Structure of a High Fidelity DNA Polymerase Bound to a Benzo[a]pyrene Adduct That Blocks Replication*

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Of the carcinogens to which humans are most frequently exposed, the polycyclic aromatic hydrocarbon benzo[a]pyrene (BP) is one of the most ubiquitous. BP is a bp product of grilled foods and tobacco and fuel combustion and has long been linked to various human cancers, particularly lung and skin. BP is metabolized to diol epoxides that covalently modify DNA bases to form bulky adducts that block DNA synthesis by replicative or high fidelity DNA polymerases. Here we present the structure of a high fidelity polymerase from a thermolabile strain of Bacillus stearothermophilus (Bacillus fragment) bound to the most common BP-derived N2-guanine adduct base-paired with cytosine. The BP adduct adopts a conformation that places the polycyclic BP moiety in the nascent DNA minor groove and is the first structure of a minor groove adduct bound to a polymerase. Orientation of the BP moiety into the nascent DNA minor groove results in extensive disruption to the interactions between the added DNA duplex and the polymerase. The disruptions revealed by the structure of Bacillus fragment bound to a BP adduct provide a molecular basis for rationalizing the potent blocking effect on replication exerted by BP adducts.

DNA replication is compromised by bulky DNA adducts that derive from the covalent modification of DNA by carcinogenic and mutagenic compounds. One of the most prevalent carcinogens that yield covalent DNA adducts are some of the diol epoxide metabolites of the environmentally ubiquitous compound benzo[a]pyrene (BP). In mammalian cells, BP is metabolically activated by cytochrome P450 and epoxide hydrolase enzymes (for review, see Ref. 1) in the liver to the major tumorogenic (2, 3) and mutagenic (4–6) metabolite, the (+)-7R,8S,9S,10R enantiomer of the diol epoxide r7,8-dihydroxy-t9,10-epoxy-7,8,9,10-dihydroxybenzo[a]pyrene ((+)-anti-BPDE). This and other stereoisomeric diol epoxides can attack guanine or adenine in DNA predominantly at the exocyclic N2 or N6 amino groups, respectively, by either trans or cis addition at the C10 position of BPDE, yielding a mixture of adducts (7, 8). The dominant adduct formed in vivo from (+)-anti-BPDE is the (+)-trans-anti-BPDE-N2-dg ([BP]dG) adduct (see Fig. 1A) (9, 10). Although bypass of [BP]dG adducts has been shown for certain polymerases of the Y-family of DNA polymerases in vitro (11–15), DNA replication by high fidelity polymerases is generally blocked by [BP]dG with only very low levels of full bypass reported (16–22).

Structural studies of DNA polymerase complexes (for review, see Refs. 23–27) in combination with extensive enzyme kinetic studies (Refs. 28–32; for review, see Ref. 33) have revealed the dominant mechanistic and structural features that contribute to accurate DNA replication, which are shared in large part by all polymerases. These features are well represented by our model system, the thermophilic Bacillus stearothermophilus DNA polymerase I large fragment (BF), that is capable of catalyzing DNA replication in a crystal. Using this system, we have previously studied replication of undamaged DNA (34, 35), damaged DNA (36, 37), and DNA mismatches (38) revealing mechanisms of replication and structural features that are not compatible with binding and replication of a bulky DNA adduct like [BP]dG. During replication, steric and geometric constraints are imposed on the template base as it moves from a pre-insertion site (see schematic, Fig. 2), where the template base is sequestered before pairing opposite an incoming dNTP, to the insertion site, where base pairs that exhibit the shape and geometry of correct Watson-Crick base pairs are selected in favor of base pairs that do not (39, 40). The steric and geometric constraints at the pre-insertion and insertion sites contribute to the accuracy of replication achieved by high fidelity polymerases and would not be expected to accommodate [BP]dG without significant distortion of the polymerase active site. After nucleotide incorporation at the insertion site, the newly formed base pair translocates to the post-insertion site. Here and throughout the DNA duplex binding region, the polymerase engages in extensive interactions with the DNA minor groove, whereas the DNA major groove is predominantly solvent-exposed. Interactions between the DNA duplex and the polymerase are inevitably compromised by [BP]dG, a minor groove adduct.

