3 M NaCl) of both pH 7.0 and pH 3.8 (results not shown). Such a spectral difference between the B and Z forms has been noted by others (Sutherland et al., 1981), thus lending some support to the acidic Z DNA assignment although the opacity of NaCl in this spectral region makes the comparison less definitive.

Plots of ellipticities at 290 nm as well as A_{295}/A_{260} (ranging from 0.17 to 0.40 as compared to 0.12–0.31 reported by Pohl & Jovin for the neutral solutions) vs. NaCl concentrations are shown in Figure 2 for the pH 3.8 titrations from which a transition midpoint of 2.0–2.1 M NaCl is estimated for the B–Z transformation of protonated poly(dG-dC)·poly(dG-dC). This is to be compared with the B–Z transition midpoint of 2.5 M in a neutral solution as obtained by Pohl & Jovin (1972). Thus, it appears that base protonation does indeed slightly facilitate the salt-induced B to Z conversion in a thermodynamic sense. Such facilitation, however, is not as pronounced as that of the N7 methylation of guanine. This

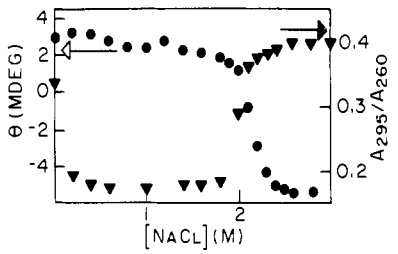

FIGURE 2: Salt titration of 23 μM poly(dG-dC)·poly(dG-dC) solution of pH 3.8. Ellipticities at 290 nm (●) and A_{295}/A_{260} (▼) are plotted against NaCl concentrations by adding the appropriate amount of solid directly into the spectrophotometric cells. A 2-cm cylindrical cell was used in the CD measurements, and molar ellipticities can be obtained by multiplying 2174 to the millidegree values in the figure.

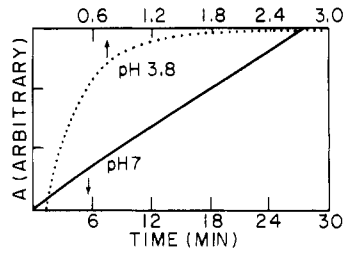

FIGURE 3: Comparison of the B to Z transition kinetics of 23 μM poly(dG-dC)·poly(dG-dC) solutions at pH 3.8 (···) and pH 7 (—). Absorbance changes are monitored at 290 nm as a function of time after an appropriate amount of poly(dG-dC)·poly(dG-dC) solution of 0.01 M NaCl is added to a 5 M NaCl solution of the same buffer to achieve a 2.6 M NaCl concentration.

may be due to the fact that only a small fraction of the guanine base is being protonated due to its low pK value (pK = 2.5 for dG but may be slightly different in polynucleotides). The more dramatic effect, however, is manifested in the kinetic aspect of such interconversion as to be described below. It is interesting to note in passing that in the pH 3.8 solution the A_{295}/A_{260} value is quite large at 0.01 M NaCl but decreases sharply to a typical B-DNA value with only a slight increase in salt concentration. This observation is consistent with the large CD changes observed in the 250-nm region (Figure 1), possibly the consequence of partial denaturation in the very low salt solution.

Kinetics of B to Z Transition. The striking pH dependence in the kinetic behavior of the salt-induced B to Z conversion of poly(dG-dC)·poly(dG-dC) is demonstrated by a relaxation time of 20–30 s at pH 3.8 vs. that of 5000 s at pH 7 as the NaCl concentration is jumped from 0.01 to 2.6 M. This difference is graphically illustrated in Figure 3, where it is apparent that within 3 min the equilibrium has been reached in the acidic solution whereas no sign of settling is evident in the neutral solution even at 30 min. Although the relaxation time at pH 7 is reduced to about 500 s (results not shown) in 4 M NaCl, it is still more than 1 order of magnitude longer than that of pH 3.8 solution. In fact, the formation of the negative 293-nm CD band in the acidic solution of 4 M NaCl is almost instantaneous, less than the manual mixing time of 10 s. This is immediately followed by a slight magnitude decrease of this 293-nm negative band as a result of progressive increase of the positive 272-nm band with a characteristic time of about 3 min (results not shown). This fact, along with a large difference in intensities, suggests that these two bands are not entirely coupled by exciton splitting in the acidic solution.

