Effects of Benzo[*a*]pyrene DNA Adducts on *Escherichia coli* DNA Polymerase I (Klenow fragment) Primer−Template Interactions: Evidence for Inhibition of the Catalytically Active Ternary Complex Formation†

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ABSTRACT: Benzo[*a*]pyrene diol epoxide (B[*a*]PDE) adducts are strong blocks of DNA replication in vitro, allowing the rare incorporation of a nucleotide across from the lesion and negligibly small extent of further bypass. To study the mechanistic details of this process, a gel-retardation assay was used to measure the dissociation constants for the binding of DNA polymerase I (Klenow fragment) (KF) to the primer-templates containing a (+)-*trans*- or (+)-*cis*-B[*a*]P-N^2-dG adduct. When the primer was terminated one nucleotide before the adduct, the presence of a (+)-*trans*-B[*a*]P-N^2-dG adduct did not affect the binding while a (+)-*cis*-B[*a*]P-N^2-dG adduct caused a slight decrease in affinity. The presence of any dNTP decreased the affinity of KF to the modified primer-templates. (In contrast, a strong increase of the affinity to unmodified primer-templates was observed in the presence of the next correct dNTP.) Limited protease digestion experiments indicated that a closed ternary complex of KF with the modified primer-templates was not detectable in the presence of any dNTP, whereas it was clearly observed with unmodified template in the presence of the next correct nucleotide. These findings suggest that these adducts may interfere with the conformational change to the catalytically active closed ternary complex and/or cause significant destabilization of this complex. When the primers extended to the position across from the adduct, the affinity of KF was significantly decreased irrespective of the identity of the base across from the adduct, possibly explaining the low bypass of the lesion. Interestingly, the stability of these DNA-polymerase complexes correlated with nucleotide insertion kinetics for the unmodified and (+)-*trans*-B[*a*]PDE-modified templates.

Several lines of evidence, including pre-steady-state kinetics (*1*−*5*), fluorescence (*5*, *6*), and crystallographic (*7*−*9*) analysis, suggest that the remarkable fidelity displayed by DNA polymerases results from discrimination between the correct and incorrect nucleotide during a number of discrete steps of nucleotide incorporation. For example, these studies have shown that most polymerases bind the next correct nucleotide much more strongly than an incorrect one when the polymerase is complexed with a primer-template in the "open" state. Interestingly, *Escherichia coli* DNA polymerase I binds both complementary and noncomplementary dNTPs approximately equally, indicating that for this enzyme much of the discrimination must occur at a later step (*10*). It has also been shown that the presence of a correctly pairing dNTP induces a conformational change from this open state to a closed complex, a process that is necessary to allow the successful completion of the chemical step (reviewed in ref *9*). In this closed conformation, the correctly paired incoming dNTP is positioned in the proper orientation that allows the formation of the phosphodiester bond. Although a crystal structure of the closed catalytically active ternary complex of DNA polymerase I is not yet available, this conformational change from a binary open to a ternary closed complex was recently detected in the presence of the next correct dNTP using a limited protease digestion method (*11*). Other indirect evidence for this dNTP-induced conformational change was obtained from binding studies that showed that the presence of a correctly paired dNTP substantially increases the strength of DNA polymerase I (Klenow fragment) (KF)[1] binding to the primer-template compared with that occurring in the presence of an incorrect dNTP or in the absence of a dNTP (*12*). A similar result was obtained with HIV-1 RT, which formed a stable complex with the primer-template only when a base-paired dNTP was present (*13*).

The mutagenic effects of bulky adducts, such as those induced upon exposure to the carcinogen benzo[*a*]pyrene (B[*a*]P), have been extensively studied (*14*, *15*). Prior to binding with DNA, B[*a*]P must be metabolically activated to a diol epoxide form that subsequently can react with the N2 position of guanine to form four sterioisomers as the major products. These adducts are very strong blocks to the replication in vitro (*16*−*19*), and the incorporation of a

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[1] Abbreviations: KF, Klenow fragment; W/C, Watson−Crick; B[*a*]P, benzo[*a*]pyrene; *anti*-B[*a*]PDE, r7,t8-dihydroxy-t9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (+)-*anti*-B[*a*]PDE, 7(R),8(S),9(S),10(R) absolute configuration; (−)-*anti*-B[*a*]PDE, 7(S),8(R),9(R),10(S) absolute configuration; B[*a*]P-N^2-dG, benzo[*a*]pyrene adduct at N-2 position of guanine; AAF, *N*-acetyl-2-aminofluorene; AF, *N*-2-aminofluorene; dNTP, 2′-deoxynucleotide triphosphate; K_d, dissociation constant.

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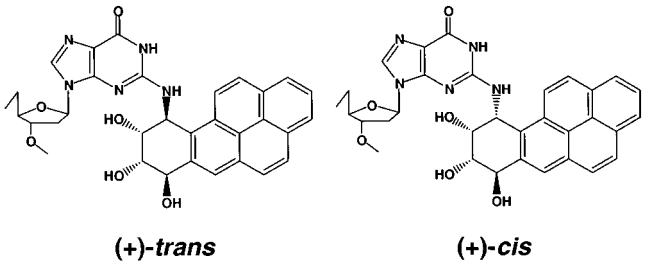

FIGURE 1: (A) Structures of the (+)-*anti*-B[*a*]P-N^2-dG adducts.

nucleotide across from these lesions has been shown to be slow, with purines being the preferred bases incorporated (*16*−*18*, *20*, *21*). Bypass appears to occur more readily in cellular systems and results in significant levels of deletions, insertions, and base substitution mutations. However, in most cases replication at the adduct site is not mutagenic, presumably because of the polymerase proofreading activity, the involvement of other replication proteins, and error-free DNA repair (*22*−*25*).

