Kinetic Flow Dichroism Study of Conformational Changes in Supercoiled DNA Induced by Ethidium Bromide and Noncovalent and Covalent Binding of Benzo[*a*]pyrene Diol Epoxide†

Hiroko Yoshida and Charles E. Swenberg*

Department of Radiation Sciences, Division of Physical Radiobiology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814

Nicholas E. Geacintov

Chemistry Department, New York University, New York, New York 10003

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ABSTRACT: The dynamic conformational changes due to the noncovalent intercalative binding of ethidium bromide and racemic *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), and the covalent binding of BPDE to supercoiled ϕX174 DNA, have been studied by gel electrophoresis and a novel application of a kinetic flow linear dichroism technique. The magnitude of the linear dichroism (ΔA) of the DNA oriented in the flow gradient is sensitive to the hydrodynamic shape of the DNA molecule which is affected by the binding of the drug or the carcinogen BPDE. While the linear dichroism of ethidium bromide supercoiled DNA is time independent, the ΔA spectra of BPDE–DNA reaction mixtures vary on time scales of minutes, which correspond to the reaction rate constant of BPDE to form 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene hydrolysis products and covalent DNA adducts. The rapid noncovalent intercalation of BPDE causes an initial large increase in ΔA (up to 250%, corresponding to the dichroism observed with relaxed circular DNA), followed by a slower decrease in the linear dichroism signal. This decrease in ΔA is attributed to the removal of intercalated diol epoxide molecules and the resulting reversible increase in the number of superhelical turns. The kinetic flow dichroism spectra indicate that the noncovalent BPDE–DNA complexes are intercalative in nature, while the covalent adducts are characterized by a very different conformation in which the long axes of the pyrenyl residues are oriented at a large angle with respect to the average orientation of the planes of the DNA bases. These results suggest that conformations of carcinogen–DNA adducts, other than intercalative ones, can cause the unwinding of superhelical DNA. The flow dichroism method is capable of following kinetically changes not only in the shapes, and thus conformations of supercoiled DNA molecules, but also in the conformations of drugs or carcinogens causing these changes.

The polycyclic aromatic hydrocarbon benzo[*a*]pyrene, a known environmental pollutant, is metabolically converted in living cells to a variety of oxygenated derivatives (Gelboin, 1978; Singer & Grunberger, 1983; Conney, 1982; Harvey, 1981). The diol epoxide *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE)[1] is known to be the ultimate mutagenic and tumorigenic metabolite of benzo[*a*]pyrene. It is widely believed that the covalent binding of BPDE to DNA plays an important role in the initiation of the carcinogenic process and in mutagenesis (Singer & Grunberger, 1983; Brookes & Osborne, 1982). The primary covalent adduct formed involves the 10-position of BPDE and the exocyclic amino group of guanine (Osborne et al., 1976; Weinstein et al., 1976; Meehan et al., 1977; Koreeda et al., 1978). Other minor adducts have also been identified (King et al., 1976; Jeffrey et al., 1977; Osborne et al., 1981).

There are striking differences in the biological activities of the different stereoisomers of BPDE (Conney, 1982) which have been, at least in part, attributed to differences in the spatial orientation of the covalently bound pyrenyl aromatic ring system (Brookes & Osborne, 1982). Such differences have indeed been found experimentally by utilizing linear dichroism techniques (Geacintov et al., 1984a,b; Jernstrom et al., 1984; Undeman et al., 1983; Shahbaz et al., 1986). Generally, two types of conformations have been identified; type I involves an intercalative binding mode in which the aromatic pyrene residue tends to be oriented parallel to the planes of the bases, while in type II binding the plane of the aromatic residue tends to be tilted away from the planes of the bases (Geacintov et al., 1982a, 1985). The reaction mechanism of the tumorigenic (+) isomer of BPDE with double-stranded DNA in aqueous solution involves first a rapid noncovalent intercalation (site I binding) followed by a slower covalent binding step in which the pyrene residue undergoes reorientation to a type II binding site (Geacintov et al., 1984a). However, covalent binding constitutes a minor reaction pathway since, typically, about 90% of the BPDE molecules are hydrolyzed to tetraols.

On the basis of the effect of BPDE on the superhelicity of supercoiled DNA, other models describing the conformations of covalent BPDE–DNA adducts have been proposed. Drinkwater et al. (1978) and Kakefuda and Yamamoto (1978)

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[1] Abbreviations: BPDE, *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; tetraol, 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; THF, tetrahydrofuran.

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found that the covalent binding of BPDE to SV40 DNA causes an unwinding of supercoiled DNA. Since these changes are similar to those produced by the intercalating agent ethidium bromide, a model of covalent intercalative binding for the BPDE–DNA adducts was suggested (Drinkwater et al., 1978). A similar conclusion was reached by Agarwal et al. (1983) based on their observations that BPDE causes a rapid positive supercoiling of relaxed circular pBR322 DNA; however, it is not totally clear whether the observed effects in the work of Agarwal et al. (1983) were due to the noncovalent intercalation or to the covalent binding of BPDE. Meehan et al. (1982) observed that the noncovalent intercalation of BPDE causes an unwinding of SV40 DNA, but not as effectively as in the case of ethidium bromide. The results of Gamper et al. (1980), also obtained with SV40 DNA, are consistent with the formation of a noncovalent intercalative BPDE–DNA complex prior to the formation of the covalent adducts (Meehan & Straub, 1979; Geacintov et al., 1981; MacLeod & Selkirk, 1982). However, Gamper et al. (1980) concluded that the unwinding associated with the covalent alkylation of DNA is also consistent with an external binding mode, as suggested by the linear dichroism experiments of Geacintov et al. (1978). The increased mobility of covalent BPDE-nicked circular DNA adducts on agarose gels was attributed to the existence of flexible "hinges" at the covalent alkylation sites (Gamper et al., 1980). On the basis of linear dichroism and other data, a wedge-shaped covalent intercalation model was proposed for linear DNA covalently modified with BPDE (Hogan et al., 1981; Taylor et al., 1983); however, alternate interpretations of these and other experimental data have been discussed (Geacintov et al., 1985; MacLeod & Tang, 1985).