The observed relaxation times in neutral solutions are considerably longer than those of Pohl and Jovin (100–1000 s), which most likely arise from the high molecular weight polymers used in the present study. The longer chain length

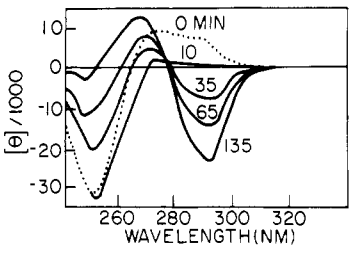

FIGURE 4: CD spectra of 20 μM poly(dG-dC)·poly(dG-dC) solution of pH 7 at various time intervals after the NaCl concentration is changed from 0.01 to 2.6 M NaCl.

manifests itself in the extreme cooperativity observed in our system. For example, a Z formation is not induced in neutral solution at 2.2 M NaCl but can be initiated with 2.4 M and is essentially complete for an overnight wait (a single exponential decay with a relaxation time of slightly over 2 h has been measured), suggesting that the transition midpoint for our neutral solution is between 2.2 and 2.4 M NaCl. This is in accord with a previous observation (Pohl, 1983) that various transition curves of different chain lengths intersect each other at 2.25 M NaCl and the current best estimate of a 2.35 M NaCl transition midpoint from Jovin's group (T. M. Jovin, private communication). The extent of the Z conversion is judged by the CD comparison with the poly(dG-dC)·poly(dG-dC) solution of 4 M NaCl.

The unusually long relaxation time for the pH 7 solution of poly(dG-dC)·poly(dG-dC) in 2.6 M NaCl enables the CD measurements to be made at various time intervals, which are presented in Figure 4. An isoelliptic point is apparent for spectra at various times after the salt addition, suggesting the coexistence of possibly two species during the process. Although the rapid kinetics in pH 3.8 prevents us from similar measurements, the above-mentioned differing spectral characteristics in the two different pH solutions for both B and Z forms can partially be seen by comparing Figures 1 and 4.

Kinetics of Ligand-Induced Z to B Transition. It has been shown that intercalators such as ethidium bromide can induce Z to B transitions with high cooperativity when more than threshold concentration of the drug is added (Pohl et al., 1972). The effect of the intercalating drugs on the kinetics of the B to Z transition has also been investigated to indicate that low levels of actinomycin D (less than 1 per 450 base pairs) can reduce the transition rate (Mirau & Kearn, 1983). We have found that the binding of actinomycin D and ethidium bromide also inhibit the B to Z transition of poly(dG-dC)·poly(dG-dC) in acidic solutions and only a very small concentration of actinomycin D is needed to induce the reverse Z to B transition. This drug is thus used to carry out comparative kinetic studies of the ligand-induced Z to B transition in neutral and acidic solutions.

At pH 7 and 4 M NaCl, the Z to B transformation induced by the addition of 5 μM actinomycin D is seen to be dominated by a slow relaxation time on the order of 4000 s with a minor component apparent at the beginning of a slightly faster rate. In contrast, the same process in a pH 3.8 solution clearly exhibits at least two relaxation times, a fast one of 10–15 s that takes up more than 50% of the ellipticity change at 290 nm and a slower one of about 350 s. These differences are more clearly illustrated in Figure 5, where a 10-fold expansion of the time scale for the acidic solution is also included to show more clearly its multiexponential nature. Notice that even on this expanded scale the slower relaxation curve in the acidic solution still has a steeper slope than that of the corresponding neutral solution, confirming the values given earlier indicating the rate is 1 order of magnitude faster in the acidic solution.

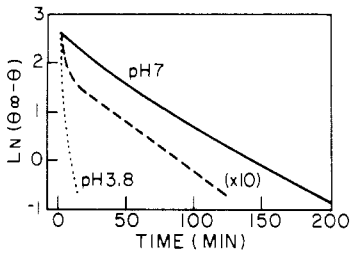

FIGURE 5: Kinetics of actinomycin D (5 μM) induced Z to B transition of poly(dG-dC)·poly(dG-dC) solutions containing 4 M NaCl at pH 3.8 (···) and pH 7 (—). Time-dependent ellipticities at 290 nm are measured after 18 μL of 0.51 mM drug is added to 1.8 mL of 23 μM polynucleotide solution in a 2-cm cylindrical cell. The dashed curve is the expanded plot for pH 3.8 with the time scale reduced by a factor of 10.