Prior studies have attempted to determine which step or steps of DNA synthesis is affected by the presence of a DNA adduct on the template strand. Using a gel shift analysis to measure DNA binding (*12*) and tryptic digestion studies (*26*) to detect the polymerase conformational change, we showed that the DNA helix distorting adduct *N*-acetyl-2-aminofluorene (AAF) completely inhibited the formation of a stable ternary closed complex while the less distorting *N*-2-aminofluorene adduct (AF) did not. These effects correlated with the ability of DNA polymerase I to bypass these adducts since it is well established that the AAF adduct is a strong block of DNA synthesis while the AF adduct is easily bypassed (*27*, *28*).

In the present study, we have extended these experiments to primer-template junctions containing (+)-*anti*-B[*a*]PDE adducts. Equilibrium dissociation constants (K_ds) were determined for primer template complexes containing either a (+)-*trans*-B[*a*]P-N^2-dG or (+)-*cis*-B[*a*]P-N^2-dG adduct (Figure 1) in which the primer terminated either before or across from the adduct. In the former case, we found that binding to the primer-templates in the absence of a dNTP is not affected by the presence of a (+)-*trans*-B[*a*]P-N^2-dG adduct in the active site, and is only slightly decreased by the (+)-*cis*-B[*a*]P-N^2-dG adduct in comparison with the unmodified primer-templates. The addition of any of the four dNTPs to the incubation mixture decreased the binding to the adducted template, whereas with an unmodified template the addition of the next correct dNTP significantly increased the binding strength. The tryptic digestion pattern of KF bound to the modified primer-templates was independent of the presence of a dNTP. Taken together, these results suggest that the B[*a*]PDE adducts are affecting the conformational change that leads to the formation of a stable ternary complex. Finally, when the primer extended to the position across from the adduct there was a substantial reduction in the stability of the polymerase complex, a factor that may contribute to the inability of this polymerase to synthesize past these adducts.

MATERIALS AND METHODS

Materials. Klenow fragment of *E. coli* DNA Polymerase I (exonuclease deficient) was purchased from Amersham

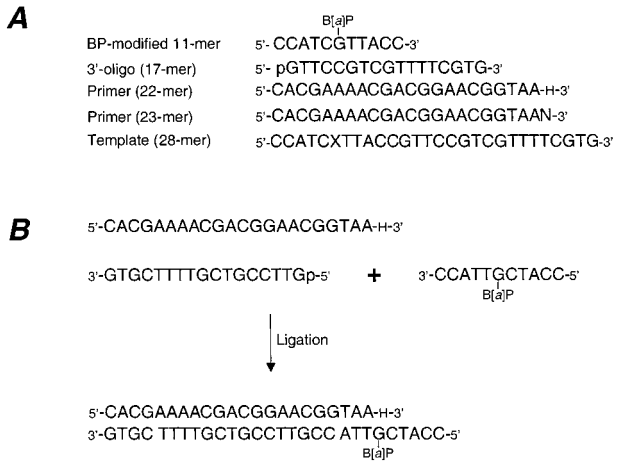

FIGURE 2: (A) Sequences of the oligonucleotides used in this study. The 22-mer and the 23-mers (N stands for one of the four possible nucleotides) were used as primers in the primer extension and binding studies with the 28-mer templates (X stands for either unmodified guanine or (+)-*cis*- or (+)-*trans*-B[*a*]P-N^2-dG). (B) To obtain the 28-mer templates the modified and unmodified 11-mers were ligated with the phosphorylated 17-mer using a complementary 22-mer as a scaffold as described in Materials and Methods.

Pharmacia Biotech. The protein had been over-produced and purified from a strain carrying a double mutation D355A, E357A which results in about 10^5 fold reduction of endogenous 3′-5′ exonuclease activity (*29*). T4 polynucleotide kinase and T4 DNA ligase were also purchased from Amersham Pharmacia Biotech. Trypsin and terminal deoxynucleotide transferase were from Roche Molecular Biochemicals.

Oligonucleotides were obtained from Midland Certified Inc. dNTPs were purchased from Promega. [γ-^{32}P]ATP was from ICN Biomedicals. The racemic (±)-*anti*-B[*a*]PDE was purchased from National Cancer Institute Chemical Reference Standard Repository (Kansas City, MO). All other general reagents and chemicals were obtained from Fisher and VWR.

Synthesis and Purification of the Unmodified and B[*a*]PDE-*Modified Oligonucleotides.* The sequences of oligonucleotides that were used in this study are shown in Figure 2A. All oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis as described (*30*) and desalted with centricon-3 microconcentrators (Amicon) according to the manufacturer protocol. The 22-mer primer lacking 3′-OH was obtained by extension of the corresponding 21-mer with ddAMP using terminal deoxynucleotide transferase. The primer (4.2 mM) was incubated with ddATP (0.1 mM) in 100 mM cacodylate buffer, pH 6.8, containing 1 mM $CoCl_2$, 0.1 mM DTT, and 62.5 units/mL terminal deoxynucleotide transferase. The reaction was carried out at 37 °C for 3 h and the 22-mer product was purified by electrophoresis in 20% denaturing gel (*30*) and desalted. The absence of a 3′-OH on the primer terminus of the resulting oligonucleotides was confirmed by lack of primer extension using the procedure described previously (*12*) (data not shown).

The 11-mer was modified by racemic (±)-*anti*-B[*a*]PDE and the four stereoisomers were separated by reverse-phase HPLC, gel-purified and characterized as described in detail (*31*, *32*). The 11-mers with (+)-*trans*- and (+)-*cis*-B[*a*]P-N^2-dG adducts, as well as unmodified 11-mers were ligated

to the phosphorylated 17-mer, using a fully complementary 22-mer as a template (Figure 2B) as described previously (*21*). The ligated 28-mers were separated and purified from the ligation mixtures on a 20% denaturing polyacrylamide gel as described (*30*) and desalted with centricon-3 microconcentrators (Amicon) according to the manufacturer protocol.

