Modification of DNA Dynamics by Platinum Drug Binding: A Time-Dependent Fluorescence Depolarization Study of the Interaction of *cis*- and *trans*-Diamminedichloroplatinum(II) with DNA†

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ABSTRACT: The interaction of calf thymus DNA with the antitumor drug *cis*-diamminedichloroplatinum(II), and with the clinically ineffective trans isomer, is studied by time-dependent fluorescence depolarization spectroscopy of intercalated ethidium. The effect of the platinum compounds on the rapid torsional motions of DNA in solution is observed via depolarization of the ethidium fluorescence. The depolarization data are successfully analyzed with an elastic model of DNA dynamics and yield a value for the product of the torsional rigidity of the DNA and the friction factor for DNA twisting. The dependence of this quantity on the degree of platination of the DNA is determined for each isomer. At low levels of platination, the cis isomer increases the solute–solvent friction acting on the DNA torsional motions, which we attribute to local kinking of the helix axis at the sites of platination. At high levels of platination, the cis isomer decreases the torsional rigidity of the DNA, indicating that disruption of DNA duplex structure occurs under these conditions. The binding of the trans isomer to DNA has no effect on the torsional rigidity or the friction. The present results are compared with other findings on the interaction of these platinum compounds with DNA.

The platinum complex *cis*-diamminedichloroplatinum(II) (*cis*-DDP)[1] is a widely used antitumor drug which binds covalently to cellular DNA and inhibits DNA replication (Hacker et al., 1984). The stereoisomer *trans*-DDP also binds covalently to DNA but is clinically ineffective and less cytotoxic than *cis*-DDP (Roberts & Thompson, 1979). This remarkable difference has prompted an investigation of the nature of, and differences between, the DNA–platinum adducts formed by each isomer in their reaction with DNA (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). These studies have implicated a particular adduct, the 1,2-intrastrand cross-link, as being responsible for the selective antineoplastic activity of *cis*-DDP [reviewed by Pinto and Lippard (1985)]. However, the molecular mechanisms by which this and possibly other adducts inhibit DNA replication are presently unknown. The possible structural basis for these mechanisms is being pursued by a variety of techniques aimed at assessing the alterations in DNA structure that are induced by adduct formation [reviewed by Sherman and Lippard (1987)].

Studies of the effect of *cis*- and *trans*-DDP on the physical properties of DNA can provide additional information which may be useful in elucidating the molecular mechanisms underlying the selective drug action of *cis*-DDP. The flexibility of DNA is a pertinent physical property in the present context, since it is likely to be involved in processes occurring during replication, such as protein binding and helix unwinding. The effect of *cis*- and *trans*-DDP binding on the flexibility of DNA is unknown and is addressed in the present study by monitoring the rapid internal motions of DNA in solution by time-dependent fluorescence depolarization of intercalated ethidium (Barkley & Zimm, 1979; Thomas et al., 1980; Millar et al., 1980, 1981, 1982). The steady-state fluorescence of intercalated ethidium has previously been used to probe changes in the structure of DNA induced by *cis*- and *trans*-DDP (Butour & Macquet, 1977). TDFDP of intercalated ethidium, however, probes a different property of the DNA–platinum complexes than steady-state fluorescence, namely, the dynamics of torsional and bending motions of the double helix occurring in solution on the nanosecond time scale. The technique provides an accurate value for the product of the torsional rigidity of DNA and the friction factor for twisting of DNA in solution. The torsional rigidity is an important mechanical property controlling the energetics of DNA winding (Barkley & Zimm, 1979) and is relevant to a variety of biological processes, including supercoiling (Horowitz & Wang, 1984; Benham, 1985), conformational transitions (Benham, 1985), and DNA–protein interactions (Hogan & Austin, 1987). Furthermore, the magnitude of the torsional rigidity depends on the secondary and tertiary structure of the DNA (Millar et al., 1980, 1981, 1982) and so may serve as a novel probe of these structural features of DNA in solution. The friction factor depends on the shape of the DNA molecule in solution and will be sensitive to changes in tertiary structure that may occur upon binding of the platinum compounds. A study of the effect of *cis*- and *trans*-DDP on the internal motion dynamics of DNA should thus provide information on possible structural changes in DNA, as well as having potential implications for the biological processes that are sensitive to DNA flexibility.

In this paper, we report measurements of the TDFDP of ethidium intercalated in DNA that is platinated by various

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); NMR, nuclear magnetic resonance; TDFDP, time-dependent fluorescence depolarization.

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amounts of *cis*- or *trans*-DDP. We find that the binding of *cis*-DDP to DNA significantly alters the internal motion dynamics in a way that depends on the level of binding. The TDFDP data are consistent with an elastic model of DNA internal motion dynamics, which allows the variation in TDFDP to be attributed to changes in the torsional rigidity or solute–solvent friction induced by platinum binding. Binding of low levels of *cis*-DDP causes the friction to increase, which we attribute to formation of DNA–platinum adducts which kink the helix axis. Binding high levels of *cis*-DDP causes the torsional rigidity to decrease, which is attributed to a disruption of DNA duplex structure. The binding of *trans*-DDP does not elicit these changes in the internal motion dynamics. We compare our results with other findings on DNA–platinum interactions.

Experimental Procedures

Samples. Calf thymus DNA was obtained from Sigma and used as supplied. A stock solution was prepared containing 200 μg/mL DNA in 10 mM $NaClO_4$. TDFDP measurements of this solution gave the correct value for the torsional rigidity of calf thymus DNA (Millar et al., 1982). *cis*-DDP and *trans*-DDP (both 99.9%), obtained from Ventron, were added in appropriate amounts to portions of the DNA stock solution and incubated in the dark at 25 °C for 42 h. The solutions were then dialyzed to removed unreacted platinum complexes. The DNA and Pt concentrations of these solutions were determined by UV absorbance and flameless atomic absorption, respectively, from which the molar ratio of bound platinum per nucleotide, r_b, was calculated.