In order to help resolve these discrepancies, we introduce here a new application of flow dichroism techniques which constitutes a first attempt to measure changes in the conformational states of supercoiled DNA kinetically, on the time scale of seconds, produced by the interaction of the carcinogen BPDE with RF I ϕX174 DNA. Utilizing the intercalator ethidium bromide, we have found that the linear dichroism signal within the DNA absorption band is remarkably sensitive to the extent of supercoiling and parallels the well-known behavior of the sedimentation coefficient (Waring, 1970). Because the linear dichroism technique is especially useful for following the kinetics of the changes in the supercoiled state of DNA qualitatively, the effects of the rapid noncovalent intercalation of BPDE can be distinguished from the slower effects produced by the covalent binding of this diol epoxide to DNA.

Materials and Methods

The ϕX174 DNA dissolved in 5 mM Tris buffer at pH 7.4, and containing 1 mM EDTA, was obtained from Bethesda Research Laboratories (Bethesda, MD); utilizing agarose gel electrophoresis, it was determined that the initial content of supercoiled RF I DNA was at least 65–70%. Racemic BPDE was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, and stock solutions (3%) were prepared in THF. Nicked circular ϕX174 DNA was prepared by exposing the supercoiled DNA solutions to ^{60}Co γ irradiation (total dose of 400 Gy); agarose gel electrophoresis indicated that the concentration of supercoiled DNA in these irradiated solutions was negligible, while approximately 20% of the DNA was in the linear form. Calf thymus DNA (type I, Sigma Chemical Co., St. Louis, MO), dissolved in 5 mM Tris, 3 mM EDTA, and 0.1 M NaCl solution, was sonicated with a Heat Systems–Ultrasonics, Inc. (Plainview, NY) sonicator for a total time of 30 min (5-min periods interspersed with 5-min rest periods) under an atmosphere of nitrogen at 0 °C. After extensive dialysis against a 5 mM Tris and 1 mM EDTA solution, the sonicated DNA was subjected to polyacrylamide gel electrophoresis, and the chain length was found to be 800 ± 300 base pairs.

Small aliquots of the BPDE–THF stock solutions were added to the aqueous DNA solutions, and the concentration of THF was always less than 1%. The concentration of BPDE was determined by utilizing an extinction coefficient of 29 000 M^{-1} cm^{-1} at 344 nm. Only samples of BPDE containing less than 10% of the hydrolysis products (tetraols) were utilized. The concentration of the ethidium bromide (Sigma Chemical Co.) was determined by absorption spectrophotometry using an extinction coefficient of 5800 M^{-1} cm^{-1} at 480 nm in buffer solution and 4800 M^{-1} cm^{-1} for the DNA complexes (absorption maximum at 520 nm).

Electrophoresis on agarose gels (1% by weight) was performed by utilizing vertical slabs and TEA electrophoresis buffer (40 mM Tris, 1 mM EDTA, and 5 mM sodium acetate solution, pH 8.2) at 17 °C. Mixtures representing 20 μL of the DNA sample and 5 μL of a bromophenol–ficol solution were loaded onto the gels, and electrophoresis was performed at 40 V for 12 h in the absence of light. The gels were then placed in TEA buffer containing ethidium bromide (1 μg/mL) for staining for 1 h. After being stained, the gels were photographed in UV light. The negatives were then analyzed, and the relative optical densities as a function of migration distance were determined by utilizing a P1000 photoscanner (Optronics International, Inc., Chelmsford, MA) and a digitizer interfaced to a computer.

The flow linear dichroism experiments were performed as described previously (Geacintov et al., 1984a) utilizing a Couette cell. The latter consists of two concentric quartz cylinders, a stationary outer cylinder, and a rotating inner cylinder (400–600 rpm). The diameter of the outer cylinder is about 26 mm, and the aqueous DNA solution is contained in the 0.6-mm annular space. As the inner cylinder is rotated, the DNA molecules tend to align along the flow lines so that the planes of the bases are tilted partially in a vertical direction with respect to the flow lines. The linear dichroism is defined by

$$\Delta A = A_{\parallel} - A_{\perp} \quad (1)$$

where $A_{\parallel}$ and $A_{\perp}$ are the absorbances of the solutions measured with the polarization vector of the light beam oriented either parallel or perpendicular to the direction of the flow. This flow linear dichroism system requires a sample volume of 1 mL and is capable of following changes in the linear dichroism signals with a time resolution of about 100 ms with a reasonable signal/noise ratio, as long as the DNA concentration is at least 10 μg/mL. In this work, DNA concentrations of 30 μg/mL were utilized. In general, ΔA is determined as a function of wavelength. Thus, the orientations of noncovalently or covalently bound carcinogen and drug molecules relative to those of the DNA bases can be determined. Because the planes of the bases in linear DNA tend to be tilted perpendicular to the flow lines, the ΔA signal within the DNA absorption band (below 300 nm) is negative in sign. Intercalated planar molecules also display negative ΔA values within their absorption bands, while molecules whose planes tend to be oriented vertically with respect to the planes of the DNA bases are characterized by positive ΔA values.

Results

Incubation with BPDE and Levels of Covalent Binding. In all experiments, the DNA concentration was 7.6×10^{-5} M

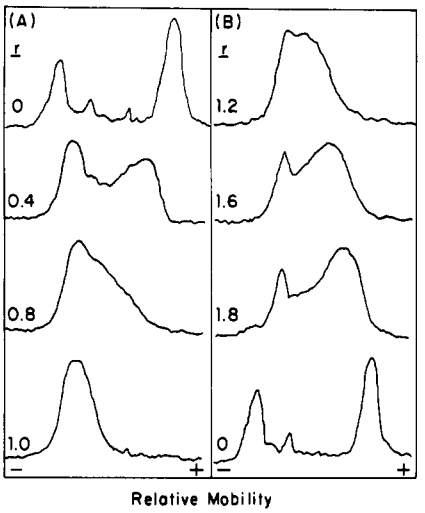

FIGURE 1: Microdensitometry traces of agarose gels of covalent BPDE–ϕX174 adducts prepared at different reaction ratios, r (initial moles of BPDE per mole of nucleotides). The reaction mixtures (7.6 $\times 10^{-5}$ M DNA, 5 mM Tris buffer, and 1 mM EDTA, pH 7.8) were incubated for 2 h at the indicated values of r and then electrophoresed for 12 h at 40 V, 17 °C, in the absence of light.