Structures of [BP]dG adducts at single strand double strand junctions in template-primer complexes in aqueous solution have been determined by NMR methods. These structures re-
veal that [BP]dG can adopt either a syn conformation (41) or an anti conformation (42) depending on its position in the duplex and whether it pairs with an opposing base. Because these structures are determined in the absence of protein, they do not address [BP]dG conformation in the context of the polymerase active site. Recently, structures of DNA polymerases bound to the bulky major-groove DNA adducts (+)-cis-[BP]-adename (43), a less prevalent product of BPDE attack on DNA, and aminofluorene (37) have been reported. However, the structure of a DNA polymerase bound to a bulky minor-groove DNA adduct has yet to be determined.

Here we report a high resolution structure of the minor groove (+)-trans-[BP]dG adduct, the major product of the reaction of (+)-anti-BP with cellular DNA at the BF active site. The [BP]dG adduct is observed in an anti conformation at the post-insertion site, where it forms Watson-Crick base pairs with an opposing cytosine. The polycyclic aromatic BP ring system protrudes into the nascent minor groove and induces distortions to the polymerase and both strands of the DNA duplex. Such distortion has not previously been observed in structures of DNA mismatches or lesions at the polymerase active site. The extensive nature of these distortions accounts for the blocking effect of [BP]dG on DNA replication.

EXPERIMENTAL PROCEDURES

Preparation of BP-modified DNA Duplexes—A Biosearch Cyclone automated DNA synthesizer was used to synthesize the oligonucleotides for this study. The sequence 5’-d(ACCTGACCATCCCT) phosphorylated at the 3’-end was synthesized by automated methods using standard phosphoramidite derivatives of 2’-deoxynucleotides and 3’-phosphate controlled-pore glass supports. The direct synthesis approach (44, 45) that was initially used to generate the structural features of the (+)-trans-anti-BP-N2-dG adduct in an oligonucleotide duplex by NMR methods (46) was used here to prepare modified sequence 5’-d(ACCT-[BP]-dG-CACCATCCCT). The oligonucleotide was dissolved in 3.2 ml of 0.05M triethylamine acetate, 0.3M NaCl, pH 10, buffer solution and treated with 0.02M BPDE tetrahydrofuran solution (initial molar ratio of [BP]dG/oligonucleotide) was 2:1) overnight at room temperature and neutralized to pH 7.0 by the addition of 20 mM sodium phosphate. Modified and unmodified oligonucleotides were separated by reverse-phase HPLC on a 4.6 × 250-mm C18 Microsorb-MV-59 column and a 13–25% 1:1 acetonitrile: methanol/triethylamine acetate (20 mM, pH 7.0) gradient. The modified oligonucleotides eluted in the order (10R)-trans, 10S)-cis, 10S)-trans, 10R)-cis, and (10S)-trans-anti-BP-N2-dG as described (45). The oligonucleotide containing the single (+)-trans-anti-BP-N2-dG adduct was purified by repeated HPLC cycles with similar protocols. The stereochemical properties of the modified oligonucleotides containing the (+)-trans adduct were verified as described (7, 44).