Ethidium bromide was obtained from Sigma and used as supplied. A small aliquot of a concentrated ethidium bromide stock solution was added to the DNA–Pt solution at a concentration of 2.8×10^{-6} M, corresponding to a nucleotide to dye ratio of 220:1. The fluorescence measurement was performed immediately following the addition of ethidium bromide.

TDFDP Measurements. The samples were excited at 532 nm by the frequency-doubled output of an actively mode-locked and CW-pumped Nd:YAG laser (Quantronix 116). The duration of the excitation pulses was 35 ps. The repetition rate of the laser pulse train was reduced from 100 MHz to 40 kHz by an electrooptic pulse selector (Ware et al., 1983). Fluorescence was collected at right angles and was observed through a polarizer and a color filter passing wavelengths longer than 570 nm. Time-resolved fluorescence profiles were recorded by using time-correlated single photon counting (O'Connor & Phillips, 1984) as follows. A portion of the excitation pulse train was incident on a photodiode connected to a constant-fraction discriminator (CFD, Ortec 934), which provided the start pulse for a time to amplitude convertor (TAC, Ortec 457). Fluorescence photons detected by a photomultiplier (Hamamatsu R928 with modified biasing; Ware et al., 1983) and a second CFD provided stop pulses for the TAC. The TAC output was processed by a multichannel analyzer (MCA, Nucleus Spectrum 88) operated in the pulse–height–analysis mode, which produced a digital representation of the time-resolved fluorescence profile. The contents of the MCA memory were transferred to a computer (PDP 11/23+) for storage and data analysis.

The excitation pulses were vertically polarized, and the polarizer in the detection optics was set either parallel or perpendicular to this direction. In TDFDP measurements, these two polarization components of the fluorescence were alternately acquired in two segments of the MCA memory for equal time periods, the laser power being constant throughout.

The impulse response of the apparatus was measured by scattering the laser pulses from a scattering solution in place of the sample and with the color filter removed. The response function had a width of 400 ps, which was sufficiently narrow that deconvolution of the data was not required for the time scales used in the experiments.

All experiments were performed at 20 °C in a temperature-controlled cell.

Data Analysis. In the TDFDP experiments, the time-resolved intensities of the parallel and perpendicular polarization components of the fluorescence, $I_\parallel(t)$ and $I_\perp(t)$, were measured. These were combined to form the experimental sum and difference decay curves, defined as

$$S(t) = I_\parallel(t) + 2I_\perp(t) \tag{1a}$$

$$D(t) = I_\parallel(t) - I_\perp(t) \tag{1b}$$

The sum curve $S(t)$ is determined solely by excited-state population decay processes. In this study, it was found necessary to represent $S(t)$ by a two-component decay, in order to account for a small fraction of free ethidium present in our samples:

$$S(t) = A(a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}) \tag{2}$$

where a_1 and a_2 are the fractions of the two components, τ_1 and τ_2 are the two lifetimes, and A is an amplitude parameter. Note that $a_1 + a_2 = 1$.

The difference curve $D(t)$ contains information on both excited-state decay processes and reorientation of the fluorescence transition dipole during the excited-state lifetime:

$$D(t) = r(t)S(t) \tag{3}$$

where $r(t)$ is the fluorescence anisotropy, which is proportional to a time correlation function of the dipole reorientation angle (Gordon, 1966). The information on reorientation dynamics of the fluorophore is contained in $r(t)$.

Ethidium intercalated in DNA undergoes reorientation primarily due to torsional motions of the double helix, with a smaller contribution due to bending motions (Barkley & Zimm, 1979; Allison & Schurr, 1979; Millar et al., 1980, 1981, 1982; Thomas et al., 1980; Hard & Kearns, 1986). Considering these motions only, the anisotropy decay of a chromophore attached to DNA can be written as (Schurr, 1984)

$$r(t) = r_0 \sum_{n=0}^{2} A_n(\epsilon) F_n(t) G_n(t) \tag{4}$$

where r_0 is the initial value of the anisotropy, $F_n(t)$ and $G_n(t)$ are torsional and bending correlation functions, respectively, and $A_n(\epsilon)$ values are functions of the angle ϵ between the chromophore's transition moment and the helix axis, as follows: $A_0(\epsilon) = {}^1/_4(1 - 3\cos^2\epsilon)^2$, $A_1(\epsilon) = {}^3/_4 \sin^2 2\epsilon$, and $A_2(\epsilon) = {}^3/_4 \sin^4\epsilon$. For ethidium intercalated in B DNA, $\epsilon = 73°$ (Hogan et al., 1979). The theoretical value of r_0 for an electric dipole transition is 0.4, although the apparent value is frequently smaller due to the presence of rapid motions that are not resolved in the experiments.

The nanosecond internal motions of DNA can be treated with an elastic model of DNA dynamics, wherein the DNA is described as a uniformly elastic rod immersed in a viscous fluid. The rod is completely characterized by its torsional and bending rigidities, C and EI, respectively (Barkley & Zimm, 1979). The correlation functions then take the form (Barkley & Zimm, 1979)

$$F_n(t) = \exp(-n^2 A t^{1/2}) \tag{5}$$

$$G_n(t) = \exp\left(\frac{-6 + n^2}{4} B t^{1/4}\right) \tag{6}$$

where $A = k_B T/(\pi f C)^{1/2}$, $B = (3.466 k_B T/\pi^{5/4}\eta^{1/4} EI^{3/4}) g(z_{max})^{1/4}$, f is the solute–solvent friction per unit length for twisting DNA in solution, η is the solution viscosity, k_B is the Boltzmann constant, and T is the temperature. The function $g(z_{max})$ is defined elsewhere (Barkley & Zimm, 1979).