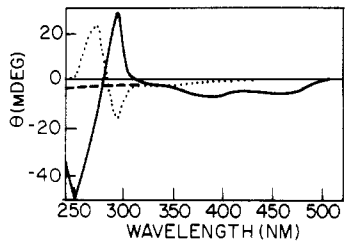

FIGURE 6: CD spectra of 23 μM poly(dG-dC)·poly(dG-dC)/4 M NaCl/pH 3.8 solution before (···) and 0.5 h after (—) the addition of 5 μM actinomycin D. These spectra are not base-line corrected, and the slight base-line drift is indicated by the dashed curve. A 2-cm cell was used, and multiplication of 2174 will convert millidegrees into molar ellipticities.

Thus, it is apparent that base protonation also facilitates the ligand-induced Z to B conversion. The differing kinetic behavior exhibited in these two solutions may be the consequence of greater rate difference in the rapid B–Z transition and the slower actinomycin D binding to B DNA in the acidic solution but of similar orders of magnitude for these two processes in the neutral solution.

The CD spectra of poly(dG-dC)·poly(dG-dC) in pH 3.8 and 4 M NaCl solution before and after the addition of actinomycin D are shown in Figure 6. The conversion to B DNA is evidenced by the large 292-nm positive and the even greater 250-nm negative bands in the DNA region and the appearance of the characteristic B DNA bound actinomycin D spectral features in the long-wavelength region. The spectrum resulting from the drug addition for the corresponding pH 7 solution is almost identical with that of the acidic solution. These observations appear to be in line with the allosteric model of Pohl et al. (1972).

Thermal Stability of Z DNA. No thermal denaturation measurement of Z DNA in NaCl has thus far been reported. This is due in part to the fact that even at a low salt concentration of 0.01 M NaCl poly(dG-dC)·poly(dG-dC) melts around 95 °C (Wells, 1970). This renders it impractical for melting studies at a higher salt concentration. The 40 °C decrease in melting temperature upon protonation as stated earlier, however, offers us the opportunities for such studies in acidic solutions. For this purpose, the salt concentrations are to be kept as low as practical for the Z formation so as not to unnecessarily increase the melting temperature.

A thermal melting curve of poly(dG-dC)·poly(dG-dC) in a pH 3.8 and 2.6 M NaCl solution was measured from 45 to 95 °C by monitoring the absorbance change at 280 nm with the result shown in Figure 7. An extremely rapid increase in absorbance occurs at 60.5 °C, which is immediately accompanied by a slower decrease until around 77 °C whereupon a steady absorbance increase sets in and levels off around 95

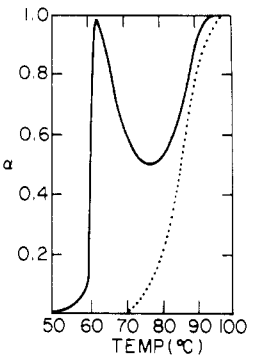

FIGURE 7: Thermal denaturation profile of poly(dG-dC)·poly(dG-dC) in a pH 3.8 and 2.6 M NaCl solution as monitored by the absorbance change at 280 nm (solid line). The dotted line represents a separate measurement starting at 64 °C after the completion of the Z-DNA melting and a 3-h wait at this temperature. A heating rate of 0.3 °C/min was maintained, and data points were acquired every 15 s. α denotes the fraction melted and is defined as $(A_t - A_l)/(A_h - A_l)$, where A_h, A_l, and A_t are absorbances at high, low, and temperature t, respectively.