Primer Extension Assays—In vitro primer extension reactions were carried out with 43-mer template strands where the (+)-trans-anti-BP-N2-dG lesions are positioned at the 25th nucleotide (counting from the 3’-side) as described (14). The 43-mer templates were annealed with a 32P-labeled 19-mer primer (Fig. 1B). A time course of primer extension assays of the adducted templates with BF was determined at 37 °C in 10 μl of buffered solutions containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol, 50 μM bovine serum albumin, 4% glycerol, 4.5 mM primer-template complexes, 100 mM dNTPs, and 20 μM of the polymerase. Corresponding unmodified primer/template complexes were extended as controls. The reaction conditions for the unmodified DNA substrates were the same as those for the modified templates, except that the enzyme concentration was 0.025 mM. The reactions were stopped after preselected time intervals by the addition of a 7 μl of 0.1M solution (20 mM EDTA, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). Characterization of dNTP incorporation preference opposite [BP]dG was performed using DNA duplexes identical to those used for crystallization. Corresponding unmodified primer-template complexes were extended as controls. The 15-mer BP-modified and unmodified templates were annealed with a 32P-labeled 9-mer primer (Fig. 1C). Reaction conditions were identical to those described above for extension of the 19-mer primer except that 5 mM dCTP, dTTP, dATP, or dATP and 10 mM BF (for BP-modified duplex) or 200 μM dNTP and 0.1 mM BF (for unmodified duplex) and 100 mM DNA duplex (both BP-modified and unmodified) were used. Reactions were performed at 25 °C to prevent the duplex from unannealing and allowed to proceed for 10 min before quenching with the stop solution. All reactions were heated to 90 °C (5 min) and chilled on ice (1 min). Reaction products were separated by gel electrophoresis (20% denaturing polyacrylamide gel containing 7 mM urea), visualized by autoradiography, and quantitated by a Storm 840 PhosphorImager using Storm ImageQuant software.

RESULTS

[BP]dG Blocks DNA Replication by BF in Solution—Primer extension assays using unmodified and BP-modified oligonucleotides as templates demonstrate the blocking potential of the [BP]dG adduct on replication by BF as shown in Fig. 1. Replication of the unmodified DNA templates is highly efficient at low enzyme concentration (0.025 mM) with full extension products observed within 2 min of reaction initiation. By contrast, replication of BP-modified templates is blocked either before (position 24, Fig. 1B) or after base incorporation opposite [BP]dG (position 25) despite a ~1000-fold higher enzyme concentration (20 mM). At this enzyme concentration, full primer extension does not exceed a few percent even after an incubation time of 30 min (Fig. 1B, right-hand panel). Insertion of dNTP opposite [BP]dG (primer-template) at the post-insertion site (BP2, PDB entry 1IXC) with the structure of BF bound to unmodified duplex DNA and an incoming dNTP (PDB entry 1LV5). All superpositions were done using the Ca atoms of BF residues 646–655, 823–838, and 863–869.

Structures of [BP]dG at the Pre-insertion and Post-insertion Sites—A co-crystal structure of BF bound to a DNA duplex where [BP]dG is positioned as the n template base was determined to 1.9 Å of resolution (complex BP1, Table I). This structure represents BF before nucleotide incorporation oppo-
BPdG blocks DNA replication by BF in primer extension assays. A, chemical structure of BPdG. B, extension of primers (22-mer) annealed to templates that contain unmodified guanine or BP-modified templates (15-mer) with each of the four dNTPs. Incorporation opposite BPdG is detected only with dCTP. Despite high BF concentration (10 nM), high dNTP concentration (5 mM), and long reaction time (10 min.), full incorporation is not observed (only ~11% of primers are extended under these conditions).

Table I

| Crystal   | BP1 | BP2 |
|-----------|-----|-----|
| Position of lesion | Pre-IS | Post-IS |
| Wavelength, Å | 1.000\(^a\) | 1.000\(^a\) |
| Resolution range, Å | 50–1.9 | 50–1.9 |
| Outer shell | 1.94–1.9 | 1.94–1.9 |
| \(R_{cryst}\), % | 4.8 (16.9) | 4.2 (18.5) |
| Unique reflections | 70,445 | 71,966 |
| Total reflections | 1,345,500 | 1,665,410 |
| Mean \(I/\sigma_I\), | 22.5 (3.5) | 20.5 (2.9) |
| Completeness, % | 98.9 (87.6) | 96.8 (73.4) |
| \(R_{cryst}\), % | 22.1 | 22.1 |
| \(R_{free}\), % | 24.6 | 25.5 |
| r.m.s. deviation bond length, Å | 0.006 | 0.006 |
| r.m.s. deviation bond angle, ° | 1.2 | 1.1 |
| Average B-value, Å\(^2\) | 25.3 | 35.1 |
| \(\sigma\) A coordinate error, Å\(^2\) | 0.10 | 0.22 |

\(^a\) Data were collected at beamline X12B, National Synchrotron Light Source. \(^b\) Values in parentheses correspond to those in the outer resolution shell.