Due to the near-perpendicular orientation of the ethidium transition dipole relative to the helix axis, the TDFDP on the nanosecond time scale is dominated by the torsional modes of the helix, and the time dependence predicted by eq 5 has been verified in earlier experiments (Thomas et al., 1980; Millar et al., 1980). Note that the rate of depolarization due to torsional motion is governed by the product of the torsional rigidity, C, and the friction factor, f. This product is treated as a single unknown parameter whose value is to be determined from the TDFDP data. The time dependence of the much smaller bending contribution predicted in eq 6 has not been directly verified by experiment but has been successfully tested against the results of a Brownian dynamics simulation of DNA bending (Allison, 1986). We therefore used eq 6 to represent the effect of the bending motion on the TDFDP, thereby obtaining a better estimate of the torsional rigidity than if bending were to be ignored in the analysis. We do note, however, that it was not possible to separately determine the value of the bending rigidity from the TDFDP data. The bending rigidity was instead calculated from the persistence length of DNA, with the result $EI = 2.15 \times 10^{-19}$ erg cm (Millar et al., 1982).

Equations 2 and 3, in conjunction with eq 4–6, represent a theoretical model of the TDFDP experiment. The unknown parameters A, a_1, τ_1, τ_2, r_0, and fC were adjusted so as to simultaneously achieve a best fit of the theoretical model to both the experimental sum and difference decay curves. This was performed by a nonlinear least-squares procedure using the Marquardt algorithm (Bevington, 1969). In some cases, a_1 was so small that τ_1 could not be determined with sufficient precision, in which case τ_1 was fixed at a particular value (see later) and the remaining parameters were adjusted for best fit. The quality of all the fits was judged by the value of the reduced χ squared, χ_r^2, and by inspection of the weighted residuals. A value of χ_r^2 less than 1.2, together with randomly distributed residuals, was considered to represent an acceptable fit.

Results

It is useful to first consider the TDFDP experiments on native calf thymus DNA before dealing with the changes that accompany platination of the DNA. The experimental sum and difference decay curves for ethidium intercalated in calf thymus DNA were well fit by the theoretical model defined by eq 2–6, with $\chi_r^2 < 1.1$ and no evidence of systematic deviations in the residuals. The best-fit parameter values were $a_1 = 0.05 \pm 0.03$, $\tau_1 = 2 \pm 1$ ns, $\tau_2 = 23.4 \pm 0.3$ ns, $r_0 = 0.36 \pm 0.01$, and $fC = (5.96 \pm 0.11) \times 10^{-34}$ erg^2 s.

As reported previously (Millar et al., 1982), the sum curve $S(t)$ is best represented by a two-component decay. The major component, with a 23-ns lifetime, is due to intercalated ethidium molecules (Millar et al., 1982). The lifetime of the minor component is poorly determined because of its small amplitude, but it is comparable to the lifetime of ethidium in aqueous solution, $\tau = 1.76 \pm 0.05$ ns. The minor component was previously attributed to nonintercalated ethidium species, these being free in solution and/or loosely associated with the helix exterior (Millar et al., 1982). In some of the platinated DNA samples, the fraction of the short lifetime component is larger than for native DNA (see below), and the short lifetime is more precisely determined, $\tau_1 = 1.8 \pm 0.2$ ns. This value agrees well with the free dye lifetime and supports the assignment of the short lifetime component to free ethidium. Thus, in the samples where τ_1 was poorly determined by curve fitting, its value was fixed at the free dye lifetime, and the data were reanalyzed by adjustment of the remaining parameters. This procedure improved the precision of the a_1 parameter but had no effect on the other variable parameters.

It was not necessary to include an additional term in the anisotropy decay to account for the free dye. Presumably, this occurred because the free dye motion was faster than the instrumental response time and so was not resolved in the experiments.

The values of τ_2 and r_0 agree well with previous determinations for ethidium and calf thymus DNA (Millar et al., 1980, 1981, 1982; Thomas et al., 1980). The r_0 value is smaller than 0.4 due to the presence of an unresolved rapid motion of limited amplitude, associated with the intercalated species. This motion, which we refer to as "wobble", probably results from a limited motional freedom of ethidium in its intercalation site (Barkley & Zimm, 1979; Millar et al., 1982). The recovered value of the initial anisotropy is indicative of the root mean square amplitude of the wobble motion.

The torsional friction per unit length acting on a long DNA molecule can be estimated from hydrodynamics as $f = 4\pi\eta b^2$, where b is the hydrodynamic radius of DNA modeled as a smooth rod (13.4 Å; Millar et al., 1982). The result is $f = 2.26 \times 10^{-15}$ dyn s. This value should be appropriate for unmodified DNA, and so we can calculate the torsional rigidity of our calf thymus DNA sample from the best-fit value of the fC parameter. This procedure gives $C = (2.64 \pm 0.05) \times 10^{-19}$ erg cm, which is significantly larger than reported previously for high molecular weight DNA (Millar et al., 1980, 1981, 1982; Thomas et al., 1980) due to a different data analysis procedure used here. In the previous analyses, a different formula for $r(t)$ was used (Barkley & Zimm, 1979). The use of the revised formula, eq 4 (Schurr, 1984), leads to the higher torsional rigidity value reported here. The previous value is retrieved when the present data are analyzed by the earlier procedure, indicating that there are no inherent differences in the present sample of calf thymus DNA. The revised value of the torsional rigidity, $C = (2.64 \pm 0.05) \times 10^{-19}$ erg cm, agrees well with an estimate obtained from the TDFDP data of Millar et al. (1980), when analyzed by using an empirical representation of the bending correlation function, $C = 2.5 \times 10^{-19}$ erg cm (Hard, 1987). Estimates of the torsional rigidity are also obtained by analysis of supercoil linking number distributions (Shore & Baldwin, 1983; Horowitz & Wang, 1984) and DNA cyclization probability (Shore & Baldwin, 1983). To compare these techniques with the present work, the contribution of DNA bending (writhe) should be accounted for in the analysis, in which case $C = 3.4 \times 10^{-19}$ erg cm is obtained from topoisomer distributions and $C = 3.8 \times 10^{-19}$ erg cm from cyclization probability (Levene & Crothers, 1986). These estimates are somewhat larger than obtained from TDFDP of intercalated ethidium.