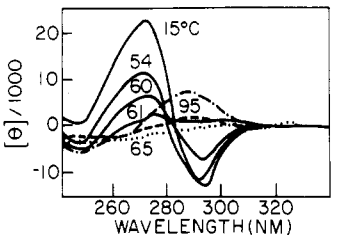

FIGURE 8: CD spectra of 23 μM poly(dG-dC)·poly(dG-dC)/2.6 M NaCl/pH 3.8 solution at various temperatures. (-·-) Spectrum after an overnight cooling back to 15 °C subsequent to complete denaturation.

°C. This rather peculiar biphasic melting profile is distinctly different from the other observed multiphasic curves that usually do not exhibit absorbance decrease in between phases.

This observed strange melting profile was initially rationalized in terms of strand separation of the duplex Z DNA at 60.5 °C followed by a partial renaturation to a protonated B-form DNA (which has a lower absorbance at 280 nm compared to that of the Z form) and an eventual denaturation of the B variety at higher temperatures with a midpoint around 87 °C. This interpretation, however, is only partially supported by the temperature-dependent CD spectra as shown in Figure 8.

A temperature increase from 15 to 54 °C greatly reduces the intensity of the 272-nm positive CD band with little effect on the negative 290-nm maximum, again suggesting that these two bands are not completely coupled. The disappearance of the characteristic 290-nm negative CD band between 54 and 61 °C with the presence of an isoelliptic point confirms that the Z DNA does denature at 60.5 °C in 2.6 M NaCl. However, no CD spectral enhancement corresponding to the absorbance decrease in the 63–77 °C region is observed as would be expected if renaturation to the B form is occurring. In fact, little or no CD intensities are apparent above 63 °C until a second denaturation is completed at 95 °C. A weak positive CD band then reappears around 288 nm that is enhanced but without the reappearance of the negative 290-nm band upon being cooled back to 15 °C.

To elucidate the basis for such peculiar melting behavior, an absorption spectrum at 63.5 °C was measured right after the first denaturation. The absorbance decrease was monitored at 280 nm as a function of time at this temperature until it stabilized, and then another spectrum was taken. The ab-

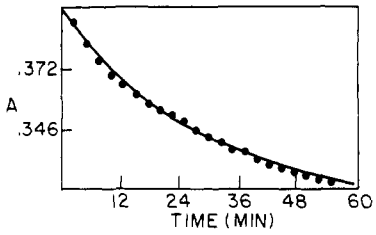

FIGURE 9: Time-dependent absorbance decrease monitored at 280 nm and with temperature maintained at 63.5 °C after melting of the protonated Z DNA. Representative experimental points are indicated by the solid circles, and the solid curve is that of a single exponential fit with a characteristic time of 2000 s.

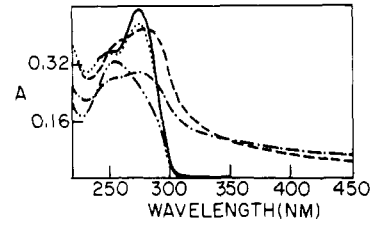

FIGURE 10: Absorption spectra of 40 μM poly(dG-dC)·poly(dG-dC)/2.6 M NaCl/pH 3.8 solution in a 1-cm cell at different temperatures: (–···–) 24 °C, (––) 63.5 °C immediately after the melting of the protonated Z DNA, (–·–) 63.5 °C after 3 h, (···) 98 °C, and (—) after being cooled back to 24 °C.

sorbance decay can be nicely fitted by a single exponential with a characteristic time of about 2000 s as shown in Figure 9. The absorption spectra measured at the end of the first-stage melting and after reaching equilibrium at 63.5 °C, as well as spectra at 24, 98, and 24 °C after cooling back, are presented in Figure 10.

The spectra at 63.5 °C are characterized by broad absorption bands, as well as a large tail at long wavelength, indicative of aggregate formation. This is confirmed by the visible Tyndall scattering in the cuvette. The second spectrum at this temperature after equilibrium exhibits a hypochromic effect at the short-wavelength region but is accompanied by a larger long-wavelength tail. The spectrum at 98 °C where the second denaturation is complete shows narrower absorption bands and the absence of the long-wavelength tail. The features of these absorption spectra taken together clearly suggest that the absorbance decrease after the Z-DNA melting and the progressive absorbance increase at higher temperatures are due to aggregate formation and melting, respectively. The structures of the aggregates are not clear and most likely consist of random multiplex, as no optical activities due to differential absorbance or differential light scattering are apparent. It is also interesting to note that upon being cooled back to room temperature the long-wavelength tail does not reappear, suggesting no aggregate reformation. It is likely that the acid-catalyzed hydrolysis of the aggregates and/or the denatured strands into nucleotides occurs at these high temperatures.