Gel Retardation Assay. Equilibrium dissociation constants (K_d) for the polymerase-primer-template complexes were determined as described in ref *12*. Increasing amounts of KF (typically 0−200 nM) were incubated with ^{32}P-labeled primer-templates (5−50 pM) for 30 min at room temperature in 50 mM Tris-HCl, pH 7.5, containing 10 mM Mg Cl_2, 1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, 4% glycerol, and 0.4 mM dNTP (if present). The reaction mixtures were analyzed in native 7% polyacrylamide gel preequilibrated with 36 mM Tris borate buffer, pH 8.3). Quantification was performed using Molecular Dynamics PhosphorImager Storm and ImageQuant. The amount of protein−DNA complex formed at equilibrium was calculated as the difference in the band intensities of the initial primer-templates without polymerase addition and unbound primer-templates. To determine K_d, the fraction of the DNA bound to the protein was plotted against the initial protein concentrations and the data analyzed using Ultrafit (Biosoft, Cambridge, UK) by fitting to the equation for single-site ligand binding. Each determination represents the average of at least three independent experiments.

Tryptic Digestion of KF Bound to the (±)-*anti-B[a]PDE-Modified Primer-Templates.* The polymerase−DNA complexes were formed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, and 1 mM dithiothreitol. The binding was carried out at room temperature for 15 min in a 12 μL reaction containing 0.6 μM annealed primer-template, 0.3 μM of KF (exo-) and 10 mM dNTP (if present). Two microliters of trypsin solution in water (15 μg/mL) was added to each reaction mixture and the digestion was terminated after 6 s by addition of 6 μL of SDS sample buffer containing 0.125 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, and 10 μg/mL bromophenol blue. The samples were loaded on a 10% SDS gel and the electrophoresis was performed according to standard procedure (*33*). Gels were fixed and stained using the GELCODE Color Silver Stain (Pierce) according to the manufacturer's protocol. To further increase the sensitivity of silver staining and detect polypeptide bands containing less than a nanogram of the material, the gels were washed with water three times and the staining procedure was repeated once or twice. The molecular weights of the proteolytic fragments were estimated by electrophoretic mobility using the Low Molecular Weight Electrophoresis Calibration Kit (Amersham Pharmacia Biotech).

RESULTS

Binding of KF to Primer-Templates Containing (+)-anti-B[a]PDE Adducts. To determine how B[*a*]PDE adducts affect the DNA-polymerase complex during nucleotide incorporation, the dissociation constants for the interaction of KF with modified and unmodified primer-templates in the absence and presence of dNTPs were determined using a gel-retardation analysis. These measurements cannot be made if nucleotide incorporation is occurring, so the primers that were used in these experiments were terminated with a dideoxy nucleotide. We have previously shown that the absence of the 3′-OH group in the primer does not affect binding of KF to unmodified or AAF- or AF-modified primer-templates (*12*). Here we find that the same is true for the B[*a*]PDE-modified primer-templates: in the absence of a dNTP the K_d values for dideoxy-terminated and normal primers were identical (data not shown). The K_d values were determined for the binding of KF to a template containing either a (+)-*trans*- or (+)-*cis*-B[*a*]P-N^2-dG adduct in the active site as described in detail (*12*) and representative gels are shown in Figure 3A. The fraction of the DNA bound to KF at a given protein concentration was calculated from the ratio of band intensities of the free primer-template at this protein concentration to the intensity of the total primer-template band without addition of polymerase ($[DNA]_0$): $[\text{bound DNA}]/[DNA]_0 = 1 - [\text{free DNA}]/[DNA]_0$. This presumably allows the measurement of the amount of the complex formed in solution at equilibrium before loading on the gel without the possible error introduced by the dissociation of the complexes in the gel during electrophoreses. The fraction of bound primer-template was plotted against total enzyme concentration (Figure 3B) and the apparent K_d values were determined from fitting these data into single-site ligand binding equation (see Table 1). Our experimental conditions yielded no multiple complexes and in contrast to other studies (*34*) the curves were hyperbolic with a good fit to the single ligand binding equation. Finally, KF did not interact with the single-stranded modified or unmodified template or with the primer (data not shown). The results for binding to the unmodified primer-templates were consistent with previous studies where primer-templates having a different DNA sequence were used (*12*). In the absence of dNTPs, the K_d was determined to be 0.4 nM, which is in good agreement for these values determined using this method (*12*, *35*) and compares with values ranging from 0.2 to 8 nM determined using a kinetic analysis (*2*, *4*, *36*). As shown previously (*12*), the addition of the next correct dNTP significantly increased (about 10-fold in terms of the K_d values) the binding strength (Table 1), while the addition of the noncomplementary dNTPs (especially the purines) resulted in a substantial decrease in binding strength (Table 1).

The binding of KF to the primer-template was not affected by the presence of (+)-*trans*-B[*a*]P-N^2-dG adduct on the template when dNTPs were not present, while the binding strength to the primer-template containing a (+)-*cis*-B[*a*]P-N^2-dG adduct was slightly decreased (Table 1). Most significantly, the addition of the next correct nucleotide (dCTP) was found not to stabilize the KF−DNA complex in comparison with the absence of a dNTP, a result that contrasts that found here and in prior studies (*12*) using unmodified templates where the presence of the next correct nucleotide resulted in enhanced binding. The addition of any dNTP was found to weaken the binding in comparison with the absence of dNTPs. An examination of Table 1 indicates that purines had a stronger inhibitory effect compared with pyrimidines for both adducts. The same trend is observed for the unmodified primer-templates, but the differences in K_d values are more pronounced when either of the adducts are present. Finally, binding was slightly weaker in all cases when the template contained the (+)-*cis*-B[*a*]P-N^2-dG adduct.