The TDFDP data for ethidium intercalated in calf thymus DNA that was platinated with various amounts of *cis*-DDP and *trans*-DDP were analyzed in the same manner as for native DNA. Excellent fits of the theoretical model to the data were obtained in all cases, with χ_r^2 values comparable to those for native DNA. The elastic model of DNA dynamics described the internal motions of the platinated DNA samples regardless of the amount or identity of the platinum complex bound to the DNA. The results found for DNA platinated by *trans*-DDP are presented in Table I and Figure 1. The

Table I: Effect of Platination on the Fluorescence Decay Parameters of Ethidium

r_b (±0.001)	τ_1 (ns)	a_1 (±0.02)	τ_2 (ns) (±0.3)	r_0 (±0.01)
0	2.0 ± 1.2	0.06	23.4	0.36
		cis-DDP		
0.0034	1.76[a]	0.05	23.8	0.36
0.0060	1.76[a]	0.05	23.6	0.35
0.012	1.76[a]	0.04	23.3	0.35
0.026	1.76[a]	0.07	23.6	0.34
0.040	1.76[a]	0.05	23.5	0.35
0.055	1.76[a]	0.04	23.7	0.35
0.079	1.9 ± 0.6	0.12	23.7	0.34
0.093	1.5 ± 0.6	0.12	23.8	0.36
0.104	2.0 ± 0.4	0.16	24.1	0.36
0.123	1.8 ± 0.4	0.16	22.8	0.37
0.135	1.7 ± 0.3	0.20	23.5	0.37
0.147	1.7 ± 0.2	0.17	23.7	0.37
0.156	1.8 ± 0.2	0.19	23.2	0.37
		trans-DDP		
0.090	1.76[a]	0.03	23.4	0.36
0.209	1.76[a]	0.05	23.1	0.36

[a] τ_1 was held fixed at 1.76 ns, and the remaining parameters were adjusted for best fit.

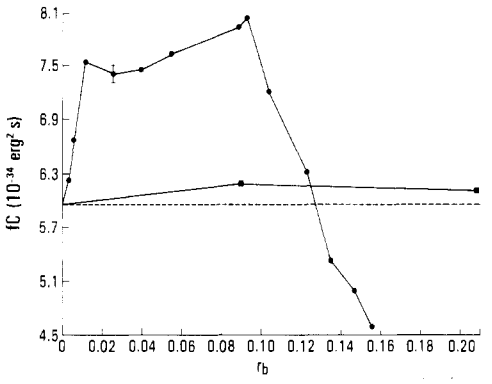

FIGURE 1: Dependence of the fC parameter for ethidium intercalated in calf thymus DNA on the molar ratio of bound *cis*-DDP (circles) and *trans*-DDP (squares). The samples contained 200 μg/mL calf thymus DNA and 2.8×10^{-6} M ethidium bromide (nucleotide to dye ratio = 220) in 10 mM $NaClO_4$ at 20 °C, in addition to the bound platinum complex. r_b is defined as the number of moles of platinum bound per mole of nucleotide. The error bar is representative of the error in all the data points. The horizontal line indicates the value of fC for native calf thymus DNA and is included for comparison.

best-fit values of a_1, τ_1, τ_2, r_0, and fC are equal, within experimental error, with the values found for native DNA, regardless of the amount of bound *trans*-DDP. The fluorescence depolarization behavior of ethidium intercalated in DNA platinated by *trans*-DDP is thus identical with native DNA, showing that the binding of *trans*-DDP has little effect on the internal motion dynamics of DNA.

The results for DNA platinated by *cis*-DDP are presented in Table I and Figure 1. The values obtained for τ_1, τ_2, and r_0 are again equal, within experimental error, to the values for native DNA, at all levels of *cis*-DDP binding. However, the a_1 and fC parameters are different than for native DNA and vary with the molar ratio of *cis*-DDP bound to the DNA.

Discussion

Variation of the fC Parameter. The primary experimental observation is that the rate of fluorescence depolarization of intercalated ethidium is modified by binding of *cis*-DDP to DNA, as reflected in the apparent variation of the fC parameter shown in Figure 1. There are several possibilities to consider in explaining this trend: (1) clustering of ethidium molecules caused by the disruption of ethidium intercalation sites by *cis*-DDP; (2) inapplicability of the elastic model of DNA dynamics; (3) changes in the ethidium intercalation geometry or other helix parameters; (4) a real change in fC due to binding of *cis*-DDP, which could be due to a change in the solute–solvent friction and/or the torsional rigidity of the platinated DNA.

