Interactions of Benzo(a)pyrene Diol-Epoxides with Linear and Supercoiled DNA

Michael C. MacLeod* and Moon-shong Tang

University of Texas System Cancer Center, Science Park-Research Division, Smithville, Texas 78957

ABSTRACT

Previous spectroscopic studies of the major adduct formed by reaction of (±)-7α,8β-dihydroxy-9β,10β-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-I) with linear DNA have been interpreted to suggest that the adduct is not intercalated in the double helix. However, studies of the electrophoretic mobility of supercoiled DNA treated with BPDE-I suggest that the adduct is intercalated. To resolve these interpretations, we have studied the reaction of BPDE-I with supercoiled and linear DNA. The kinetics of DNA-catalyzed hydrolysis and of covalent binding are similar for the two DNAs; supercoiled DNA exhibits a 20% increase in the rate of hydrolysis of BPDE-I at low DNA concentration compared to linear DNA. Fluorescence excitation spectra and fluorescence quenching experiments provide no support for a model in which BPDE-I adducts are intercalated in supercoiled DNA. When deoxyribonucleoside adducts were analyzed by high-performance liquid chromatography, identical distributions of BPDE-I adducts were found for supercoiled and linear DNA. These data are consistent with a previously proposed model (Hogan, M. E., Dattagupta, N., and Whitlock, J. P., Jr. J. Biol. Chem., 256: 4504-4513, 1981; Taylor, E. R., Miller, K. J., and Bleyer, A. J. Biol. Chem., 260: 1-13, 1985), in which the major BPDE-I adduct in both linear and supercoiled DNA exists in a conformation which allows stacking with the neighboring base pair and introduces a "kink" into the path of the helical axis. Although this model provides an explanation for all available experimental data, there are undoubtedly other DNA adduct conformational models which are also consistent with the data.

INTRODUCTION

The environmental procarcinogen B(a)P* is metabolized by mammalian cells to electrophilic derivatives which bind to cellular macromolecules, initiating the process of carcinogenesis. Among these derivatives is the ultimate carcinogen, BPDE-I, which is the major DNA-binding metabolite in cells treated with B(a)P (1, 4, 14, 16). The interactions of BPDE-I with DNA, which may be of critical importance for initiation of carcinogenesis, have been studied in vitro using purified DNA by various investigators (8, 9, 12, 17, 18, 20, 26). Since the patterns of DNA adduct formation in these systems mimic patterns found in treated cells, there is reason to believe that in vitro data may help us to understand processes which occur in intact cells.

Many planar, aromatic molecules including BPDE-I bind noncovalently to DNA by intercalation. This allows stacking interactions with base pairs on either side of the intercalant which leads to a sizable (10 nm) red shift in the absorbance spectrum of the molecule (13, 15). In addition to noncovalent binding, BPDE-I undergoes several covalent reactions in the presence of DNA. One pathway leads to covalent adduct formation, primarily but not exclusively with the exocyclic amino group of dGuo. The second pathway is hydrolysis of the diol-epoxide to tetros (10, 18), a detoxification pathway which we have recently shown is catalyzed by DNA. At near neutral pH, the hydrolysis pathway predominates and is therefore of utmost importance in determining the ultimate level of adduct formation. Both of these covalent pathways as well as intercalation are inhibited by increasing concentrations of divalent cations (8, 10, 17, 18), and it has been suggested that intercalation may be a prerequisite for hydrolysis (10, 21), covalent binding (19), or both, although our recent data suggest that hydrolysis does not depend on intercalation.

Since the ability of a cell to repair a given DNA adduct may be as important as the initial absolute level of adducts formed, much interest has centered on determining the conformation of BPDE-I-DNA adducts (5, 8, 9, 23, 24, 28). Data obtained by spectroscopic means using linear DNA molecules have been interpreted to show that the major BPDE-I adduct lies in the minor groove of the DNA helix, not in an intercalated position (9, 23, 24, 28). On the other hand, studies of the electrophoretic properties of supercoiled DNA molecules containing varying amounts of BPDE-I adducts have demonstrated an apparent relaxation of superhelical stress consistent with the adducts maintaining an intercalated conformation (5, 8). It seemed possible that differences in the interaction of BPDE-I with linear and supercoiled DNA molecules could have caused this discrepancy. As part of our studies of the effects of various diol-epoxide-DNA adducts on the biological activity of ϕX174 RF I DNA, we report here a comparison of the interactions of BPDE-I with linear and supercoiled DNA molecules.