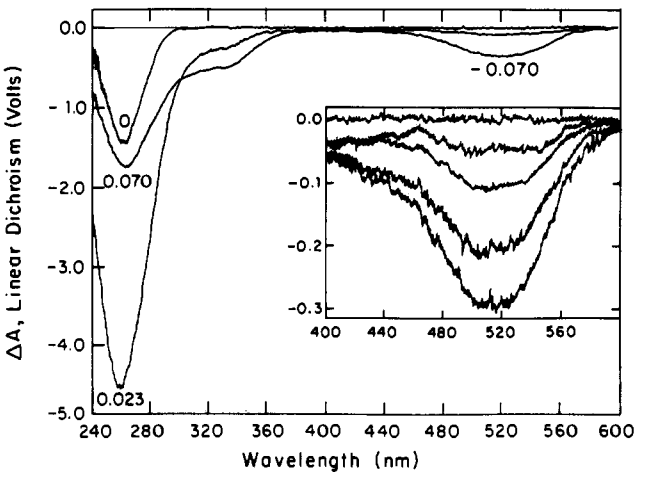

FIGURE 2: Flow linear dichroism spectra of supercoiled DNA and of ethidium bromide–ϕX174 complexes at different ratios, r_b (moles of drug bound per mole of DNA nucleotides). The inset represents expanded dichroism spectra in the region of the visible absorption band of ethidium bromide; the r_b values are (from the top to the bottom trace) 0.00, 0.010, 0.023, 0.050, and 0.070. The vertical ΔA signal is expressed in volts (output of the lock-in amplifier).

(expressed in terms of the concentration of nucleotides). The incubations were carried out at 25 °C and at different molar reaction ratios, r (initial moles of BPDE per mole of DNA). The reactions reached completion after about 10 min, and the level of covalent binding of the diol epoxide was determined spectrophotometrically after the tetraol hydrolysis products were extracted with ether (Geacintov et al., 1980; Yoshida, 1984). At molar ratios of r = 0.1, 0.2, 0.6, and 1.8, the level of covalent binding corresponded to molar binding ratios, r_b (generally defined as moles of drug or carcinogens bound to the DNA per mole of nucleotides), of 0.005, 0.007, 0.01, and 0.015, respectively. These ratios translate to 28, 38, 52, and 82 BPDE molecules per molecule to ϕX174 DNA, respectively.

Gel Electrophoresis. Some typical densitomer traces of ϕX174 DNA incubated with various amounts of BPDE are shown in Figure 1. The top trace in Figure 1A (and the bottom trace in Figure 1B) represents the electrophoretic gel patterns of DNA samples without addition of BPDE. The high-mobility band is due to the supercoiled RF I form, while the low mobility band is attributed to relaxed closed circular RF II DNA, which was present in varying amounts in the original DNA samples, depending on the batch furnished by the supplier.

The r = 0.4 and other traces show the effects of covalent binding of BPDE to DNA and are consistent with the results of Drinkwater et al. (1978) and Gamper et al. (1980). The r = 0.4 trace shows that the mobility of RF II DNA is increased, probably because of increased flexibility of the DNA at the alkylation sites as proposed by Gamper et al. (1980). The mobility of the RF I DNA decreases upon modification with BPDE which is attributed to a loss in superhelicity resulting from a local unwinding of the DNA superhelix as discussed by Drinkwater et al. (1978). The broadening of the RF I band for r = 0.4 is presumably due to the heterogeneity in the number and spatial location of covalently bound BPDE molecules per DNA molecule, and the resulting heterogeneity in the superhelicity of the DNA. At r = 1.0, only one peak is apparent which suggests that the BPDE-modified RF I and RF II forms have equivalent mobilities and thus comigrate. Another possible interpretation is that the supercoiled DNA is converted to the RF II form by the introduction of at least one single-strand break due to the binding of BPDE. However, this interpretation seems unlikely since at higher initial BPDE concentrations ($r > 1.2$) a broadened, higher mobility band reappears, while the low-mobility peak attributed to the relaxed circular form appears to remain nearly stationary (Figure 1B). At the molar reaction ratio r = 1.8, the leading edge of the fastest band has a mobility which is almost comparable to that of the unmodified RF I form. Such an effect was also observed by Drinkwater et al. (1978). The increased electrophoretic mobility at high r values is attributed to a rewinding (in the opposite sense) of the modified supercoiled DNA at high concentrations of the covalently bound carcinogen. This effect appears to be analogous to the unwinding and rewinding effects induced by increasing concentrations of ethidium bromide (Waring, 1970).

Flow Linear Dichroism. (A) Ethidium Bromide–DNA Complexes. We first investigated whether the flow dichroism technique was capable of revealing changes in the superhelicity of ϕX174 DNA induced by the well-known intercalator ethidium bromide. Some typical linear dichroism spectra obtained at different r_b (=moles of ethidium bromide bound per mole of DNA) values are shown in Figure 2. The linear dichroism at 500–520 nm due to the drug molecule is negative in sign, as expected for an intercalative mode of binding, and increases with increasing ethidium concentration. However, within the absorption band of DNA below 300 nm, the dependence of ΔA on r_b is quite different. The negative dichroism increases in magnitude as the drug concentration is increased from zero to r_b = 0.023 and remains constant up to r_b = 0.04. At these values of r_b, the magnitude of the dichroism at 260 nm is higher by the remarkably large factor of 3.5. Above this concentration of ethidium, the magnitude of the dichroism decreases with increasing r_b until a constant value, approximately equal to the original r_b = 0 value, is reached (Figure 3). In contrast to the behavior of supercoiled DNA, the linear dichroism signal of linear DNA at 260 nm increases monotonically as the ethidium bromide concentration is increased (Figure 3).