The binding of *cis*-DDP to DNA is known to reduce the number of intercalation sites available to ethidium (Butour & Macquet, 1977). The local concentration of ethidium in the unplatinated regions of DNA will thus be higher than suggested by the nucleotide to dye ratio. A high local concentration of ethidium could perhaps alter the intrinsic torsional rigidity of the DNA, which may explain the increase in fC seen in Figure 1. However, this possibility is unlikely for several reasons. First, in previous experiments, the torsional rigidity of calf thymus DNA was found to be independent of ethidium concentration, over a wide range of nucleotide to dye ratios (Millar et al., 1982). In a related experiment, the fluorescence depolarization of intercalated ethidium was shown to be unaffected by the binding of high concentrations of a second intercalator, proflavin (Genest et al., 1985). Second, there was very little *cis*-DDP bound to the DNA in the binding regime where the increase in fC was observed, and the reduction in the number of ethidium intercalation sites is very minor under these conditions (Butour & Macquet, 1977). Third, if clustering of ethidium intercalators did occur, it should have resulted in a smaller apparent value of fC, due to faster fluorescence depolarization arising from nonradiative electronic energy transfer among the intercalated ethidium molecules. Finally, if clustering of the intercalators was the dominant effect responsible for the apparent increase of fC, then similar changes would also be expected for binding of *trans*-DDP, since it produces a similar reduction in ethidium intercalation sites to *cis*-DDP (Butour & Macquet, 1977). Thus, we believe that clustering of the ethidium intercalators is not responsible for the apparent increase in fC at low levels of bound *cis*-DDP. At higher binding levels, where r_b is in the range 0.1–0.15, the apparent value of fC does decrease, perhaps due to the effect of energy-transfer depolarization. However, appreciable energy transfer among ethidium intercalators is only expected at near-saturation binding levels of ethidium, which was not the case for any of the platinated DNA samples under the conditions of our experiment (Butour & Macquet, 1977). Furthermore, a similar decrease was not observed with similar levels of bound *trans*-DDP. Since the binding of *trans*-DDP has no effect on the rate of fluorescence depolarization of ethidium, the apparent changes in the fC parameter that are induced by *cis*-DDP must result from the formation of specific adducts which are only formed by this isomer.

The disruption in DNA structure caused by the binding of *cis*-DDP could violate the assumption of uniform elasticity of the DNA and possibly lead to apparent changes in the fC parameter. However, some degree of disruption in DNA structure can be accommodated within an effectively uniform elastic rod model. Provided that the length scale of the disruptions is small compared with the wavelength of the predominant torsional modes of the DNA, then the structure will behave as effectively uniform and be characteized by a value of fC which is an average over the entire molecule, thus encompassing both intact and disrupted regions. This argument was previously invoked to explain the good fits of the elastic model to the TDFDP of ethidium intercalated in partially denatured DNA (Millar et al., 1982). The predominant torsional modes of a long DNA molecule have wavelengths

on the order of 100 base pair separations (Barkley & Zimm, 1979; Millar et al., 1982). Since *cis*-DDP has a strong binding specificity for certain short sequences, such as d(GpG) and d(ApG) (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985), which are randomly distributed, the platinated DNA should satisfy the condition for effective uniformity, which is confirmed by the excellent fits of the theoretical model to the data for all the DNA samples platinated by *cis*-DDP. Furthermore, the constant value of fC obtained for the DNA samples platinated by *trans*-DDP suggests that the presence of disruptions in the DNA due to adduct formation need not in itself cause an apparent change in this quantity.

The value of the fC parameter obtained from the fit of the elastic model to the TDFDP data depends on the values chosen for the fixed parameters—the ethidium transition dipole angle, ϵ, and the bending rigidity, EI. Changes in these parameters due to platination of DNA could, if not explicitly included in the analysis, result in apparent changes in the recovered fC parameter. A change in ϵ could result from a change in the intercalation geometry, perhaps resulting from a change in the local DNA structure due to binding of *cis*-DDP. However, the lifetime and wobble amplitude of intercalated ethidium remain constant at all levels of bound *cis*-DDP, which suggests that the ethidium is always intercalated at a "normal" site possessing the native DNA structure, since these parameters are sensitive to the local environment of the intercalator (Millar et al., 1981, 1982). Thus, ethidium appears not to intercalate at the site of an adduct, yet it is nonetheless sensitive to their effect on the internal motion dynamics of DNA by virtue of the long range of the torsional motions responsible for the nanosecond TDFDP. Another possibility is that the torsional dynamics are actually unaffected by platination with *cis*-DDP and that the apparent variation of fC is really due to a change in the bending rigidity with r_b. However, the TDFDP of intercalated ethidium is dominated by the torsional motions of the double helix, and the changes we monitor in the rate of depolarization must primarily reflect changes in the torsional friction or the torsional rigidity of the DNA.

The most likely interpretation of the data in Figure 1 is that they represent real changes in the quantity fC resulting from the binding of *cis*-DDP to DNA. Three distinct regions are apparent in the dependence of fC on r_b. At the lowest binding levels, $r_b < 0.02$, fC increases rapidly with r_b. At intermediate levels, $0.02 < r_b < 0.09$, fC is larger than for native DNA but depends only weakly on r_b. At the highest binding levels, $r_b > 0.1$, where saturation binding is being approached, fC decreases with r_b and eventually falls below the value characteristic of intact native DNA. The variation in fC could reflect changes in the torsional rigidity or the solute–solvent friction, or a combination of the two. In discussing these possibilities, we will separately consider the different regimes of *cis*-DDP binding.

Interaction of cis-DDP with DNA at Low r_b: Evidence for Duplex Kinking. At low levels of bound *cis*-DDP, the quantity fC increases with r_b as shown in Figure 1. Since the TDFDP of ethidium senses the average value of this quantity over the entire molecule, the increase with r_b must result from increased torsional rigidity and/or solute–solvent friction in the platinated regions of the molecule. These changes are evidently associated with adducts which are only formed at low levels of *cis*-DDP, since fC does not increase due to platination by *trans*-DDP. The distribution of adducts formed in the reaction of *cis*-DDP with random-sequence DNA has been reported by several authors (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). The major adduct is a 1,2-intrastrand cross-link that results from the bifunctional attachment of *cis*-DDP at the N7 positions of two adjacent guanine bases on the same strand, in a d(GpG) sequence. This adduct accounts for 65% of the platinum-containing adducts at $r_b < 0.01$ (Eastman, 1986), which is considerably in excess of the prediction for random binding of *cis*-DDP to DNA, thus demonstrating the high specificity of the drug for the d(GpG) sequence. The minor adducts are a 1,2-intrastrand cross-link formed at the d(ApG) sequence (23%) and a 1,3-intrastrand cross-link formed at the d(GpXpG) sequence (7%), which results from bifunctional attachment of *cis*-DDP to two guanines separated by any intervening base X (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). Monofunctional adducts and interstrand cross-links are relatively rare for *cis*-DDP, accounting for less than 5% and 1% of the adduct population, respectively (Fichtinger-Schepman et al., 1985; Roberts & Friedlos, 1981).