The co-crystal structure of BF bound to C:[BP]dG (primer-template) at the post-insertion site (Fig. 2) reveals extensive distortions to both the DNA duplex and the polymerase. Distortions to the DNA duplex extend from the n-1 (post-insertion site) to the n-4 position (the DNA duplex region) (Fig. 3B). At each of these positions, Watson-Crick hydrogen bonds between the bases are retained, but the base pair parameters are distorted (Table II). At the n-1 position, [BP]dG adopts an anti conformation \(\gamma = 269°, \delta = 277°\), \(\alpha' = 196°\), \(\beta' = 278°\) (42). In the context of BF,
[BP]dG forces polymerase side chains to adopt alternate conformations such that the interactions between the protein and the C:[BP]dG base pair are lost (Fig. 3C). Rather than form direct hydrogen bonds with the minor groove of the newly formed base pair, protein residues Arg-615 and Gln-797 are confined to the floor of the active site, far removed from the primer and template strands, which lift away from the surface of the polymerase. This displacement of the primer strand results in loss of coordination between the primer 3'-OH and the universally conserved catalytic Asp-830. The hydrogen bond distance between the primer 3'-OH and Asp-830 is normally 2.5 Å; in the BP2 complex, this distance is 6.9 Å. The 3'-OH is, therefore, no longer in position for in-line attack during nucleotide addition.

The BP Moiety Prevents Binding of the Incoming dNTP—To assess the effect of [BP]dG on binding of an incoming dNTP, the structure of BF bound to C:[BP]dG at the post-insertion site was superimposed with the structure of BF bound to duplex DNA and an incoming dNTP (PDB entry 1LV5) (Fig. 3D). In this modeled structure, the adduct moiety overlaps with the bound dNTP and is, therefore, sterically incompatible with extension beyond the adducted base without significant structural distortion to the polymerase or DNA. With binding of the incoming dNTP prevented by the presence of the BP adduct, replication is, therefore, effectively blocked.

**DISCUSSION**

Structures of [BP]dG at the BF polymerase active site that represent the adducted base before and after incorporation of dCTP reveal structural distortions that provide a structural rationale for the block to replication exerted by [BP]dG. [BP]dG induces distortions that promote stalling of the polymerase at either the pre-insertion or post-insertion site and dissociation of the polymerase from the duplex, thereby promoting subsequent excision or translesion synthesis by repair polymerases.

Upon encountering [BP]dG as the n template base, BF pauses, and replication is stalled (as shown by primer extension assays, Fig. 1). During replication of unmodified DNA, the n template base transitions from the pre-insertion site to the insertion site, where it pairs opposite the incoming dNTP. Because of the bulk of the BP moiety, [BP]dG cannot occupy the pre-insertion site and is, therefore, not poised for nucleotide incorporation even though the polymerase active site is not disrupted. The bulk of the BP moiety and the limited conformational degrees of freedom of [BP]dG relative to unmodified guanine may also impair the transition from pre-insertion site to insertion site, which involves a 90° bend in the template strand. Nucleotide incorporation opposite [BP]dG is, therefore, significantly impaired.

Although limited nucleotide incorporation opposite [BP]dG is possible, extension past [BP]dG is essentially not observed.
This inability to extend beyond the lesion in the template strand can be rationalized by the observation that [BP]dG adopts an anti conformation at the BF post-insertion site, where the BP residue positioned in the nascent minor groove points toward the 5'-end of the template strand. [BP]dG also adopts anti conformations in structures of BP-modified DNA duplexes in the absence of protein as determined by NMR (42, 56). However, comparison of these NMR structures with the present structure reveals significant differences. One component of these differences arises from distortion to the DNA duplex observed when the duplex is bound to polymerase. Deviation of the polymerase-bound DNA duplex from normal B-form DNA is observed even in the absence of [BP]dG as the duplex is partially unwound to an A-form-like conformation as