These results suggest that the changes in superhelicity are accompanied by changes in the hydrodynamic shapes of the DNA molecules which are detectable by the flow linear dichroism technique. The more compact, highly supercoiled RF I DNA is characterized by a lower overall dichroism; this

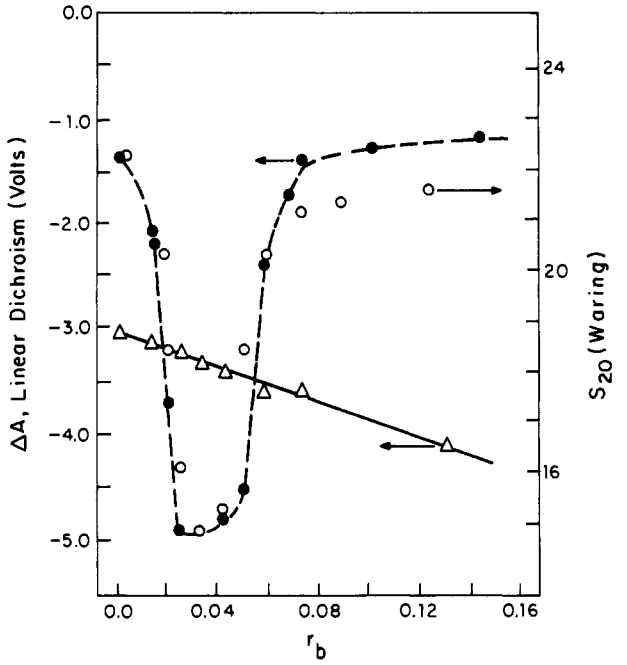

FIGURE 3: Flow linear dichroism signals measured within the DNA absorption band at 260 nm at different ethidium bromide concentrations expressed in terms of the molar binding ratio r_b, and constant ϕX174 DNA (●) or linear calf thymus DNA (△) concentration (7.6×10^{-5} M). The open circles (○) represent the sedimentation coefficients, s_{20}, determined by Waring (1970) and superimposed on the ΔA data.

indicates either (1) an average lower degree of orientation of the DNA molecule in the flow gradient or (2) a lower average degree of orientation of the bases perpendicular to the flow lines, or both.

There is a remarkable correlation between the magnitude of the linear dichroism signal at 260 nm, and the sedimentation coefficient (s_{20}) determined by Waring (1970), as a function of increasing ethidium bromide concentration. These data are compared to one another in Figure 3. It is evident that the flow linear dichroism results provide data which are similar to those obtained by the sedimentation method, except with the following advantages: the relative orientations of the polycyclic molecules and the DNA bases can be determined simultaneously, and changes in the superhelicity of the DNA molecule can be followed kinetically with a time resolution as high as 100 ms at a particular fixed wavelength.

As in the case of the sedimentation data (Waring, 1970), the ethidium bromide concentration-dependent changes in the linear dichroism are interpreted in terms of the following: (1) as the drug concentration is increased from $r_b = 0$ to $r_b = 0.023$, the negatively supercoiled DNA unwinds and reaches a minimum (closed circular DNA) and the magnitude of the dichroism increases; (2) as r_b is increased still further, the formation of a positively supercoiled helix results in a more compact and twisted DNA molecule characterized by a lower dichroism.

(B) BPDE–DNA Reaction Mixtures. The flow linear dichroism spectra of a RF I DNA solution taken just prior to the addition of BPDE, and at two different time intervals after the addition of BPDE ($r = 0.1$), are depicted in Figure 4. Before addition of BPDE, the highest linear dichroism signal is observed at 258 nm, as expected, since the magnitude of the dichroism is proportional to the absorption spectrum within a homogeneous absorption band (Frederick & Houssier, 1972).

When BPDE is added to a DNA solution, the BPDE binds noncovalently to the DNA by an intercalation mechanism

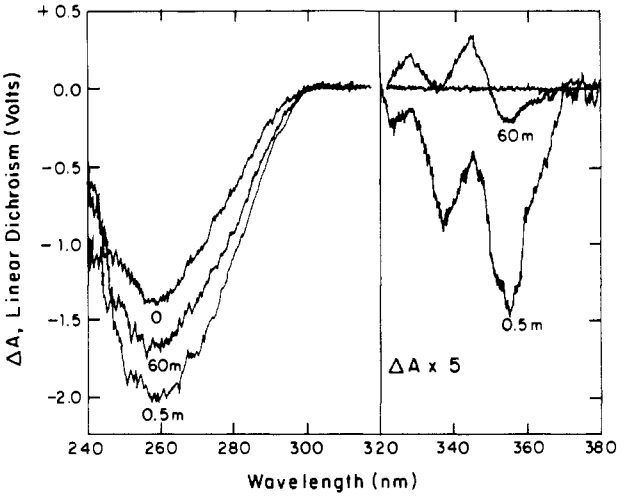

FIGURE 4: Linear dichroism spectra of reaction mixtures containing BPDE (8.3×10^{-6} M) and ϕX174 DNA (7.6×10^{-5} M) measured 0.5 and 60 min after mixing. The initial spectrum is characteristic of noncovalently intercalated BPDE molecules, while the final spectrum is characteristic of mostly covalent adducts (see text). The linear dichroism of the DNA sample before addition of BPDE is also shown.

within several milliseconds (Geacintov et al., 1981). The negative linear dichroism spectrum in the 320–370-nm region is attributed to the intercalated pyrenyl chromophore of BPDE (Geacintov et al., 1984a). On the time scale of minutes, over 90% of the BPDE molecules are converted to tetraols, while less than 10% are converted to adducts bound covalently to DNA (Yoshida, 1984). After a 10-min reaction time, the linear dichroism spectrum can be attributed solely to covalent adducts and intercalated tetraol molecules. The noncovalent association constant K of the tetraols is lower than that of BPDE by a factor of 3–4 (Geacintov et al., 1981, 1982; Yoshida, 1984); therefore, the linear dichroism signal due to intercalated tetraols is lower than that of BPDE and gives rise to the negative ΔA signal at about 353 nm in the equilibrated reaction mixture (Figure 4). This negative band disappears upon extraction of the tetraols with ether, and only the positive ΔA bands at about 328 and 345 nm due to the covalently bound (+) enantiomer of BPDE remain (Geacintov et al., 1984a; Yoshida, 1984).