MATERIALS AND METHODS

Labeled and unlabeled diol-epoxides were obtained from the National Cancer Institute Chemical Repository. Stock solutions were made by dissolving the diol-epoxide in absolute ethanol, determining concentration by spectrophotometry (ε₅₄₀ = 48,770 at 344 nm), and storing the appropriate dilution at −20°C. Linear salmon sperm DNA was obtained from Sigma Chemical Co. (St. Louis, MO) and was further purified by treatment with RNase, proteinase K, and sodium dodecyl sulfate followed by RNase. 5 M. C. MacLeod and K. Zachary, manuscript in preparation.
BPDE-DNA INTERACTIONS

extraction with phenol and precipitation with ethanol at -20°. The DNA was dissolved in 20 mM Tris-HCl (pH 7.4), sheared by passage through a 25-gauge needle, and stored at 4° for a few drops of chloroform. DNA prepared in this way was of relatively high molecular weight, most molecules being in the range of 5,000 to 10,000 base pairs as determined by electrophoresis. Thermal hyperchromicity of this DNA was 38%, exhibiting a single transition with a melting temperature of 75° in 10 mM NaCl-20 mM Tris (pH 7.4). Similar results were obtained with calf thymus DNA (Sigma) which was used to prepare deoxyribonucleoside adducts.

Closed circular, supercoiled DNA was prepared from Escherichia coli C sup E cells infected with ϕX174am3cs70 phages by the cleared lysate method and was purified by isopyknic centrifugation (2). Each preparation was used as checked for the presence of relaxed circular molecules by gel electrophoresis. All preparations used contained greater than 85% supercoiled molecules as determined by densitometry of gels stained with ethidium bromide or 4',6-diamidino-2-phenylindole. The ethanol-precipitated DNA was dissolved in 20 mM Tris-HCl (pH 7.4) and stored at -20°. Concentrations of both kinds of DNA were determined spectrophotometrically using 1 A₂₆₀ = 50 μg/ml; the base compositions of similar and ϕX174 DNA are very similar; therefore, no corrections due to base composition were necessary. Absorbance spectra were collected with a Hewlett-Packard Model 8450 spectrophotometer.

To estimate association constants by the fluorescence-quenching method (13, 25), tetrols were prepared by hydrolysis of BPDE-I overnight in 20 mM Tris-HCl (pH 7.4). The fluorescence of a solution of tetrols (λₑₓ = 345; λₑᵦ = 400) was determined before and after sequential additions of small volumes of DNA solutions on a Farrand Mark I spectrofluorimeter using 10-nm slits. Tetrrol concentrations (40 to 120 nM) were kept low enough to ensure at least a 300-fold molar excess of DNA at all DNA concentrations. Fluorescence spectra were measured on the same machine with 2.5-nm slits. The rate of hydrolysis of BPDE-I was measured fluorometrically as described previously (18), and pseudo-first-order rate constants for hydrolysis were determined by nonlinear regression; in all cases, the experimental data points closely followed pseudo-first-order kinetics. As shown in Chart 2, for both linear and supercoiled DNA, Kₒₑᵦ increased linearly with DNA concentration up to about 40 μM. In 4 independent determinations, the supercoiled DNA was more active in catalyzing hydrolysis than was the linear DNA. The average ratio of the slopes of the linear portions of the curves was 1.21 ± 0.11 (S.D.), which represents a statistically significant difference (p < 0.025).

Covmtal Binding. By measuring the DNA concentration dependence of covalent binding, one obtains a measure of the relative overall rate constants for DNA-catalyzed hydrolysis and adduct formation (17). In the present work, we have measured constant measured spectrophotometrically (13). Typical quench curves for linear and supercoiled DNA are shown in Chart 1. The ratio F₀/F increased linearly with DNA concentration up to at least 300 μM. As determined from the slope of these lines, there was a consistent and statistically significant 40% increase (p < 0.001, t test) in the association constant for supercoiled DNA relative to linear DNA. Three independent preparations of ϕX174 DNA exhibited this behavior as did a preparation of supercoiled PM2 DNA (kindly provided by Dr. R. Naim, data not shown). However, a preparation of ϕX174 DNA which had been " nicked" during handling yielded >90% relaxed circles (RF II) as determined by gel electrophoresis was indistinguishable from salmon DNA in tetrrol association constant measurements (data not shown). A similar increase in intercalation of ethidium bromide in supercoiled DNA has been noted (11). Absorbance spectra of the DNA plus-tetrrol solutions demonstrated the 10 nm red shift, characteristic of intercalation (data not shown).