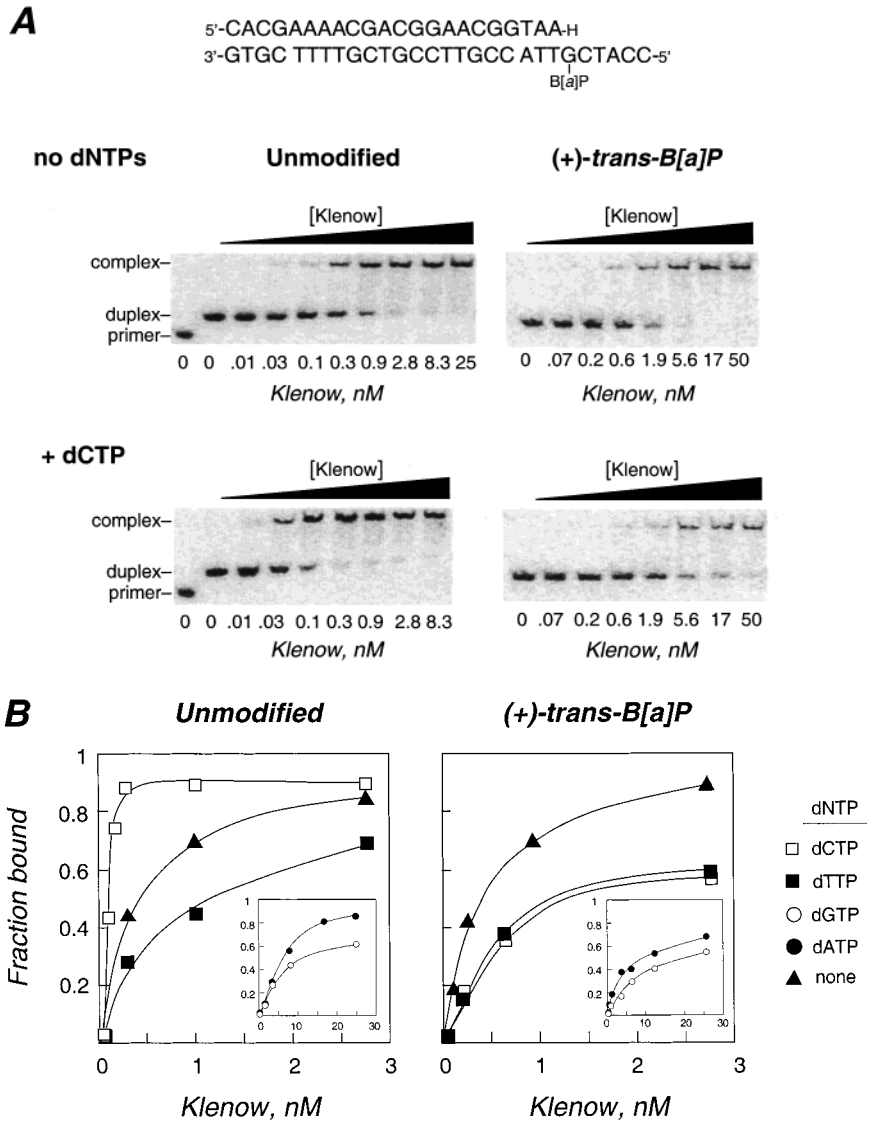

FIGURE 3: Gel-shift assay used to determine the dissociation constants for the binding of KF to the modified and unmodified primer-templates. The ^{32}P-labeled dideoxyterminated 22-mer primers (P) were annealed to 28-mer templates and incubated with increasing concentrations of KF in the absence or presence of one of the four dNTPs under conditions described in Materials and Methods. The incubation mixtures were analyzed on a 7% native polyacrylamide gel. (A) Examples of the gel-shifts of the primer-templates containing unmodified guanine (left) and (+)-*trans*-B[*a*]P-N^2-dG (right). The upper gels represent the results obtained in the absence of the nucleotides; the lower gels show the results obtained in the presence of 0.4 mM dCTP. (B) Binding curves for the interaction of KF with the primer-templates in the presence or in the absence of dNTPs. The fraction of the bound DNA determined as described in the Materials and Methods was plotted vs the initial concentration of KF. Wider ranges of KF concentration are shown in the inserts for the binding in the presence of dGTP and dATP.

Table 1: Dissociation Constants (nM) for Klenow 22-mer/28-mer Primer Template Complexes[a]

template	−	dCTP	dTTP	dATP	dGTP
unmodified	0.4 ± 0.2	0.05 ± 0.02	1.2 ± 0.6	4.8 ± 2.4	10 ± 5
(+)-*trans*	0.8 ± 0.3	1.5 ± 0.8	1.2 ± 0.5	11 ± 5	20 ± 10
(+)-*cis*	1.7 ± 0.8	3.2 ± 1.4	4.6 ± 2.1	23 ± 11	21 ± 10

[a] Dissociation constants were determined using the gel-retardation assay as described in Materials and Methods using the primer-templates shown in Figure 3A.