The increase of fC induced by *cis*-DDP binding thus occurs in a regime where 90% of the adducts are in the form of 1,2-intrastrand cross-links. It seems reasonable to associate the increase of fC with this class of adduct, especially since the effect is absent for binding of *trans*-DDP, which is unable to form this bifunctional adduct due to the sterically unfavorable trans geometry (Stone et al., 1976; Cohen et al., 1982). The formation of 1,2-intrastrand cross-links by *cis*-DDP could well increase the local torsional rigidity of the region of DNA in which they form, since a directional cross-link between neighboring base pairs would be expected to resist the change in mutual orientation required upon rotation about the helix axis. Furthermore, the X-ray structure of a 1,2-intrastrand cross-link formed by *cis*-DDP at a d(GpG) site reveals the presence of hydrogen bonds formed between the coordinated ammines in *cis*-DDP and the oxygen atoms of the phosphate groups on the 5′ side of the attachment site (Sherman et al., 1985). These additional hydrogen bonds may further reduce the conformational mobility of the DNA–platinum adduct and contribute to an increase in torsional rigidity. Thus, the increase in fC may be due to an increase in torsional rigidity. We can test this possibility by estimating the increase in torsional rigidity which can be expected upon formation of 1,2-intrastrand cross-links in DNA. The overall torsional rigidity, C, of a heterogeneous rod composed of N subunits (base pairs) is given by

$$1/C = \sum_{i=1}^{N} (1/C_i) \tag{7}$$

where C_i is the torsional rigidity of subunit i (Hogan & Austin, 1987). An upper limit on the torsional rigidity of the platinated DNA can be calculated by assuming that all the adducts are 1,2-intrastrand cross-links, each of which causes a complete loss of torsional flexibility at one base pair. Then the torsional rigidity measured at binding level r_b will be given by $C = C_0/(1 - 2r_b)$, where C_0 is the torsional rigidity of unmodified calf thymus DNA. Now, the maximal increase in fC is about 30% and occurs at $r_b = 0.01$. According to the previous formula, the average torsional rigidity will increase by at most 2% under these conditions. Thus, the expected maximal increase in the average torsional rigidity due to the formation of 1,2-intrastrand cross-links is much less than the observed increase in fC. The average torsional rigidity could only increase by 30% if the binding of one *cis*-DDP molecule were to immobilize 15 base pairs to twisting, which appears very unlikely. We conclude that the increase in fC at low r_b cannot be attributed to an increase in the torsional rigidity of the DNA.

The increase of fC at low r_b must therefore be mostly due to an increase in the solute–solvent friction brought about by

binding of *cis*-DDP. Since it is unlikely that the hydrodynamic radius will be appreciably increased by the formation of cross-links, the increase in friction must occur by kinking of the helix axis. Several authors have proposed that the formation of a 1,2-intrastrand cross-link at the d(GpG) sequence causes local kinking of the helix axis (den Hartog et al., 1985; Kozelka et al., 1985, 1987). Molecular mechanics calculations suggest that the helix is bent through an angle of 50–60° in a direction toward the major groove (Kozelka et al., 1985, 1987). As discussed previously, the internal torsional deformations of DNA ("speedometer cable motions") will not be transmitted by a directional cross-link between neighboring base pairs, and the segment of DNA adjacent to the kink must consequently rotate about an axis other than its own. Twisting of a kinked DNA molecule in solution will thus be subject to considerably more solute–solvent friction than twisting of a straight molecule of equal length. This effect should be important when the kinks are separated by a distance on the order of the wavelength of the predominant torsional modes responsible for the TDFDP (~100 base pair separations), which is indeed observed. The 30% increase in fC at $r_b = 0.01$ is a surprisingly large effect for so few adducts but is expected if the adducts kink the helix axis. Thus, TDFDP of intercalated ethidium is well-suited to detect kinking in a long DNA molecule by *cis*-DDP, or other covalent adducts, in the clinically relevant low binding regime.

At intermediate levels of *cis*-DDP binding, fC increases much more weakly with r_b (Figure 1). The proportion of cross-links at d(GpG) sites decreases in this regime as they become saturated with *cis*-DDP (Eastman, 1986). The weaker dependence of fC on r_b in this intermediate regime could be associated with this saturation. However, 1,2-intrastrand cross-links continue to form at d(ApG) sites (Eastman, 1986) and should also increase the friction. The incremental contribution of these adducts may be small because of a smaller kink angle, or it may be partially offset by a competing effect which tends to decrease fC. We note that such an effect dominates the behavior of fC at higher r_b values. We discuss the origin of this effect in the next section.