**TABLE II**

**DNA base pair parameters for BP-modified DNA duplex**

| Base pair | $d_{C1-C1}$ | $\lambda_{\text{primer}}$ | $\lambda_{\text{template}}$ | Shear | Stretch | Stagger | Buckle | Propeller | Opening |
|-----------|-------------|----------------|----------------|-------|---------|---------|--------|-----------|---------|
|           | Å           | degrees        | degrees        | Å     | degrees  | Å       | Å      | Å         | Å       |
| C:[BP]dG (n - 1) | 10.2 (10.3) | 61.3 (59.7) | 56.6 (57.9) | 0.48 (0.26) | -0.09 (0.02) | -0.66 (0.53) | 31.85 (29.53) | -15.75 (-8.45) | 3.81 (4.40) |
| G:C (n - 2) | 10.5 (10.2) | 55.6 (55.3) | 65.8 (59.8) | -0.71 (0.01) | 0.28 (-0.02) | 0.89 (0.25) | 18.74 (20.76) | -2.75 (-8.72) | 11.08 (7.15) |
| T:A (n - 3) | 10.3 (10.5) | 57.9 (56.4) | 63.7 (55.8) | -0.33 (0.08) | 0.04 (-0.10) | 0.49 (-0.17) | 8.71 (17.03) | -21.06 (-15.50) | 9.43 (0.03) |
| G:C (n - 4) | 10.5 (10.7) | 58.3 (60.0) | 58.6 (54.4) | -0.25 (-0.12) | 0.07 (-0.07) | 0.56 (0.01) | 5.45 (6.51) | -6.68 (8.45) | 7.55 (1.12) |

| Base pair | Shift | Slide | Rise | Tilt | Roll | Twist |
|-----------|-------|-------|------|------|------|-------|
|           | Å     | Å     | Å    | Å    | Å    | Å     |
| C:[BP]dG (n - 1) | -0.54 (-0.19) | 0.50 (-1.05) | 3.29 (3.23) | 14.44 (3.69) | 17.60 (10.09) | 24.94 (30.84) |
| G:C (n - 2) | 0.63 (-0.26) | 0.19 (-1.47) | 3.07 (3.31) | 0.11 (-5.74) | 3.31 (11.07) | 32.47 (26.24) |
| T:A (n - 3) | -0.77 (-0.17) | -0.42 (-0.47) | 3.13 (3.16) | -2.40 (-2.40) | 3.16 (7.13) | 32.02 (35.59) |
| G:C (n - 4) | -0.48 (0.30) | -0.71 (-0.16) | 3.33 (3.07) | -4.67 (1.36) | 6.83 (6.88) | 30.79 (28.86) |

*Values for cognate base pairs are shown in parentheses.*
it enters the polymerase active site (47). This underwound conformation is stabilized by a network of interactions between the polymerase and both the DNA minor groove and the sugar-phosphate backbone. Although many of these interactions are retained in the presence of [BP]dG, the adduct induces distortions to the duplex beyond what is typically observed for unmodified DNA duplexes bound to BF. A second component of the differences between NMR structures and the present one is the constrained environment of the polymerase, which in addition to the internal steric constraints imposed by the BP moiety itself, results in differences to the local conformation of [BP]dG.

By adopting the anti conformation, [BP]dG blocks replication in obvious ways. [BP]dG induces further distortions to the polymerase and disrupts all of the interactions between the minor groove of the n-1 base pair. Protein side chains that normally read the DNA minor groove of correctly formed Watson-Crick base pairs retreat to the floor of the active site. Normally read the DNA minor groove of correctly formed DNA polymerases bound to BF, a second component of the lesion. By contrast, the error-prone Y family polymerases are more open and capable of accommodating DNA lesions that block further DNA synthesis even in the rare instance that incorporation opposite [BP]dG does occur. Loss of protein-DNA interactions and steric block to dNTP incorporation at the insertion site reveal how steric constraints imposed by the active site of a high fidelity polymerase present an architecture that is clearly incompatible with DNA replication of certain types of damaged DNA.

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Structure of a High Fidelity DNA Polymerase Bound to a Benzo[a]pyrene Adduct That Blocks Replication
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