The distinctive characteristic of the denatured absorption spectra of pH 3.8 at 98 °C is the dominant prominence of the 275-nm maximum, which cannot even be discerned as a shoulder in the native state. Even for the denatured spectrum at pH 7, only a broad shoulder at this wavelength is exhibited (Wells et al., 1970). It is also worth noting that upon a cooling back to room temperature, not only is there no sign of renaturation or aggregate reformation, but the 275-nm band is even more enhanced. Such intensity enhancement is in line with the above suggestion that acid-catalyzed hydrolysis of polynucleotides occurs at high temperatures. This maximum is most likely due to the protonated cytosine because deoxycytidine has a pK value of 4.3 and exhibits a 50% increase in absorptivity and red shift of the maximum from 271 to 280 nm upon complete protonation at pH 1 (Fasman, 1975). Thus, our experimental observations seem to indicate that poly(dG-dC)·poly(dG-dC) if thermally denatured above 95 °C in an acidic solution cannot be renatured (Z or otherwise), although partial renaturation to Z form is observed if it is only heated up to 70 °C (CD results not shown).

The extremely cooperative melting of Z DNA at 60.5 °C is evidenced by a narrow melting half-width of about 1 °C. A van't Hoff enthalpy change of 1.1 Mcal/mol of cooperative melting units is estimated from the expression (Pohl, 1974) $\Delta H° = 5.1RT_1T_2/(T_2 - T_1)$, where T_1 and T_2 are the temperatures at the half-height of the differential melting curve. The total absorbance change due to this phase transition (see Figure 7) is about the same as that after the second melting, indicating that this polynucleotide exists almost exclusively in the Z form at 2.6 M NaCl below the first melting temperature. The second stage melting is less cooperative, as can be seen in Figure 7 where a separate curve for a melting experiment starting at 64 °C after 3 h at this temperature is also included.

The melting temperature of 60.5 °C for the Z DNA in 2.6 M NaCl pH 3.8 solution should be compared with that of the protonated B form in 1 M NaCl solution, which exhibits a melting temperature of 76 °C and a melting half-width of about 6 °C. As the melting temperature is expected to increase as the NaCl concentration is increased, these results seem to indicate that formation of the Z form lowers the melting temperature of DNA at least in the acidic solution.

The comparison with the features exhibited by the corresponding pH 7 solution will be particularly pertinent. Unfortunately, the much higher melting temperatures exhibited by the neutral solutions, coupled with the limitation of the temperature accessory in our spectrophotometer, have prevented us from performing melting measurements in the NaCl solutions although we have pushed our CD measurements to around 110 °C to indicate that the Z DNA melts or converts into another form (presumably A form as judged by the CD spectra) in that region but with lesser cooperativity for the 2.6 M NaCl, pH 7 solution. It is noted that the melting temperature for the B-form poly(dG·dC)·poly(dG-dC) at this ionic strength is about 129 °C as extrapolated from the expression (Wells, 1970) t_m (°C) = 14 × log [Na^+] (mM) + 81.

Discussion

The evidence presented thus far clearly suggests that protonation facilitates the B–Z interconversion, especially in the kinetic sense. Similar rate increase has also been reported in the N7-methylated poly(dG-dC)·poly(dG-dC) system (Moller et al., 1981). This observation coupled with the resemblance in spectral characteristics exhibited by the Z DNA of the protonated and the N7-methylated poly(dG-dC)·poly(dG-dC) makes Guschulbauer's suggestion on the N7 protonation of guanine to be eminently attractive although our results cannot prove its validity. Recent Raman spectroscopic studies with native DNA (O'Connor et al., 1982), however, have demonstrated cytosine and adenine as the primary protonation sites, and significant protonation of guanine occurs only during acid denaturation (pH <2.5). Although Gushulbauer's model consists of rearrangement of the N7-protonated guanosine from an anti to a syn conformation and sharing the proton with the N3 of cytosine through Hoogsteen's base pairing, it is not apparent that it is compatible with the Raman evidence. It is also to be noted that this model is different from the B–Z isomerization process as suggested by Wang et al. (1979),

which consists of base-pair hydrogen-bond breakage and subsequent rotation of the entire dC residue and a concomitant anti–syn conformational change of the guanosine.