Interaction of cis-DDP with DNA at High r_b: Evidence for Duplex Disruption. At the highest levels of bound *cis*-DDP, $r_b > 0.1$, fC decreases with increasing r_b. The formation of adducts characterized by a reduced torsional rigidity could be responsible for this behavior. While we previously argued that the presence of a few regions of increased torsional rigidity cannot appreciably affect the average rigidity measured in the TDFDP experiments, the situation is different when regions of decreased rigidity are present, since eq 7 shows that the overall torsional rigidity of a heterogeneous rod is dominated by its most flexible elements. In previous experiments, the torsional rigidity of calf thymus DNA measured by TDFDP was observed to decrease after heat denaturation or destabilization of the DNA under conditions of low ionic strength (Millar et al., 1980, 1982). The presence of flexible nonduplex regions in a DNA molecule can thus cause an observable decrease in the average torsional rigidity. We propose that the decrease in fC observed with *cis*-DDP at $r_b > 0.1$ is due to a decrease in torsional rigidity, which is associated with a progressive loss of duplex structure as increasing amounts of the drug bind to the DNA. This is consistent with the increase in the proportion of free ethidium fluorescence (a_1) in this regime, since the locally melted DNA should bind less ethidium (Butour & Macquet, 1977). There is considerable evidence from a variety of other experiments which suggests that the binding of *cis*-DDP disrupts the duplex structure of DNA. These experiments include DNA melting studies (Macquet & Butour, 1978), circular dichroism (Macquet & Butour, 1978), electric dichroism (Houssier et al., 1983), Tb^{3+} fluorescence enhancement (Housier et al., 1983), intrinsic viscosity (Munchausen & Rahn, 1975), buoyant density (Shooter & Merrifield, 1972), and writhing changes in closed circular DNA (Cohen et al., 1979). These experiments suggest that ~50% of the bases are unpaired at saturation binding levels of *cis*-DDP (Houssier et al., 1975; Scovell & Capponi, 1982). Direct evidence for disruption of base pairing comes from biochemical experiments that employ reagents which specifically recognize single-stranded DNA or unpaired bases. Thus, the S_1 nuclease activity of DNA is greatly enhanced by high levels of bound *cis*-DDP (Scovell & Capponi, 1982). In fact, the onset of enhanced activity occurs at approximately the same r_b value as the onset of the torsional rigidity decrease (Scovell & Capponi, 1982). In an interesting recent study (Sundquist et al., 1986), anti-nucleoside antibodies were used to detect the presence of unpaired bases in DNA that had reacted with *cis*-DDP. The extent of base pair disruption was found to be a nonlinear function of r_b. At low r_b, the disruption was largely confined to GC base pairs, which reflected the formation of d(GpG) cross-links, whereas at high r_b there was a large, general disruption of base pairing which was suggestive of cooperative DNA melting (Sundquist et al., 1986). These findings are consistent with the results of the present study, in which the general disruption of base pairing is manifested as a decrease of torsional rigidity at high r_b values.

Interaction of trans-DDP with DNA. The data for DNA platinated by *trans*-DDP provide a contrast to the behavior exhibited by *cis*-DDP. Although our data for *trans*-DDP are less extensive than for *cis*-DDP, the parameter fC is equal, within experimental error, to the value for native DNA, at r_b values where *cis*-DDP-treated DNA had either a significantly larger or a smaller value, respectively. It appears that *trans*-DDP affects neither the torsional rigidity of DNA nor the solute–solvent friction. The absence of any increase in fC shows that no appreciable population of adducts is formed which kink the helix axis. This is consistent with other studies which show that *trans*-DDP does not form 1,2-intrastrand cross-links in its reaction with DNA (Stone et al., 1976; Cohen et al., 1982). The absence of a substantial decrease in fC suggests that *trans*-DDP causes significantly less duplex disruption than *cis*-DDP under the conditions of our experiment. This hypothesis is consistent with the smaller proportion of free ethidium found for DNA treated with *trans*-DDP as compared with *cis*-DDP (Table I). The results of other studies have produced mixed conclusions concerning the extent of duplex disruption by *trans*-DDP in comparison with *cis*-DDP. For example, *trans*-DDP causes less helix unwinding (Scovell & Kroos, 1982) and buoyant density change (Stone et al., 1976) than *cis*-DDP, while the S_1 nuclease activity of DNA saturated by *trans*-DDP is 3–4 times smaller than for the same amount of *cis*-DDP (Scovell & Capponi, 1982). However, anti-nucleoside antibodies bind more strongly to DNA modified by *trans*-DDP than to DNA modified by an equal level of *cis*-DDP (Sundquist et al., 1986). These differences are not yet fully understood since the adducts formed by *trans*-DDP are less well characterized than for *cis*-DDP. The formation of monofunctional adducts by *trans*-DDP may explain our results since these will not kink the helix axis and should induce less duplex disruption than the bifunctional adducts. Recent experiments have shown that a large proportion of monofunctional adducts are formed in the reaction of *trans*-DDP with DNA, although these disappear slowly due to

chelation with non-nearest-neighbor purine bases (Butour & Johnson, 1986). The proportion of monofunctional adducts in our *trans*-DDP-platinated DNA samples is unknown.

Conclusions

Our study of the TDFDP of intercalated ethidium has revealed that the internal motion dynamics of DNA in solution are significantly altered by the covalent binding of *cis*-DDP to DNA. Furthermore, the nonmonotonic dependence of the depolarization rate on the level of binding demonstrates that *cis*-DDP acts on DNA by more than a single mechanism. A variety of DNA–platinum adducts are known to form when *cis*-DDP binds to DNA (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). The TDFDP experiment separates the contribution of different adducts by virtue of their differing effect on the internal motion dynamics of DNA.

When low levels of *cis*-DDP bind to calf thymus DNA, our results suggest that adducts are formed which kink the helix axis, thereby increasing the solute–solvent friction and slowing the torsional motions. Other studies have shown that the 1,2-intrastrand cross-link at the d(GpG) sequence is the preferred adduct under these conditions (Eastman, 1983, 1986; Fichtinger-Schepman et al., 1985). Our results support the kinked duplex models proposed by Kozelka et al. (1985, 1987) on the basis of molecular mechanics calculations of an oligonucleotide containing a 1,2-intrastrand cross-link at a d(GpG) site, and are consistent with NMR evidence for duplex kinking at d(GpG) sites (den Hartog et al., 1985). The TDFDP of intercalated ethidium appears to be a suitable technique for detecting widely separated kinks in a long DNA molecule. In contrast, binding of the clinically ineffective trans isomer to DNA has no effect on the friction, showing that this isomer does not form adducts which kink the helix axis.