Hydrolysis Rate. Next, we determined the concentration dependence of DNA-catalyzed hydrolysis of BPDE-I by measuring the time course of the appearance of tetrrol fluorescence. The pseudo-first-order rate constants for hydrolysis, Kₒₑᵦ, were determined by nonlinear regression; in all cases, the experimental points closely followed pseudo-first-order kinetics. As shown in Chart 2, for both linear and supercoiled DNA, Kₒₑᵦ increased linearly with DNA concentration up to about 40 μM. In 4 independent determinations, the supercoiled DNA was more active in catalyzing hydrolysis than was the linear DNA. The average ratio of the slopes of the linear portions of the curves was 1.21 ± 0.11 (S.D.), which represents a statistically significant difference (p < 0.025).

Covalent Binding. By measuring the DNA concentration dependence of covalent binding, one obtains a measure of the relative overall rate constants for DNA-catalyzed hydrolysis and adduct formation (17). In the present work, we have measured

RESULTS

Association Constants. We have previously presented measurements of association constants for B(a)P-diol-epoxides obtained by quantitating the absorbance shift which accompanies adduct formation (17). In the present work, we have measured the ability of the DNA preparations to quench the fluorescence of B(a)P tetrols since only small quantities of the supercoiled DNA were readily available. An alternative, we have measured the ability of the DNA preparations to quench the fluorescence of B(a)P tetrols. The fluorescence of a solution of B(a)P tetrols (λₑₓ = 345; λₑᵦ = 400) of a solution of B(a)P tetrols was determined before (F₀) and after (F) sequential additions of linear salmon DNA (•) or supercoiled μX174-DNA (Δ). The values were corrected for dilution and F₀/F plotted versus the DNA concentration (Stem-Volmer plots); typical determinations are shown. The quenching constants as determined from the slopes of the Stern-Volmer plots were: ϕX174 DNA, Kₒₑᵦ = 2495 ± 231 liters/mol (n = 3); salmon DNA, Kₒₑᵦ = 1780 ± 221 (n = 10).
BPDE-DNA INTERACTIONS

Chart 2. Catalysis of BPDE-I hydrolysis by DNA. Hydrolysis rates were determined from the increase in fluorescence after addition of BPDE-I to aqueous solutions of linear salmon DNA (⧫) or supercoiled φX174 DNA (▲); final [BPDE-I] = 80 nM. Data points taken at 30-sec intervals were used to determine the pseudo-first-order rate constant, *k*, by nonlinear least-squares regression (6). Data from duplicate determinations are plotted and normalized to *k*, the rate constant observed in the absence of DNA. The relative abilities of supercoiled and linear DNA to catalyze BPDE-I hydrolysis are given by the slopes of the *k*/*k* versus [DNA] plots; the experimentally determined ratio (supercoiled/linear) was 1.22 ± 0.11 (n = 4).

Chart 3. Covalent binding of BPDE-I to DNA. [3H]-BPDE-I (470 Ci/mol) was added to aliquots of linear salmon DNA (⧫) or to supercoiled φX174 DNA (▲) to a final concentration of 3.24 μM and allowed to react to completion (>2 hr) at room temperature. Noncovalently bound hydrolysis products were removed by extraction 4 times with 2.5 volumes of ethyl acetate and then twice with 2.5 volumes of ether. Radioactivity bound covalently to the DNA was determined by liquid scintillation counting of the aqueous phase. Control experiments showed that nonspecific binding of B(a)P tetrols to DNA under these conditions was negligible (<0.5% of input). Duplicate determinations are shown. In one experiment, calf thymus DNA was substituted for salmon DNA with similar results. The data points were fitted to the theoretical equation (17)

\[ f_a = \frac{D_0}{k_d/k_e + (1 + k_d/k_e) D_0} \]

by nonlinear least-squares regression, where *f* is the fraction of added diol-epoxide which binds to DNA, *D* is the DNA concentration, *k* is the rate constant for DNA-catalyzed hydrolysis, and *k* is the rate constant for adduct formation.