The kinetics of the changes in the ΔA signals at 260 nm taken from successive spectral linear dichroism scans are depicted in Figure 5 for $r = 0.1$. Immediately after addition of BPDE to RF I ϕX174, the magnitude of the ΔA signal increases by about 30%. This change is attributed to two factors: (1) an unwinding of DNA induced by the noncovalent intercalation of BPDE, and (2) a contribution of intercalated BPDE to the linear dichroism signal at 260 nm; the ΔA signals of the DNA and intercalated carcinogen are expected to be additive, since the planes of the bases and BPDE are parallel to one another. The latter effect produces an increase in the ΔA_{260} signal upon addition of BPDE in the cases of nicked circular RF II and linear DNA (Figure 5).

In all three cases, using supercoiled, relaxed circular, and linear DNA, the magnitude of the ΔA signal decreases as the reaction of BPDE progresses and reaches an equilibrium value after approximately 8 min. In contrast, when ethidium bromide is added to supercoiled DNA, the absolute value of ΔA increases immediately by a factor of 3.5 ($r_b = 0.03$) and remains constant as a function of time. The decrease in ΔA in the case of linear DNA modified covalently with BPDE is attributed to the formation of a kink or bend at the alkylation site (Hogan et al., 1981). An increase in the electrophoretic mobility of RF II DNA upon covalent binding of BPDE has

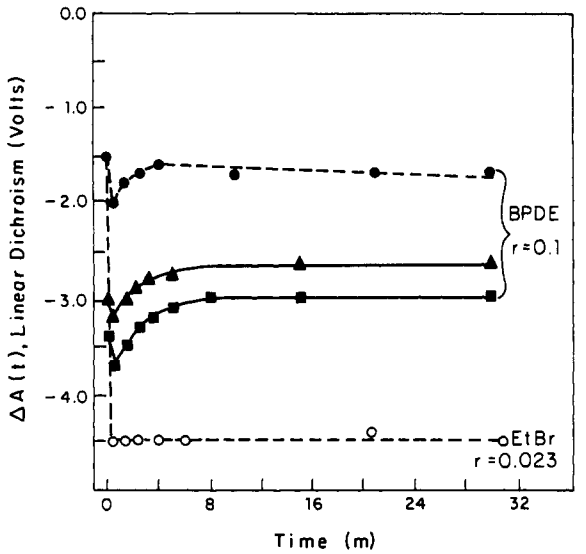

FIGURE 5: Time dependence of the linear dichroism measured within the DNA absorption band at 260 nm at a BPDE molar reaction ratio of $r = 0.1$, and in the presence of ethidium bromide [(○) $r_b = 0.023$]. All DNA concentrations were 7.6×10^{-5} M. Supercoiled RF I DNA (●); calf thymus linear DNA (▲); nicked circular ϕX174 DNA (■).

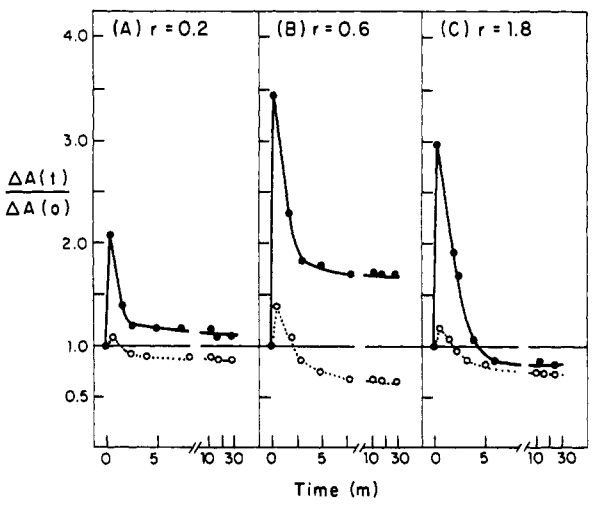

FIGURE 6: Kinetics of the ratio $\Delta A(t)/\Delta A(0)$ at different initial concentrations of BPDE expressed in terms of the molar reaction ratios r, where $\Delta A(0)$ is the linear dichroism signal measured at 260 nm just after the addition of BPDE, while $\Delta A(t)$ is the dichroism measured at time t after the addition of BPDE. Supercoiled ϕX174 DNA (●); nicked circular ϕX174 DNA (linear DNA exhibits analogous behavior; data not shown) (○). All DNA concentrations were 7.6×10^{-5} M.

been observed (Drinkwater et al., 1978; Gamper et al., 1980), which has been attributed by Gamper et al. to the formation of "flexible hinges" at the BPDE covalent binding sites. This effect also gives rise to a diminished overall degree of alignment of the DNA bases in relaxed circular DNA in the flow gradient, thus causing a decrease in the magnitude of ΔA as the covalent binding reaction progresses.

The decrease in the magnitude of ΔA after addition of BPDE in the case of supercoiled DNA can, in principle, be due to the kinking effect, or due to a rewinding of the DNA as the BPDE molecules are hydrolyzed and leave the intercalation sites. It is not possible to distinguish between these two effects on the basis of the experimental data alone depicted in Figure 5. However, at higher values of the reaction ratio r, the effect of rewinding can be observed more easily. In order to accentuate the differences in behavior between supercoiled and relaxed circular DNA, we have plotted the ratios $\Delta A(t)/\Delta A(0)$ as a function of time in Figure 6, where $\Delta A(0)$ is the initial dichroism (before the addition of BPDE) while $\Delta A(t)$ is the dichroism at 260 nm measured at different times after the addition of BPDE. The immediate sharp rise is due to the unwinding of supercoiled DNA produced by the intercalation of BPDE, while the subsequent rapid drop is attributable to the rewinding of the DNA as the BPDE molecules are hydrolyzed to tetraols and the number of intercalated diol epoxide molecules decreases. We mention in passing that the addition of equivalent amounts of tetraols produces only a negligibly small unwinding of RF I DNA (data not shown). The sharp decreases in the quantity $\Delta A(t)/\Delta A(0)$ as the reaction progresses are too large to be explained in terms of the kinking effect. The changes in the dichroism of relaxed DNA attributable to kink formation are significantly smaller than the effects observed in the case of supercoiled DNA (Figure 6).