Tryptic Digestion of KF Bound to B[a]PDE-Modified Primer-Templates. Kinetic measurements suggest that the closed ternary DNA-polymerase-dNTP complex is more stable than the open binary DNA−polymerase complex (*12*, *37*, *38*). The fact that addition of the next correct dNTP did not result in stronger binding to B[*a*]PDE-modified templates suggests that the adduct may interfere with the formation or stability of the ternary complex. To test this hypothesis, a limited proteolysis analysis was performed on KF bound to unmodified and B[*a*]PDE-modified primer-templates in the presence of each of the dNTPs. We have previously shown that this limited protease digestion analysis can be used to distinguish between the open and closed conformations of KF bound to a primer-template (*11*, *26*). Because this analysis is performed in the presence of dNTPs, a 22-mer primer that terminated with a dideoxynucleotide was used in this study to prevent primer extension. When the digestion was performed on KF bound to the unmodified primer-template in the absence of the nucleotides two major cleavage products were observed as was previously reported (*11*, *26*). (Figure 4, lane 3). The digestion pattern was not affected by the presence of a noncomplimentary nucleotide (Figure 4, lanes

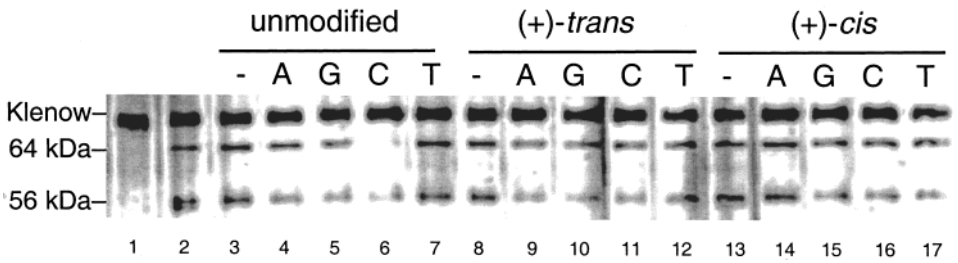

FIGURE 4: Limited tryptic digestion of KF bound to unmodified (lanes 3−7), (+)-*trans*-B[*a*]P-N^2-dG (lanes 8−12) and (+)-*cis*-B[*a*]P-N^2-dG containing (lanes 13−17) primer-templates in the absence or in the presence of the individual dNTPs. Lane 1: intact KF. Lane 2: limited tryptic digestion of KF without primer-templates and dNTPs.

Table 2: Dissociation Constants (nM) for Klenow 23-mer/28-mer Primer Template Complexes[a]

template	dC	dT	dA	dG
unmodified	1.2 ± 0.3	9.7 ± 5.1	20 ± 7	12 ± 6
(+)-*trans*	43 ± 10	14 ± 5	7.4 ± 3.6	12 ± 5
(+)-*cis*	6.2 ± 2.8	8.9 ± 4.2	15 ± 5	25 ± 9

[a] Dissociation constants were determined using the gel-retardation assay as described in Materials and Methods using the primer-templates shown in Figure 5A.

4, 5, and 7). However, in the presence of dCTP, which is the next correct nucleotide, the band at approximately 64 KDa was hardly detectable (Figure 4, lane 6). Prior studies (*11*, *26*) have concluded that the reason for the inhibition of this cleavage is that the structural rearrangement to the closed conformation that occurs in the presence of the next correct nucleotide makes this cleavage site unavailable. When tryptic digestion was carried out using KF bound to primer-templates containing either a (+)-*trans*- or (+)-*cis*-B[*a*]P-N^2-dG adduct, the cleavage that generated the 64 KDa fragment occurred in the presence of any of the four dNTPs (Figure 4, lanes 8−17). Even though it is possible that the structure of the polymerase is significantly perturbed by the adduct and is different from either that of the open or closed states, it is clear that this structure is different from that which is formed when the template is unmodified and the next correct dNTP is present suggesting that stable conversion to the closed complex did not occur.

Binding of KF to Primer-Templates in Which the Primer Extends to the B[a]PDE Adduct Position. Bulky DNA adducts have been shown not only to affect nucleotide insertion across from the damaged base, but also to interfere with incorporation of several nucleotides past the adduct position (*39*). This effect has been attributed to the fact that the adducts disturb the structure of DNA in the polymerase active site, therefore altering the polymerase−DNA interactions. To gain an understanding of the factors that influence the replication after a nucleotide is inserted across from the lesion, binding experiments were carried out in which the primers extended to the adduct position. For these experiments primers that had each of the four nucleotides at this position were annealed to unmodified, (+)-*trans*-, or (+)-*cis*-B[*a*]PDE-modified templates (Figure 5 and Table 2). In analyzing the data presented in Table 2 and in comparing it with that shown in Table 1, several points are evident. First, the KF complex with a correctly paired unmodified primer-template is somewhat less stable than the one observed for the experiment shown in Table 1. This is most likely due to the fact that the primer used in this experiment (Table 2) were one nucleotide longer, since it is known that the stability of these complexes can decrease if the length of the 5′-overhang becomes shorter (*34*). Second, having an incorrectly paired nucleotide across from an unmodified dG causes a significant reduction in the stability of the complex compared with that of the correctly paired situation (Table 2). The order of the decreasing stability for the four primers was dC > dT ≥ dG > dA, which is consistent with kinetics of the nucleotide insertion across from the unmodified guanine and the ability of KF to extend the corresponding template further (*21*). Third, consistent with previous findings (*34*), the presence of an adduct causes a significant decrease in the binding strength using any of the primers and the difference between modified vs unmodified was greatest when the primer terminated with the correct nucleotide, dC (Table 2). Finally, different order of stabilities were observed for the complexes obtained with each of the four primers annealed to the template containing a (+)-*trans*-B[*a*]P-N^2-dG vs a (+)-*cis*-B[*a*]P-N^2-dG adduct (Table 2). For the trans case the order of decreasing stability of the complex was dA > dG ≥ dT > dC while for the cis adduct the complexes in which the primers were terminated with a pyrimidine were more stable than those containing a purine at this position. This contrasts with the data shown in Table 1 where the presence of either adduct caused similar effects.