The fraction of added diol-epoxide which is bound covalently to DNA at equilibrium using [3H]BPDE-I. As shown in Chart 3, the maximal level of binding to linear DNA was slightly higher than was the binding to supercoiled DNA. When the experimental data from 6 independent determinations were fitted to the theoretical equation (see Chart 3, legend), giving the ratio of the rate constants for hydrolysis and adduct formation (*k*/*k*), this ratio was consistently higher for supercoiled DNA than for linear DNA by a factor of 1.21 ± 0.05 (p < 0.005). Since we found above that supercoiled DNA gave a 20% increased hydrolysis rate, this finding suggests that the intrinsic rate of formation of DNA adducts is essentially the same for supercoiled and linear DNAs.

Previous work has demonstrated that BPDE-I adducts in native DNA do not show a major red shift in their absorbance or fluorescence excitation spectra. However, some BPDE-II (28) and the major BPDE-III adducts (17) do show such a shift (8 to 10 nm), consistent with their existence in an intercalated conformation. Fluorescence quenching measurements support the idea that BPDE-III adducts remain intercalated (17). We have measured fluorescence excitation spectra of supercoiled DNA containing BPDE-I adducts (Chart 4). The major excitation peak occurs at 346 nm, not at the longer wavelengths (about 352 nm) characteristic of intercalated molecules. The spectra are identical to those obtained with linear DNA and provide no support for the idea that supercoiled DNA-BPDE-I adducts exist in an altered conformation.

We have also measured the ability of acrylamide to quench...
DNA-Adduct Analysis. To look for possible differences between the adducts formed with supercoiled and linear DNAs, we prepared adducts from DNA treated with BPDE-I (Chart 6, A and C) or with BPDE-II (Chart 6, B and D) and analyzed them by high-performance liquid chromatography. With supercoiled DNA, BPDE-I formed 4 adducts (Chart 6A, Peaks 1 to 4). Adduct 2 was cochromatographic with the major adduct formed in the reaction of (+)-BPDE-I with dGMP; this is the major adduct found in a number of intact cell systems (1, 4, 14, 16). By comparison with our standards, we can suggest that Adduct 1 is also a dGuo adduct and that Adducts 3 and 4 are derived from dAdo. Adducts 1, 2, 3, and 4 were also found in BPDE-I reactions with linear DNA (Chart 6C), and quantitation of the radioactivity in each peak (Table 1) revealed that the distribution of radioactivity among these peaks was essentially the same for supercoiled DNA. Adducts 5 and 6 were also found in BPDE-II reactions with linear DNA (Chart 6D), and quantitation of the radioactivity in each peak revealed that the distribution of radioactivity among these peaks was essentially the same for supercoiled DNA.

Table 1

<table>
<thead>
<tr>
<th>% of total adducts found in</th>
<th>Linear DNA</th>
<th>Supercoiled DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPDE-I adducts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>62 ± 0.6₇</td>
<td>66 ± 0.4₇</td>
</tr>
<tr>
<td>2</td>
<td>87.5 ± 1.1</td>
<td>79.2 ± 4.5</td>
</tr>
<tr>
<td>3</td>
<td>0.7 ± 0.3</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>2.2 ± 0.7</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>BPDE-II adducts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>25.1 ± 1.4</td>
<td>22.4 ± 3.3₇</td>
</tr>
<tr>
<td>6</td>
<td>47.6 ± 2.4</td>
<td>47.7 ± 5.9</td>
</tr>
<tr>
<td>7</td>
<td>4.1 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>8</td>
<td>3.9 ± 0.1</td>
<td>3.4 ± 0.5</td>
</tr>
</tbody>
</table>

₇ Mean ± S.D. (n = 3).
₈ Mean ± range (n = 2).
and linear DNAs. Although large differences in minor (<1% of total) adducts could have escaped detection in this analysis, the 4 major adducts quantitated represent at least 90% of the total adduct formation.

Reaction of BPDE-II with φX174 DNA led to a larger number of adduct peaks (Chart 6B), but again a similar distribution was seen with salmon DNA (Chart 6D). By comparison with standards, we could tentatively identify Peaks 5 and 6 as dGuo adducts and Peaks 7 and 8 as dAdo adducts. Quantitation of these peaks (Table 1) again revealed no significant differences between linear and supercoiled DNA.

