Differential Effects of *N*-Acetyl-2-aminofluorene and *N*-2-Aminofluorene Adducts on the Conformational Change in the Structure of DNA Polymerase I (Klenow Fragment)†

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ABSTRACT: The carcinogen *N*-acetyl-2-aminofluorene forms two major DNA adducts: the *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF) and its deacetylated derivative, the *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene adduct (dG-C8-AF). It is well established that the AAF adduct is a very strong block for DNA synthesis in vitro while the AF adduct is more easily bypassed. In an effort to understand the molecular mechanism of this phenomenon, the structure of the complex of an exonuclease-deficient *Escherichia coli* DNA polymerase I (Klenow fragment) bound to primer-templates containing either an AF or AAF adduct in or near the active site was probed by nuclease and protease digestion analyses. The results of these experiments suggest that positioning the AAF adduct in the polymerase active site strongly inhibits the conformational change that is required for the insertion of a nucleotide. Similar experiments with AF-modified primer-templates shows a much less pronounced effect. The inhibition of the conformational change by either adduct is not detected if they are positioned in the single-stranded part of the template just one nucleotide before the active site. These findings may explain the different abilities of these lesions to block DNA synthesis.

Bulky DNA adducts formed by *N*-acetyl-2-aminofluorene (AAF)[1] have been extensively studied as model lesions that interfere with DNA synthesis and give rise to mutations in both in vivo and in vitro systems. Among these lesions, two major ones have attracted most attention: the *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF, see Figure 1), which is known to be a strong block to DNA synthesis, and the *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene adduct (dG-C8-AF), which can be much more easily bypassed by all polymerases tested (*1*−*3*). In *E. coli*, AAF adducts lead mostly to frameshift mutations while AF adducts produce mostly base substitution mutations (*4*−*6*). The reason for the different behavior of these lesions in replication is not clear, but it is likely to involve the different conformations that the adducts assume in DNA, which presumably carry over into the active site of the DNA polymerase. Recent NMR structural studies have demonstrated that these arylamine adducts exhibit conformational heterogeneity (*7*, *8*), but the AAF adduct is much more prone to adopt the distorting base displacement structure than the AF adduct. In this structure, the guanine bearing the C8-AAF adduct rotates from the anti to syn conformation, and the fluorenyl moiety is inserted into the helix (*7*, *9*). In contrast, the AF adduct more often favors one of the less distorting external conformations and stays outside the helix with the modified guanine remaining in the anti conformation.

It is likely that the structural alterations imposed by DNA adducts influence the interaction of the modified DNA with the replication machinery. DNA polymerases are known to insert nucleotides at a primer terminus by the repetition of four general steps: identifying and binding to the 3′ end of the growing strand (open binary complex), binding the dNTP (open ternary complex), conformational rearrangement that positions the α-phosphate of the dNTP in the proper geometry for the nucleophilic attack by the 3′-hydroxyl (closed ternary complex), and the chemistry step which leads to incorporation of the dNMP, release of pyrophosphate, and translocation to the next position of the template (*10*). Each of these steps may theoretically be inhibited by a bulky DNA adduct that is bound to the DNA in the vicinity of the polymerase binding site. It was shown, however, that AF or AAF adducts have a significant effect on DNA polymerase I (Klenow fragment) (KF) binding to DNA (*11*) and activity (*12*) only if they are positioned at the primer-template junction or in the double-stranded part of the primer-template. When either of these adducts is positioned at the primer-template junction, the polymerase activity is strongly inhibited, although the binding of the enzyme to DNA is not compromised (*11*, *12*). This latter observation has recently been supported by measurements of the equilibrium dissociation constants of the interaction of KF with AAF-modified DNA that have shown that the presence of this adduct actually increases the stability of the KF−DNA complex (*11*). Analysis of the polymerase−DNA binding

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[1] Abbreviations: AAF, *N*-acetyl-2-aminofluorene; AF, *N*-2-aminofluorene; dG-C8-AF, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene; dG-C8-AAF, *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene; KF, *E. coli* DNA polymerase I (Klenow fragment); DNase I, pancreatic deoxyribonuclease I.