DISCUSSION

Bulky adducts have been shown to interfere with DNA replication either by blocking synthesis at the site of the damage or by reducing replication fidelity (*16*, *40*−*42*). The specific consequences of this type of DNA damage depend on many factors, including, for instance, the structure and stereochemistry of the adduct, the organism studied, the polymerase involved in the DNA replication, and the DNA sequence context where the damage is located. These factors and the fact that these factors may influence each other make the interpretation of the experimental results dealing with DNA synthesis and mutagenesis using templates containing bulky adducts very complex and often difficult to interpret (*22*, *41*, *42*). Studies that employ a purified DNA polymerase and templates site-specifically modified with an adduct of known stereochemistry in a specific DNA sequence significantly decrease the number of variables and can serve to provide a basic understanding of this process despite the fact that these simple systems lack the complexities that must occur during replication inside a cell (see, for example, ref *21*).

In this study we have used a 3′-5′ exonuclease-deficient Klenow fragment of *E. coli* DNA polymerase I and templates site-specifically modified with stereiochemically pure (+)-*anti*-B[*a*]PDE adducts. Even though the role of DNA

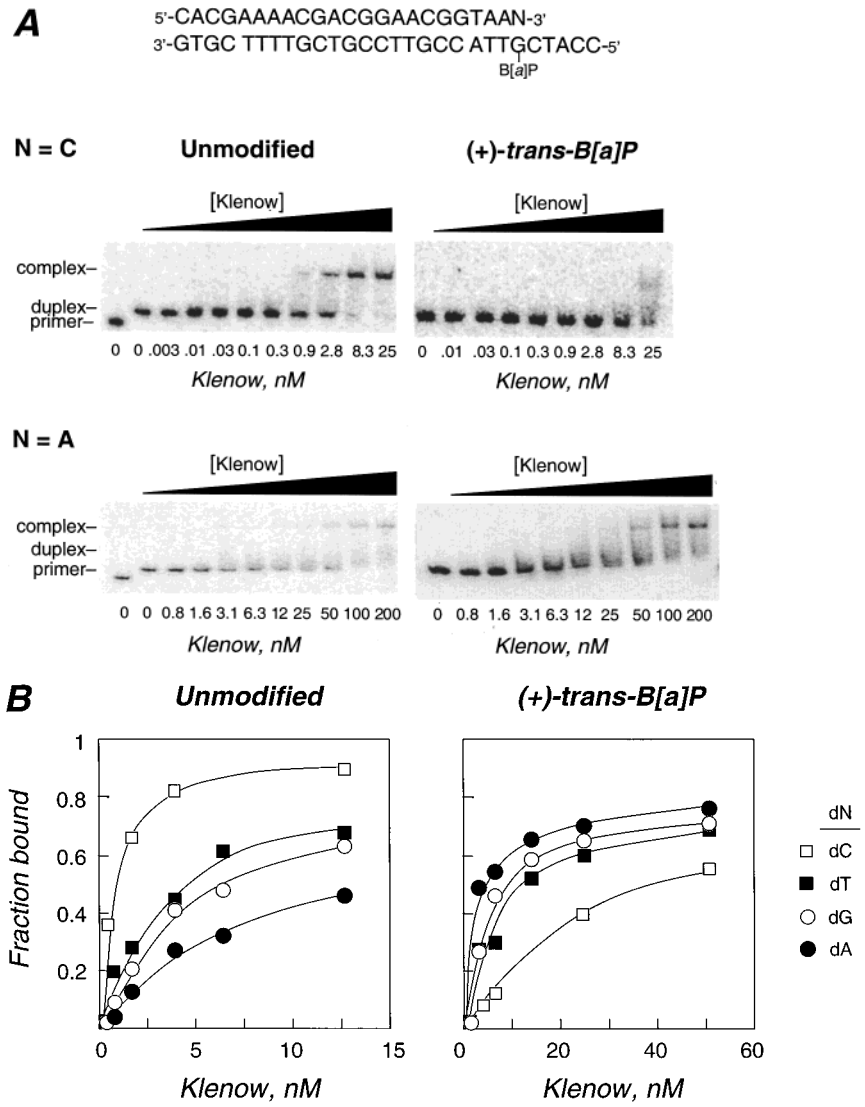

FIGURE 5: Gel-shift assay used to determine the dissociation constants for the binding of KF to the modified and unmodified primer-templates. The ^{32}P-labeled 23-mer primers (P) with one of the four nucleotides on the 3′-terminus (dN) were annealed to the indicated 28-mer template and incubated with the increasing concentrations of KF under conditions described in Materials and Methods. The incubation mixtures were analyzed in 7% native polyacrylamide gel. (A) Examples of the gel-shifts of the primer-templates containing unmodified guanine (left) and (+)-*trans*-B[*a*]P-N^2-dG (right). The upper gels show the results obtained when the primer contained dC on the 3′-terminus, the lower gels show the results obtained when the primer contained dA on the 3′-terminus. (B) Binding curves for the interaction of KF with the primer-templates in which one of the four possible nucleotides (dN) is positioned across from the adduct. The fraction of the bound DNA determined as described under the Materials and Methods was plotted vs the initial concentration of KF.

polymerase I in DNA replication is limited, it has been traditionally used as a model replication enzyme in these types of studies because of the detailed knowledge of its structure and mechanism, because of its relative simplicity compared with multi-subunit polymerases, and because it is able to incorporate nucleotides across from many DNA lesions. In addition, DNA polymerase I shares mechanistic and structural properties with many polymerases that are more directly involved in replication (*8*, *9*).