When high levels of *cis*-DDP bind to DNA, adducts are formed which act to destabilize the duplex structure of DNA, thereby introducing flexible single-stranded regions which result in faster overall torsional motion. This conclusion is in accord with other reports of duplex disruption by *cis*-DDP. Binding of the trans isomer does not appreciably decrease the torsional rigidity, indicating that it causes significantly less duplex disruption than *cis*-DDP, at least under the conditions of our experiment.

We have presented evidence for structural perturbations of DNA which are specifically induced by binding of the clinically effective isomer *cis*-DDP to DNA. Kinking of the helix axis or disruption of duplex structure may therefore be related to the antineoplastic activity of *cis*-DDP.

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Registry No. *cis*-DDP, 15663-27-1; *trans*-DDP, 14913-33-8.

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Synthesis of an *N*-Methyl-*N*-nitrosourea Linked to a Methidium Chloride Analogue and Its Reactions with ^{32}P-End-Labeled DNA†

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ABSTRACT: The synthesis and characterization of an *N*-methyl-*N*-nitrosourea (MNU) analogue that is covalently linked to a methidium nucleus (**9**) is described. At 37 °C in pH 8.0 buffer **9** hydrolyzes via pseudo-first-order kinetics, with a calculated $t_{1/2}$ = 77 min. By use of polyacrylamide sequencing gels the formation of piperidine-labile N^7-methylguanine adducts from the reaction of **9** and MNU with 5′-^{32}P-end-labeled DNA restriction fragments is reported. DNA methylation by **9** in 10 mM Tris buffer is enhanced with increasing ionic strength (50–200 mM NaCl), which contrasts to the inhibition of MNU-induced cleavage with increasing salt. In addition, **9** methylates all G sites equally, while MNU shows a clear preference for d(G)$_n$ ($n \geq 3$) runs and an asymmetrical methylation pattern within these G-rich regions. The results are discussed in terms of the delivery of the MNU moiety to the DNA target by a non-sequence-specific intercalation process and the subsequent hydrolytic generation of a nondiffusible alkylating intermediate.

The mutagenic, carcinogenic, and antineoplastic activities of *N*-alkyl-*N*-nitrosoureas are associated with their covalent modification of DNA (Druckrey et al., 1967; Magee, 1976; Lawley, 1984; Preussmann & Stewart, 1984). The conversion of *N*-alkyl-*N*-nitrosoureas to electrophilic alkylating intermediates in aqueous solution at near-neutral pH entails an initial specific-base-catalyzed hydrolysis (Garrett et al., 1965; Synder & Stock, 1980) to a transient alkanediazotic acid (White & Woodcock, 1968; Moss, 1974) followed by ionization to the corresponding alkanediazonium ion (Huisgen & Rüchardt, 1956; Streitweiser & Schaeffer, 1957; Friedman, 1970; Kirmse, 1976; Southam & Whiting, 1982; Gold et al., 1984). Depending on the nature of the alkyl substituent, the diazonium ion directly (McGarrity & Smyth, 1980; Ford, 1986) or via a nitrogen-separated ion pair or triplet (White & Woodcock, 1968; Moss, 1974; Gold et al., 1984) alkylates DNA (Figure 1). The high bond dissociation energy for methanediazonium ion (Ford, 1986) and NMR studies on the protonation of diazomethane (McGarrity & Smith, 1980; McGarrity & Cox, 1983) indicate the involvement of methanediazonium ion in DNA alkylation by MNU.[1] We have previously observed that the MNU-mediated formation of N7-MeG is sequence specific, with d(G)$_3$ runs showing heavy methylation at the central G site (Wurdeman & Gold, 1988). In d(G)$_4$ sequences the asymmetrical methylation pattern is quite striking and distinctly different from that observed for DMS. In addition, DNA methylation was dose-dependently inhibited by the addition of NaCl (50–200 mM) or by the addition of micromolar concentrations of the cationic DNA affinity binders ethidium bromide, distamycin A, or spermine. This inhibition was independent of sequence, i.e., methylation at all G sites is equally reduced. The alkylation pattern was attributed to the electrostatic attraction between d(G) stretches and a positively charged alkylating agent. The inhibition results from decreased electrostatic ion pairing of the cationic methylating agent and the polyanionic DNA molecule (Perahia et al., 1979; Cauchy et al., 1980; Pullman & Pullman, 1981). Accordingly, methylation of DNA by DMS was not affected by increasing ionic strength or by the presence of the cationic DNA affinity binders (Wurdeman & Gold, 1988).

In order to understand the basis for the sequence specificity observed for MNU and to increase the yield of DNA methylation, an MNU analogue was covalently linked to a methidium chloride intercalator. Methylation of sequence-characterized ^{32}P-end-labeled DNA restriction fragments indicates that the preference of MNU for poly-d(G) runs and the inhibition of DNA methylation at high salt are overcome by the sequence- and salt-independent intercalation process.

Materials and Methods

Materials

Solvents. DMF was passed over a column of 4-Å molecular sieves and dried by sequential azeotropic distillation with benzene and reduced-pressure distillation from BaO. Chlorobenzene and nitrobenzene were dried over 3-Å molecular sieves and distilled from P_2O_5. DMSO was freshly distilled from CaH_2 after initial drying over 4-Å molecular sieves. *N*-Ethylmorpholine and ethylenediamine were dried over KOH, and 1,1′-carbonyldiimidazole was recrystallized from anhydrous THF.

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[1] Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; DMS, dimethyl sulfate; bp, base pair(s); DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-pressure liquid chromatography; *N*7-MeG, N^7-methylguanine; THF, tetrahydrofuran; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane.

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