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in the presence of dNTPs led us to propose that the AAF adduct inhibits the nucleotide binding step and/or the conformational change of the polymerase necessary for formation of the closed ternary complex (*11*).

We have recently developed a protease digestion technique to detect the conformational rearrangement of KF that accompanies the formation of the closed catalytically competent ternary complex in the presence of the primer-template and a dNTP (*13*). We also showed that, consistent with the induced fit mechanism for polymerase fidelity, this conformational change is inhibited by the presence of nucleotides having geometries incompatible with the shape of the polymerase active site. Because DNA adducts are also expected to disturb the active site structure, it is reasonable to expect that they may interfere with this conformational rearrangement. To test this idea, the nuclease and protease digestion analyses were applied to complexes of KF with primer-templates modified with the AF and AAF adducts. The results indicate that the AAF adduct has a much stronger inhibitory effect on this conformational rearrangement when it is positioned in the primer-template junction. The effect of the AF adduct, although observable, is clearly less pronounced. This different ability to interfere with the conformational change, which is critical for nucleotide insertion, is likely to be one factor that accounts for the difference in replication properties of these adducts.

MATERIALS AND METHODS

Materials. The Klenow fragment of *E. coli* DNA polymerase I (exonuclease free) was purchased from Amersham Pharmacia Biotech. The protein had been overexpressed 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 (*14*). T4 polynucleotide kinase and bovine pancreatic deoxyribonuclease I (DNase I) also were purchased from Amersham Pharmacia Biotech. Trypsin and terminal deoxynucleotide transferase were from Boehringer Mannheim.

Oligonucleotides were obtained from Midland Certified Inc. Site-specifically-modified 12-mer (GTG ATG$^{(C8-AAF)}$ ATA AGT) used for the synthesis of the AAF-modified template was obtained as described previously (*15*). All dNTPs and ddNTPs were ordered from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from ICN Biomedicals.

Synthesis and Purification of the Oligonucleotides. The sequences of the oligonucleotides that were used in this study to create the primer-templates are shown at the top of Figures 2 and 5. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Site-specifically-modified 28-mer templates were synthesized, purified, and characterized as described in (*11*). The primers lacking 3′-OH were obtained by extension of the corresponding oligonucleotides with ddNMPs using terminal deoxynucleotide transferase as described (*13*).

DNase I Footprinting Analysis of the Polymerase−DNA Complexes. The KF−DNA binding reactions were performed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. The binding was carried out at 25 °C for 30 min in a 12 μL reaction containing 10 nM ^{32}P-labeled duplex DNA, 20 nM KF (exo−), and 0.4 mM dNTP (if present). Two microliters of DNase I solution (0.11 unit/μL in 15 mM $CaCl_2$) was then added for 6 s, and the reaction was stopped by adding 40 μL of the gel loading buffer containing 90% formamide. Aliquots of the reaction mixtures were loaded on 15% denaturing polyacrylamide gels. The gels were fixed with 7% acetic acid and 30% methanol, dried, and scanned using a Molecular Dynamics Phosphorimager.

Tryptic Digestion of KF. Protease digestion of the KF−DNA complex was performed as described (*13*). Briefly, the enzyme−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 KF (exo−), and 0.4−10 mM dNTP (if present). Trypsin solution in water (15 μg/mL final) was added to each reaction mixture, and the digestion was terminated after 6 s by the 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 (*16*). Gels were fixed and silver 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 (3 times, 10 min each wash), and the staining procedure was repeated once or twice.