To further elucidate the mechanistic details of how a polymerase is affected by a bulky DNA adduct, we have studied the molecular interactions that occur between KF and the modified primer-templates using two methods: a gel retardation assay (*12*) and limited tryptic digestion (*11*). The former allows a measurement of the apparent dissociation constants of the DNA polymerase and DNA, while the latter allows a direct detection of the conformational change from an open binary complex to a closed ternary complex that occurs during the incorporation of a nucleotide. Previously we have used these methods to study both the replication mechanism of unmodified primer-templates by KF (*11*) and the effects of positioning an AAF or AF adduct on the template in the polymerase active site (*12*, *26*). Here we have extended this study to B[*a*]PDE-modified templates and determined that these PAH adducts affect replication in a manner that is different from that determined for the aromatic amine adducts.

To allow the measurement of the effects caused by the presence of dNTPs, a primer that terminated with a dideoxynucleotide is needed to be used in these studies to prevent primer extension. Here and in prior work (*12*) we find that the absence of the 3′-OH group of the primer does not affect binding of KF to the modified and unmodified templates in the absence of the nucleotides. Although, this does not eliminate the possibility that the stability of the ternary complex might be altered, it has been shown that the 3′-OH

group of the primer does not interact with the polymerase directly (*43*) and the crystal structure of the T7 polymerase complexed with a dideoxy-terminated primer-template (*44*) showed the nucleotide to be perfectly situated for the nucleotide insertion. Consequently, we believe that the absence of the 3′-OH group does not substantially affect the structure or stability of the either the binary or ternary KF−DNA−polymerase complexes.

Two sets of binding experiments were carried out, one where the primer terminated just prior to the adduct position modeling the situation before the insertion event (see Figure 3A, top), and one where the primer terminated opposite the adduct, modeling the situation after nucleotide insertion has taken place (see Figure 5A, top). In the former case, K_ds were determined in the absence and presence of each of the four dNTPs. In the absence of a dNTP, the presence of a (+)-*trans*-B[*a*]P-N^2-dG adduct in the template had little effect on the binding strength using the shorter primers while a (+)-*cis*-B[*a*]P-N^2-dG adduct caused a slight decrease in affinity. Prior results using similar templates containing AF or AAF adduct also showed that these adducts caused either no decrease (AF) or an *increase* (AAF) in the binding strength (*12*). The implication of these result is that these bulky adducts can be well accommodated into the active site of the DNA polymerase in the open conformation of the enzyme.

Upon binding the next W/C nucleotide, it has been shown that the DNA polymerase undergoes the conformational change to the closed complex (*9*). This conformational rearrangement is thought to serve as a method to check whether the geometric shape of the incoming nucleotide substrate fits the geometry of the polymerase active site, thereby enforcing highly accurate DNA synthesis (*45*). Unlike what is observed with unmodified templates (ref *12* and Table 1), there is little change in the K_ds upon the addition of the next correct nucleotide when the primer terminates prior to either the (+)-*trans*- or (+)-*cis*-B[*a*]P-N^2-dG adduct (Table 1), suggesting that a stabilized ternary complex is not forming. This was confirmed by the tryptic digestion experiment (Figure 4), which showed that the addition of the next correct nucleotide did not result in a measurable conformational change to a closed ternary complex as is observed with unmodified templates. The reasons that could have produced the observed tryptic digestion patterns are discussed in prior work (*11*, *26*) and the same considerations are applicable here. Detection of an intermediate that forms prior to the closed complex also cannot be ruled out. However if the latter is the case, the conclusion based on these experiments is that the presence of the bulky adduct in the active site prevents the enzyme from adopting this intermediate conformation and consequently blocks the closed complex formation. On the basis of these considerations, we believe that the most reasonable interpretation of these results is that the structure of the complex bound to a B[a]PDE-modified template in the presence of any dNTP is different from that of the polymerase in the closed conformation.

Comparison of the results reported here with the results of similar experiments carried out with primer-templates modified with bulky aromatic amine adducts (*12*, *26*) shows significant differences that may indicate that different steps in the polymerase mechanism are being affected by the presence of each adduct. A possible model for this process is that the binding of any of the dNTPs triggers a conformational change in the KF−DNA complex, but that only a correctly base-paired nucleotide allows the formation of a stable closed complex. An incorrect dNTP positioned in the active site results in a measurable destabilization of the complex making the detection of the conformational change to the closed form impossible. In support of this model is the fact that more spacious purines produce a much greater destabilization of the polymerase-DNA complex (Table 1 and ref *12*) even though there is little discrimination for binding an incorrect dNTP in the open complex (*10*). The observation that the addition of a dNTP also induces a destabilization when the primer-template is modified by a (+)-*anti*-B[*a*]PDE adduct and that purines cause a stronger destabilization then pyrimidines similarly suggests that the conformational change has been initiated but that it does not lead to the formation of the stable closed complex, presumably because the presence of the adduct alters the structure of the polymerase active site in a way that effects the proper fit of the dNTP. The existence of a nonproductive ternary complexes to explain the pre-steady-state kinetics of HIV-1 reverse transcriptase at modified DNA bases has been previously suggested (*46*).

When the primer template is modified with an AAF adduct the effects are very different. In this situation, the addition of a dNTP has no effect on the stability of the complex (*12*) and does not induce a conformational change that can be detected by tryptic digestion (*26*). These results suggest that in contrast to the B[*a*]PDE adducts, the AAF adduct appears to completely inhibit the conformational change that leads to the formation of a closed complex. This model is further supported by the observation that in the presence of the easily bypassed AF adduct, KF shows a nucleotide-dependent destabilization of DNA binding (*12*) and undergoes a conformational change detectable by proteolysis (*26*) in the presence of dCTP, the nucleotide that is the most often incorporated opposite this lesion (*28*).