A detailed mechanism for the B–Z transition involving the interesting Berry pseudorotation has recently been proposed (van Lier et al., 1983). Formation of trigonal-bipyramidal P(V) intermediates in the helix backbone is suggested to be the initiating step for such transformation. The rotation of the entire dC fragment and the disruption of the base pair are accompanied by an anti–syn conformational adjustment of dG that induces a pucker change from C(2′) endo to C(3′) endo. Models that do not require the breakage of Watson-Crick hydrogen bonds have also been proposed by Olson et al. (1983) and Harvey (1983).

Although the actual mechanism for the B–Z transition is not known, the observed dramatic enhancement in the rates of B–Z interconversions of poly(dG-dC)·poly(dG-dC) upon base protonation indicates a lowering of activation energy as a consequence of H^+ binding. This activation energy lowering most likely is the result of decreased B duplex stability upon protonation, as evidenced by the striking lowering of melting temperature as stated in the introduction, which can result in easier base-pair disruption. If such disruption is present in the transition process, it must be local in nature as no large-scale hydrogen-bond breakage has been detected by NMR experiments (Sarma et al., 1983).

If the observed facilitation of B–Z transition is indeed the consequence of guanine N7 protonation, it may have important implication in the action of Z-DNA binding protein. It is noted that stabilization of Z DNA by polyarginine near physiological ionic strength has been observed recently (Klevan & Schumaker, 1982). Polyarginine is known to preferentially interact with the GC-rich regions of DNA (Leng & Felsenfeld, 1966) presumably through specific interactions of the amino side chains with the O6 and N7 positions of guanine (Seeman et al., 1976). Spectroscopic (Mansy et al., 1976) as well as theoretical (Helene, 1977) studies of arginine and arginine-rich histone binding to DNA have suggested a specificity of arginine for GC-rich regions of DNA through participation of the guanine N7 in the hydrogen-bonding interaction.

It is also interesting to note that Z conformation is also stabilized when poly(dG-dC)·poly(dG-dC) is modified by chlorodiethylenetriaminoplatinum(II) chloride, an N7-coordinating agent (Malfoy et al., 1981). On the other hand, although aflatoxin B1 reacts at the N7 position of guanine and puts a positive charge on the imidazole ring, it inhibits the salt-induced conversion of B to Z DNA (Nordheim et al., 1983). This may be the consequence of its bulk and/or stabilization of the right-handed conformation through its interaction with other components in the major groove (Nordheim et al., 1983).

Our thermal denaturation results indicate that the protonated Z DNA melts at a lower temperature than the corresponding protonated B form of lower salt concentration, which in turn melts at a temperature 40 °C lower than its neutral counterpart. This, coupled with the observed facilitation on the B–Z interconversions, makes one wonder if base protonation or a similar event is operative in the DNA replication or control processes. After submission of the original version of the manuscript, a paper appeared (Robert-Nicoud et al., 1984) that contains some findings very similar to the ones reported here, further strengthening the credibility of this work.

Acknowledgments

I thank M. Yeargin for her careful reading of the manuscript.

Registry No. Poly(dG-dC), 36786-90-0.

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Bleomycin May Be Activated for DNA Cleavage by NADPH–Cytochrome P-450 Reductase†

Robert E. Kilkuskie, Timothy L. Macdonald, and Sidney M. Hecht*

ABSTRACT: In the presence of NADPH and O_2, NADPH–cytochrome P-450 reductase was found to activate Fe(III)–bleomycin A_2 for DNA strand scission. Consistent with observations made previously when cccDNA was incubated in the presence of bleomycin and Fe(II) + O_2 or Fe(III) + C_6H_5IO, degradation of DNA by NADPH–cytochrome P-450 reductase activated Fe(III)–bleomycin A_2 produced both single- and double-strand nicks with concomitant formation of malondialdehyde (precursors). Cu(II)–bleomycin A_2 also produced nicks in SV40 DNA following activation with NADPH–cytochrome P-450 reductase, but these were not accompanied by the formation of malondialdehyde (precursors). These findings confirm the activity of copper bleomycin in DNA strand scission and indicate that it degrades DNA in a fashion that differs mechanistically from that of iron bleomycin. The present findings also establish the most facile pathways for enzymatic activation of Fe(III)–bleomycin and Cu(II)–bleomycin, provide data concerning the nature of the activated metallobleomycins, and extend the analogy between the chemistry of cytochrome P-450 and bleomycin.