Determination of K_d for KF−DNA Interactions by a Gel-Retardation Assay. Determination of the equilibrium dissociation constants for the interaction of the polymerase with primer-templates was carried out by the gel-retardation assay as described previously (*11*). In this experiment, DNA-binding reactions were performed in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, and 4% glycerol. The binding was carried out at 25 °C for 30 min in a 10 μL reaction containing 5−10 pM ^{32}P-labeled duplex DNA, increasing amounts of KF (exo−) (typically 0−81 nM), and 0.4 mM dTTP (if present). The reaction mixtures were loaded on a running native 7% polyacrylamide gel preequilibrated with 0.4 × TB buffer (36 mM Tris−borate, pH 8.3). Gels were fixed with 7% acetic acid, dried, and scanned using a Molecular Dynamics Phosphorimager. The amount of polymerase−DNA complex formed at equilibrium (bound DNA) was estimated as the difference between the intensity of the dsDNA band without addition of polymerase and the intensities of the free primer-template band separated from the complex. To obtain the K_d, the fraction of bound DNA was plotted against initial protein concentrations, and the data were analyzed using the program Ultrafit (BIOSOFT, Cambridge, U.K.) and fitted to the equation for single-site ligand binding.

RESULTS

The two major DNA lesions induced by aromatic amines (Figure 1) are known to interfere with DNA synthesis, but the AAF adduct is a much more potent inhibitor of DNA replication than the AF adduct. It therefore seems reasonable that these adducts may have different effects on the structure of the polymerase−DNA complex. To detect these structural

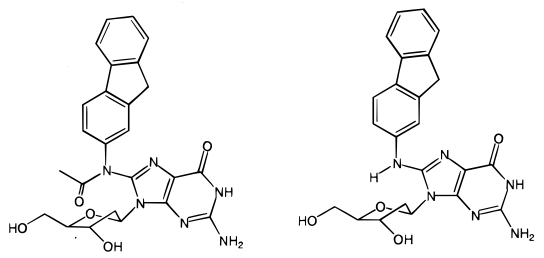

FIGURE 1: Structures of the *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF) and the *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene adduct (dG-C8-AF).

differences, nuclease and protease digestion experiments were carried out on the KF−DNA complexes in which either an AF or an AAF adduct was positioned in or near the polymerase active site in the absence or presence of each of the dNTPs. Since it was important to observe structural alterations of the protein−DNA complex in the presence of dNTPs, the primers used in this study were terminated by a ddNMP, thus inhibiting the chemical step of nucleotide insertion and allowing for formation of a ternary polymerase−DNA−dNTP complex.

DNase I Footprinting Analysis of Primer-Templates Bound to KF. DNase I footprinting was carried out to detect the structural and binding variations in the KF−DNA complexes induced by the presence of an AF or AAF adduct in the primer-template junction and dNTPs in solution. First, studies were carried out using a 5′-^{32}P-labeled primer to determine the effect of the adducts on the polymerase interactions with the primer strand. Consistent with the observation that DNase I binds in the minor groove of the DNA helix 5′ to the cleavage site and loses its activity in the regions were the minor groove ceases to exist (*17*), DNA fragments shorter than four nucleotides were not observed after the cleavage (Figure 2). When KF was bound to the unmodified primer-template, approximately 11 nucleotides were observed to be protected by the protein on the primer strand (Figure 2, lane 3), similar to what has been observed previously (*18*). Neither the cleavage pattern nor the size of the protected region changed in the presence of either the nucleotide complementary to the template base (dCTP) or a noncomplementary nucleotide (dATP) as shown in Figure 2, lanes 3−5. However, consistent with our prior studies (*11*), better protection (implying a more stable complex) was observed in the presence of the complementary dNTP, and worse protection was observed in the presence of a nonpaired dNTP (Figure 2, lanes 4 and 5), indicating a change in binding strength presumably corresponding to a change in the structure of the polymerase−DNA complex (*11*). When these experiments were repeated with the ^{32}P-labeled template strands, the same trends of binding strength were found as observed with the labeled primer strand (data not shown).