In the $r = 1.8$ case, a positive dichroism signal is observed in the 280–300-nm region (data not shown), which also disappears with increasing time of reaction. This effect, observed only at relatively high concentrations of BPDE, is attributed to the aggregation of BPDE molecules on the surface of the DNA and has not been further investigated. However, this phenomenon accounts for the fact that immediately after addition of such a high quantity of BPDE, the $\Delta A(t)/\Delta A(0)$ signal is lower in the $r = 1.8$ case than in the $r = 0.6$ experiment.

The limiting, constant values of $\Delta A(\infty)/\Delta A(0)$ at long reaction times are due to the covalent adducts. We have found that the level of covalent binding of BPDE to linear and supercoiled DNA is approximately the same [see also MacLeod & Tang (1985)]. Thus, the differences in the limiting dichroism values observed with supercoiled and relaxed or linear DNA cannot be attributed to differences in the number of covalently bound BPDE residues. In the $r = 0.2$ and 0.6 cases, $\Delta A(\infty)/\Delta A(0) > 1.0$, indicating that the modified supercoiled DNA orients better than the unmodified RF I DNA. This correlates with the apparent larger hydrodynamic volume of the modified DNA and its decreased electrophoretic mobility at these values of r (Figure 1). At still higher degrees of modification at $r = 1.8$ (about 80 covalently bound BPDE molecules per genome), $\Delta A(\infty)/\Delta A(0) < 1.0$, and the modified DNA orients less well in the flow gradient than unmodified supercoiled DNA. This may be due to the induction of the more compact positive supercoiled form at these high levels of modification (Figure 1B), or to the kinking effect, or to a combination of these two effects.

(C) Kinetics of Relaxation of Linear Dichroism. The minimum elapsed time between the addition of either ethidium bromide or BPDE to the DNA-containing solutions and the first linear dichroism measurement is about 20 s. This is the time required to achieve adequate mixing and the full rotational speed of the inner cylinder in the Couette cell. In the case of ethidium bromide, the maximum magnitude of the linear dichroism signal is achieved within less than 20 s, since there is no change in ΔA from the very first measurement to the last (after 2 days; data not shown). Thus, the intercalation of this drug and its effect on the conformational state of superhelical DNA occur on time scales faster than 20 s. In the case of BPDE, the initial effects of noncovalent intercalation and unwinding also occur on time scales which are too rapid to be resolved (less than 20 s); however, the subsequent effects of the chemical reactions of BPDE occur on time scales of minutes, and the accompanying changes in the dichroism spectra can be easily followed as a function of time.

The kinetics of the relaxation of the linear dichroism signals within the DNA absorption band (260 nm) and the absorption

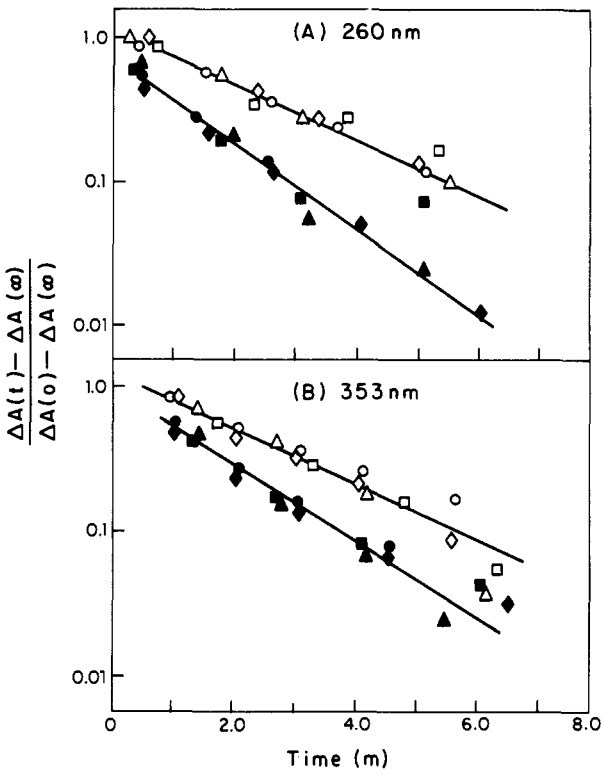

FIGURE 7: Semilogarithmic plots of eq 2. The linear dichroism data were obtained from ΔA spectra measured for reaction mixtures with different initial BPDE concentrations at 260 nm (DNA absorption) and 353 nm (absorption band of noncovalently intercalated BPDE): $r = 0.10$ (○, ●); $r = 0.20$ (□, ■); $r = 0.60$ (△, ▲); $r = 1.8$ (◇, ◆). Supercoiled ϕX174 DNA (●, ■, ▲, ◆); nicked circular ϕX174 DNA (○, □, △); linear calf thymus DNA (◇). All DNA concentrations were 7.6×10^{-5} M. The data points obtained at different r values were shifted vertically to fall on the same straight line plots for supercoiled, relaxed circular, or linear DNA. These two different sets of lines were further offset from one another along the vertical axis for clarity.