The bleomycins are a family of antitumor antibiotics elaborated by *Streptomyces verticillus* as Cu(II) complexes (Umezawa et al., 1966; Carter, 1978; Crooke, 1978; Umezawa, 1979). In the presence of Fe (Sausville et al., 1976, 1978a,b), Cu (Oppenheimer et al., 1981; Murugesan et al., 1982), or Co (Chang & Meares, 1982), the bleomycins have been shown to effect DNA strand scission; their ability to degrade DNA is believed to represent the basis of their therapeutic efficacy (Umezawa, 1979). Although substantial progress has been made in understanding the nature of the process(es) by which bleomycin may be activated for DNA cleavage (Burger et al., 1981, 1982; Giloni et al., 1981; Murugesan et al., 1982), and the explicit mechanism(s) by which cleavage occurs (Hecht, 1979; Giloni et al., 1981; Wu et al., 1983), a number of questions remain unanswered. Among these is the way in which bleomycin is activated in situ.

To date, bleomycin has been activated successfully in vitro in the presence of Fe(II) + O_2 (Sausville et al., 1976, 1978a,b), Fe(III) + H_2O_2 or ethyl hydrogen peroxide (Burger et al., 1981, 1982), Cu(I) + O_2 (Oppenheimer et al., 1981), and Co(III) + $h\nu$ (Chang & Meares, 1982). Murugesan et al. (1982) have recently shown that Fe(III)·BLM[1] and Cu(II)·BLM can be activated with oxygen surrogates such as iodosobenzene, in analogy with observations made previously for cytochrome P-450 and for a number of Fe(III)–porphyrins (Hrycay et al., 1975; Lichtenberger et al., 1976; Gustafasson et al., 1979). Moreover, the bleomycins so activated mediated chemical transformations remarkably similar to those reported for cytochrome P-450 (analogues) (Groves et al., 1980; Tabushi et al., 1980) and were also capable of effecting DNA strand scission in the absence of dioxygen (Aoyagi et al., 1982; Murugesan et al., 1982).

Cytochrome P-450, a component of the microsomal electron transport system, is activated in situ by NADPH–cytochrome P-450 reductase in the presence of NADPH and O_2. The microsomal electron transport system has been shown to activate BLM for DNA cleavage in a reaction that was dependent on NADPH and O_2 (Yamanaka et al., 1978; Trush et al., 1982a,b) and enhanced by Fe(III) (Trush, 1983). In a preliminary report, NADPH–cytochrome P-450 reductase was also shown to support DNA cleavage by Fe(III)·BLM (Scheulen et al., 1981). Presently, we confirm the activation of Fe(III)·BLM by NADPH–cytochrome P-450 reductase and demonstrate that activated Cu(II)·BLM can also be obtained enzymatically and mediates DNA cleavage. Also described are the nature of NADPH–cytochrome P-450 reductase mediated activation of the metallobleomycins, with regard to required components, preferred pathways, and time course of activation, and important characteristics of the enzymatically activated bleomycins and the processes by which they mediate DNA strand scission.

Experimental Procedures

SV40 DNA and agarose were purchased from Bethesda Research Laboratories; 2-thiobarbituric acid and calf thymus

† From the Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901. *Received March 8, 1984; revised manuscript received June 21, 1984.* This work was supported by NIH Research Grants CA-29235 (to S.M.H), awarded by the National Cancer Institute, and ES-03181 (to T.L.M.), awarded by the National Institute of Environmental Health Services.

[1] Abbreviations: BLM, bleomycin; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide.

0006-2960/84/0423-6165$01.50/0