DISCUSSION

We have observed small differences in the interaction of BPDE-I with supercoiled φX174 DNA as compared to linear DNA. The maximum observed difference was a 40% increase in the association constant for supercoiled DNA. The measured ratio of rate constants

\[ R = \frac{K_d (\text{supercoiled DNA})}{K_d (\text{linear DNA})} \]

is related to the unwinding angle, φ, of the intercalated molecules by the equation

\[ R = \exp(-A\phi/kT) \]

where A is the torsional free energy change per degree of unwinding (22); A is linearly related to the superhelical density. To estimate φ for the noncovalent intercalation of B(a)P tetrols, we adjusted the value of A/kT given by Gamper et al. [−0.0088 deg⁻¹ (8)] to reflect the higher superhelical density (in units of number of supercoils/10 base pairs) of φX174 DNA [σ = −0.0571 at 20° in low salt (3)] compared to the measured superhelical density of the SV40 DNA preparations of Gamper et al. [21 supercoils in 5400 base pairs; σ = −0.0389] giving A/kT = −0.0129 deg⁻¹. When combined with the measured value of 1.4 for R, this gives an unwinding angle of about 26 degrees, the same as that of ethidium bromide [26 degrees (29)]. Theoretical calculations of Taylor et al. (27) suggest a minimum base-pair separation for a noncovalent BPDE-I intercalation site of 7.86 Å, corresponding to an unwinding angle of 28 degrees, similar to that found for the tetrol intercalation site.

The ability of supercoiled DNA to catalyze BPDE-I hydrolysis is slightly higher than that of linear DNA; we do not have a good explanation for this phenomenon at present, although it may be related to differences in charge density for the 2 classes of DNA molecule. Covalent binding at high DNA concentration is lower for supercoiled DNA by approximately the same factor (20%). Since this parameter is directly proportional to the ratio of the intrinsic rate constant for covalent binding to the rate constant for DNA-catalyzed hydrolysis, the implication is that the intrinsic rate constants are equal. Previous workers (8) found a slight increase in covalent binding to supercoiled DNA using a single (unspecified) DNA concentration. Since the level of covalent binding at saturation is insensitive to slight differences in DNA concentration, the approach used here should yield more reliable data. Since our previous studies have suggested that covalent binding but not hydrolysis is dependent on intercalation,5 the equality of covalent binding rate constants for supercoiled and linear DNAs implies that the geometries of the intercalation complexes are very similar. This is supported by our finding that the profiles of adducts formed with the 2 DNAs by BPDE-I and BPDE-II are essentially identical (Chart 5). Changes in the geometry of intercalation would be expected to alter the distribution of adducts formed.

The spectroscopic evidence that we have presented does not suggest that the BPDE-I adducts remain intercalated in the supercooled DNA to any greater extent than in linear DNA molecules. Consistent with previous studies (5, 8), the adducts formed do cause an apparent relaxation of the supercoiled DNA molecules7 as monitored by gel electrophoresis. Thus, the discrepancy between the spectroscopic and electrophoretic evidence remains and is not explainable by differences between the adducts formed with supercoiled and linear DNAs. Hogan et al. (12) postulated a wedge-shaped (+)-BPDE-I-dGuo adduct conformation which they suggested was compatible with both lines of evidence. In a recent theoretical study, Taylor et al. (27) have derived coordinates for a similar binding site which produces a kink in the DNA. A plausible reaction pathway which leads from a classical intercalation site to the kinked site was also presented by Taylor et al. (27). The DNA kinks at the adduct sites were postulated to produce the observed "relaxation" of the molecules as assayed by gel electrophoresis. The present work lends support to the proposal of Taylor et al., although other conformations are not excluded.

ACKNOWLEDGMENTS

We thank K. Zachary and R. Doisy for expert technical assistance and P. Mutschink, J. Ing, and J. Riley for their help in preparing the manuscript. We thank Ken Miller and Pierre LeBreton for communicating results prior to publication. Some of the experiments reported herein were performed at the Center for Fast Kinetics Research at the University of Texas at Austin. The Center is supported jointly by the Biotechnology Branch of the Division of Research Resources of NIH (RR-00886) and by the University of Texas at Austin.

REFERENCES


M-S. Tang and M. C. MacLeod, manuscript in preparation.
17. MacLeod, M. C., Mansfield, B. K., and Selkirk, J. K. Covalent binding of isomeric benzo(a)pyrene diol-epoxides to DNA. Carcinogenesis (Lond.), 3: 1031–1037, 1982.
Interactions of Benzo(a)pyrene Diol-Epoxides with Linear and Supercoiled DNA

Michael C. MacLeod and Moon-shong Tang