band of intercalated BPDE molecules (353 nm) are compared in the semilogarithmic plots of the time-dependent normalized linear dichroism

$$\frac{\Delta A(t) - \Delta A(\infty)}{\Delta A(0) - \Delta A(\infty)} = \exp(-kt) \qquad (2)$$

as a function of time (Geacintov et al., 1984a) in Figure 7; $\Delta A(0)$, $\Delta A(t)$, and $\Delta A(\infty)$ are the linear dichroism signals measured just after the addition of BPDE, at a time t after addition, and for equilibrated reaction mixture (all BPDE molecules reacted), respectively. The pseudo-first-order reaction rate constant of BPDE is defined by k. In Figure 7, the data points obtained from dichroism spectra of reaction mixtures with different r values have been combined. The data points obtained with relaxed circular and linear DNA fall on lines with the same slopes with constants $k = 0.29 \pm 0.02$ min^{-1} (260 nm) and 0.30 ± 0.02 min^{-1} (353 nm). In the case of supercoiled DNA, the data points fall on lines with different slopes ($k = 0.44 \pm 0.02$ min^{-1} at 260 nm and $k = 0.40 \pm 0.02$ min^{-1} at 353 nm). Within experimental error, the relaxation kinetics are approximately the same at 260 and 353 nm for both the supercoiled and the circular and linear DNA samples. The kinetics of this rewinding parallel the decay of BPDE and are the same for all reaction ratios r (Figure 7). At the different reaction ratios used ($r = 0.1$–1.8), the levels of covalent binding vary from $r_b = 0.005$ to 0.015 and give rise to large differences in the final values of the linear dichroism (time points beyond 10 min in Figures 5 and 6). The covalent adducts are produced during the time intervals of 0–6 min, however, when the large changes in ΔA, attributed to the rewinding of the DNA, are occurring.

It has been shown previously (Geacintov et al., 1984a; Yoshida, 1984) that k obtained from plots of eq 2 using linear dichroism data at 353 nm (due mostly to physically intercalated BPDE molecules) indeed represents the decay constant of BPDE. The fact that at 260 nm the kinetics are similar to those at 353 nm suggests that the conformational changes of the DNA (rewinding in the case of supercoiled DNA, kink or bend formation in the case of relaxed circular and linear DNA) occur as soon as the BPDE molecules are hydrolyzed or converted to covalently bound moieties. There is no measurable time lag between these events and the conformational changes of the DNA molecules.

In the case of supercoiled DNA, the reaction rate constant k is about 1.3–1.5 times faster than in the cases of relaxed circular and linear DNA. This difference is consistent with the results of MacLeod and Tang (1985), who, utilizing different methods of analysis, found a ratio of 1.21; since the reaction rate constant of BPDE in DNA solution depends on the intercalative association constant K (Geacintov et al., 1982b), the higher reactivity of BPDE in the presence of supercoiled DNA can probably be attributed to a somewhat higher value of K in RF I DNA than in RF II DNA (MacLeod & Tang, 1985).

Discussion

Flow Linear Dichroism as a Technique for Monitoring Changes in Shape of Supercoiled DNA. The linear dichroism signal of supercoiled DNA oriented in a flow gradient is sensitive to the changes in shape of the macromolecule induced by the intercalative binding of ethidium bromide, or the noncovalent and covalent binding of BPDE. The more compact supercoiled DNA molecules are characterized by faster mobilities on electrophoretic gels and higher sedimentation coefficients, but lower linear dichroism signals than nicked circular, relaxed DNA. The lower dichroism measured within the DNA absorption band at 260 nm is associated with a lower average degree of alignment of the nucleic acid bases with respect to the flow lines. Such a lower average alignment may be due either to a greater rotational mobility of the compact supercoiled molecules and/or to a lower degree of orientation of the bases with respect to the axis of alignment of the macromolecule in the flow field. The noncovalent intercalation of drug or carcinogen molecules causes a partial unwinding of the supercoiled DNA leading to an apparent hydrodynamically larger molecule with reduced electrophoretic mobility and lower sedimentation coefficient, but higher linear dichroism. The magnitude of the linear dichroism of relaxed circular DNA is 3.5 times higher than that of the supercoiled DNA sample. It is interesting to note that the magnitude of the dichroism signal closely parallels the changes in the sedimentation coefficient, s_{20}, measured by Waring (1970) at different ethidium bromide concentrations. The linear dichroism method is therefore suitable for following the kinetics of unwinding (or rewinding) on the time scale of seconds. The limiting factor in measuring the kinetics is the 20-s time period required to add the drug to the DNA sample and to subsequently bring the Couette cell to its full rotational speed. Thus, the flow dichroism technique can provide information similar to that obtained by the sedimentation technique, but with the additional advantage that the kinetics of the changes in the shape of the DNA can also be followed.

Effect of Noncovalent Binding. The effect of the noncovalent binding of ethidium bromide and BPDE on the su-

perhelicity of supercoiled ϕX174 DNA can be compared by considering the magnitudes of the dichroism induced by these two molecules at the same value of r_b. The first value of ΔA, measured within less than 1 min of mixing (Figures 5 and 6), is an indication of the effect of noncovalent intercalative binding of BPDE on the shape of supercoiled DNA. The value of r_b can be estimated from the reaction ratio r, the DNA concentration, the known association constant for linear DNA (K = 12000 M^{-1}; Geacintov et al., 1982b; Yoshida, 1984), and the finding that K is only slightly larger (about 40% in the case of the tetraol derived from the hydrolysis of BPDE; MacLeod & Tang, 1985) for supercoiled than for linear DNA. At relatively low molar reaction ratios, e.g., when r = 0.1, the fraction of physically bound BPDE molecules can be estimated from the expression (Geacintov et al., 1982)

$$K[\mathrm{DNA}]/(1 + K[\mathrm{DNA}]) \quad (3)$$

At a DNA concentration of 7.6×10^{-5} M, the fraction of bound molecules is about 0.48 and r_b = 0.048. Under these conditions, the magnitude of the linear dichroism is increased by 30% immediately after the addition of BPDE, and before any substantial decomposition of BPDE has occurred (Figure 5). At a similar value of r_b, ethidium bromide causes a 250% increase in the magnitude of ΔA and complete unwinding (Figure 3). It is thus concluded that ethidium bromide is much more effective, per molecule bound, in inducing unwinding of supercoiled DNA than the noncovalent binding of BPDE. Meehan et al. (1982) reported an unwinding angle of 13° for the physical binding of BPDE to SV40 DNA as compared to a value of 30° for ethidium bromide, which is consistent with our conclusions based on kinetic flow dichroism data. This difference may be due to a larger distortion of the local structure of the DNA by the more tightly and electrostatically bound drug molecule than the distortion caused by the more weakly bound electrically neutral diol epoxide molecule.