Additional evidence for this model comes from a recent molecular modeling study (*47*) that suggested that binding of a (+)-*trans*-B[*a*]PDE-modified primer-template by polymerase β involved an interaction of the aromatic moiety of the adduct with the same amino acids that are involved in the nucleotide binding in the closed complex. This study also indicated that with modest movement of the modified base and enzyme, that the incoming dNTP could be accommodated in the active site. It is possible that the excess dNTP used in our experiment could stimulate the formation of such a structure and allow the initiation of the conversion to the closed complex, which, however, is unstable due to the presence of the adduct.

The use of primers that extended to the adduct position models the situation where a nucleotide has been incorporated across from the lesion. For these experiments, the K_ds were determined using four different primers, each terminating with a different nucleotide at the 3′ end. Interestingly, the binding strength significantly decreased (8−16-fold in terms of K_d values) when a nucleotide on the primer terminus formed a mismatch with the base on unmodified template (Table 2). Most of this decrease in stability is probably not due to a shift of the primer terminus to the exonuclease site since it has been shown that usually only about 20% of KF

molecules bind the single-mismatched primer terminus at the exonuclease site (*48*) but rather is most likely due to a structural alteration in the DNA duplex that is no longer optimal for polymerase binding. This conclusion is supported by the fact that the order of decreasing stabilities of these KF−DNA complexes exactly correlates with the trends of nucleotide insertion across from and extension past G (*21*) and with the thermodynamic stabilities of the duplex DNA of these sequences (*31*, *49*). Taken together, these results suggest that binding strength is one of the factors that contributes to the fidelity of DNA synthesis. Others have shown that HIV-1 reverse transcriptase, which is characterized by lower fidelity, binds to the primer-templates having pyrimidine-purine or purine-purine 3′-terminal mispairs with equal efficiency (*50*).

The stability of the complexes having a primer that situates a nucleotide opposite either B[*a*]PDE adduct was substantially reduced compared with both that of the unmodified correctly paired primer-templates (Table 2) and the modified primer-templates with the primers one nucleotide shorter (Table 1). NMR studies have revealed substantial differences between the structures of the (+)-*trans*-B[*a*]P-modified primer-template junctions in which the primer is terminated before the adduct (*51*) vs those where it contains a C across from the modified G on the template (*52*). Presumably these different structures play a large role in the different K_ds we observe and that these decreases in stability contributes to the inability of KF to synthesize past these bulky adducts.

A second difference is evident for the data obtained using the longer primers. While the results shown in Table 1 gave similar K_d values for the (+)-*cis*- and (+)-*trans*-B[*a*]P-N^2-dG adduct in the presence of each of the dNTPs, there were significant differences between the K_ds for the (+)-*cis*- and (+)-*trans* B[*a*]P-N^2-dG adducts determined using primers in which a nucleotide was positioned opposite the modified G (Table 2). For the trans isomer the order of stability was dA > dG ≥ dT > dC while the order for the cis isomer was dC ≥ dT > dA > dG, suggesting that once a nucleotide is incorporated opposite these lesions the adducts adopt different structures in the polymerase active site. Although there is NMR data for a (+)-*trans*-B[*a*]P-N^2-dG adduct situated at the primer-template junctions (*40*), the corresponding structure for a (+)-*cis*-B[*a*]P-N^2-dG adduct is not available so it is not known what the precise nature of these differences are. Interestingly, prior studies have shown that bypass synthesis occurred only for primers that terminated with dT in this sequence for both isomers (*21*), or terminating with dA in other sequences (*16*, *17*, *21*) suggesting that these structural differences do not always translate into differences in nucleotide selection during trans-lesion synthesis.

Prior studies have shown that the relative local destabilizing effect caused by the presence of the adduct is the least when it is paired with A, and the most when the base across from the lesion is C, even though the stability of the corresponding full duplexes follows the opposite trend (*31*, *49*). This observation was correlated with the results of in vitro studies that showed that in this (*21*) and most other sequences (*16*, *17*, *20*) the base that is preferentially incorporated across from the adduct is A, while the correctly paired C is incorporated the least efficiently. The trend observed here (Table 2) suggests that the structure in which an A is positioned in the primer across from the (+)-*trans*-B[*a*]P-N^2-dG adduct is the most suitable for the polymerase binding, especially in comparison with the W/C C, probably also suggests that this structure is least distorted. These factors and the better ability of the purines (compared with that of pyrimidines) to stack with B[*a*]P moiety (*53*) may contribute to the observed insertion kinetics for each nucleotide (*16*, *21*), despite stronger destabilization of the KF−primer-template complexes by the presence of purines versus the pyrimidines (Table 1), which probably can be explained by simple steric considerations. In addition, it is possible that the B[a]P adduct can adopt more than one conformation in the active site (*22*) and that the K_d values would represent the average for all of these while primer extension might occur from only one which structure is more suitable for dNTP insertion.

In summary, this study provides further details regarding the interaction of a DNA polymerase with DNA when a bulky adduct is located at the primer-template junction in the polymerase active site. Both the (+)-*trans*- and (+)-*cis*-B[*a*]P-N^2-dG adducts have little effect on the open complex if the primer ends before the adduct position but both reduce the stability of the complex with a primer that extends to the adduct position. A model is proposed in which the presence of a dNTP induces the conformational change to a closed ternary complex but that this structure is not stable and leads to dissociation. These results are different from those found for AAF and AF adducts, suggesting that the structures of these complexes have substantial differences. Finally, using templates in which the primer extends to the adduct position, significant differences in complex stabilities were observed in comparing the (+)-*cis*- and (+)-*trans*-B[*a*]P-N^2-dG adducts suggesting that the structures of these complexes are different.

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