Effect of Covalent Binding of BPDE. The linear dichroism signal of the fully equilibrated BPDE–DNA reaction mixtures reflects the effects of the covalently bound 7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene residues. Since the effects of the noncovalently bound tetraols on the unwinding and on the linear dichroism changes of supercoiled DNA are negligible, the variations in $\Delta A(\infty)$ are due to the covalently bound BPDE residues. In the experiments described here, the covalent binding ratio varied from r_b = 0.005 (at an initial reaction ratio of r = 0.1) to r_b = 0.015 at r = 1.8. While a detailed quantitative study of the effect of covalent binding on the linear dichroism of superhelical DNA was not the focus of this study, a rough comparison between the effects produced by ethidium bromide and by the covalent binding produced by BPDE is nevertheless possible. For example, at r = 0.6, the covalent BPDE binding ratio is r_b = 0.010, and at equilibrium, this level of covalent binding leads to a 70% increase in ΔA (Figure 6). In the case of ethidium bromide, a similar increase in ΔA is produced at r_b = 0.010–0.015. This result is consistent with the findings of Drinkwater et al. (1978), who reported that the unwinding produced by the noncovalent binding of ethidium bromide is about the same as the effect produced by the covalent binding of BPDE to supercoiled SV40 DNA. It is thus evident that the noncovalent intercalative binding of BPDE, which precedes the covalent binding reaction, causes much less of a perturbation of the local structure of supercoiled DNA, and thus less unwinding, than the covalently bound benzo[*a*]pyrene triol residues.

Orientation of Pyrenyl Chromophores and Unwinding. The linear dichroism spectra of BPDE–DNA noncovalent complexes and covalent adducts provide information on the relative orientations of the pyrenyl chromophores and the planes of the DNA bases. As in the case of linear DNA, the ΔA spectra of the noncovalent BPDE–DNA complexes, taken within 1 min of mixing, are negative in sign (Figure 4); the minima in ΔA at 337 and 353 nm coincide with the absorption bands of physically bound BPDE and are consistent with a type I intercalative conformation of the BPDE chromophore (Geacintov et al., 1984a; Yoshida, 1984).

In the case of the equilibrated reaction mixtures (the 60-min spectrum in Figure 4), the positive maxima at 328 and 345 nm are due to the covalently bound (+) enantiomer of BPDE and are attributed to type II conformations in which the pyrenyl residue is located either in an external region or in a disordered region of the DNA (Geacintov et al., 1978, 1982a, 1984a). A wedge-shaped intercalative complex is unlikely for this structure since other diol epoxides, e.g., the (−) enantiomer of BPDE, display a red-shifted negative linear dichroism which is more consistent with such a quasi-intercalative conformation (Yoshida, 1984; Geacintov, 1985). Drinkwater et al. (1978) suggested that the covalently bound residue of BPDE is intercalated since the unwinding effect produced is similar to that generated by the intercalator ethidium bromide. However, the linear dichroism spectrum shown in Figure 4 is not consistent with such an assignment, since the positive ΔA peaks at 328 and 345 nm are characteristic of type II, nonintercalated adducts.

In summary, the advantage of the kinetic flow dichroism technique is that changes in the conformation of the DNA can be monitored simultaneously with changes in the conformation of the drug or carcinogen causing the unwinding of superhelical DNA. We conclude that conformations of carcinogens, other than intercalative binding, can cause changes in the superhelicity of supercoiled DNA.

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Registry No. (±)-BPDE, 58917-67-2; ethidium bromide, 1239-45-8.

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Molecular Cloning and Sequence Analysis of Complementary DNA Encoding Rat Mammary Gland Medium-Chain *S*-Acyl Fatty Acid Synthetase Thio Ester Hydrolase

Richard Safford,* Jacquie de Silva, Clare Lucas, John H. C. Windust, James Shedden, Christopher M. James, Christopher M. Sidebottom, Antoni R. Slabas, Michael P. Tombs, and Stephen G. Hughes

Biosciences Division, Unilever Research, Bedford MK44 1LQ, U.K.

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ABSTRACT: Poly(A)+ RNA from pregnant rat mammary glands was size-fractionated by sucrose gradient centrifugation, and fractions enriched in medium-chain *S*-acyl fatty acid synthetase thio ester hydrolase (MCH) were identified by in vitro translation and immunoprecipitation. A cDNA library was constructed, in pBR322, from enriched poly(A)+ RNA and screened with two oligonucleotide probes deduced from rat MCH amino acid sequence data. Cross-hybridizing clones were isolated and found to contain cDNA inserts ranging from ~1100 to 1550 base pairs (bp). A 1550-bp cDNA insert, from clone 43H09, was confirmed to encode MCH by hybrid-select translation/immunoprecipitation studies and by comparison of the amino acid sequence deduced from the DNA sequence of the clone to the amino acid sequence of the MCH peptides. Northern blot analysis revealed the size of the MCH mRNA to be 1500 nucleotides, and it is therefore concluded that the 1550-bp insert (including G·C tails) of clone 43H09 represents a full- or near-full-length copy of the MCH gene. The rat MCH sequence is the first reported sequence of a thioesterase from a mammalian source, but comparison of the deduced amino acid sequences of MCH and the recently published mallard duck medium-chain *S*-acyl fatty acid synthetase thioesterase reveals significant homology. In particular, a seven amino acid sequence containing the proposed active serine of the duck thioesterase is found to be perfectly conserved in rat MCH.

In animals, de novo synthesis of fatty acids is catalyzed by a multienzyme complex, fatty acid synthetase, which elongates acetyl coenzyme A (acetyl-CoA) by successive additions of two-carbon units derived from malonyl-CoA. The acyl chain is covalently bound, via a thio ester linkage, to the 4′-phosphopantetheine moiety of the fatty acid synthetase (Phillips et al., 1970). Chain termination and release of the free fatty acid product is achieved by hydrolysis of the thio ester by a component of the synthetase complex, thioesterase I. The chain length of the released fatty acid is usually C16. However, in some specialized tissues, such as the mammary glands of nonruminant mammals (Libertini & Smith, 1978; Knudsen

* Author to whom correspondence should be addressed.

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