DNase I Footprinting of AF- and AAF-Modified Primer-Templates. The presence of an adduct at the primer-template junction did not change the size of the region protected by KF or the relative intensities of the resulting cleavages at different sites (Figure 2, lanes 3, 7, 11). A similar lack of effect of the adduct on the polymerase binding site and protection pattern was observed when the cleavage of template strands was analyzed (data not shown). It should

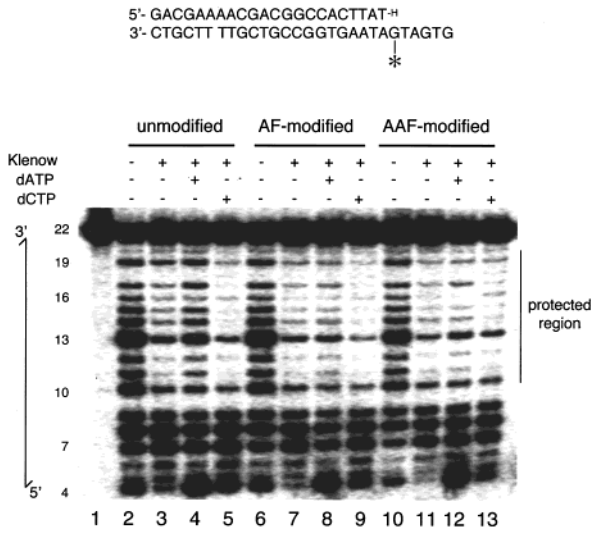

FIGURE 2: DNase I footprinting analysis of KF−DNA complexes. A ^{32}P-labeled 22-mer primer (10 nM) was annealed to an equal amount of the template, the resulting duplex was preincubated with KF (20 nM), and then the DNA was digested with DNase I in the presence or absence of dNTPs as indicated. Lane 1, uncut 22-mer primer; lanes 2−5, unmodified primer-template; lanes 6−9, AF-modified primer-template; lanes 10−13, AAF-modified primer template. The sequences of the 22-mer primer and 28-mer template are shown at the top. Positions of the 22-mer primer as well as some digestion products (19-mer, 16-mer, 13-mer, 10-mer, 7-mer, and 4-mer) are indicated to the left of the gel. The primer was modified with ddTMP as described under Materials and Methods. The 28-mer template was either unmodified or modified at the C8 position of G6 by an AF or AAF adduct as indicated.

also be noted that in the absence of the polymerase, the adducts did not significantly affect the DNase I cleavage pattern or relative band intensities of the ^{32}P-labeled primer strand (Figure 2, cf. lanes 2, 6, and 10).

Consistent with our previous results (*11*) in which we found nucleotide-independent strong binding to AAF-modified primer-templates, the presence of an AAF adduct led to a protection from nuclease digestion that was almost unaffected by the presence or nature of an added dNTP (Figure 2, lanes 11−13). Also consistent with these previous data (*11*), protection in the presence of the complementary dCTP was better than in the presence of noncomplementary dATP for the AF-modified template (Figure 2, cf. lanes 8 and 9), but the magnitude of this effect was smaller than on unmodified DNA. It has been shown that dCTP is the nucleotide that is best incorporated across from both AF and AAF adducts by DNA polymerase I (*19*).

Tryptic Digestion of KF Bound to Primer-Templates Containing Adducts Positioned at the Primer-Template Junction. Based upon the binding study results (*11*) and supported by the DNase I footprinting analysis described above, we have previously suggested that the AAF adduct may interact with the polymerase in the active site and inhibit the conformational change of the enzyme required for the incorporation of a nucleotide. It was also suggested that the effect of the AF adduct on the conformational change is weaker, presumably because of the higher fraction in the "outside binding" mode in the structures adopted by this adduct, which allows the template base to adopt a more native configuration. To test this idea, a tryptic digestion analysis was employed here to probe the conformation of the polymerase bound to primer-templates modified with

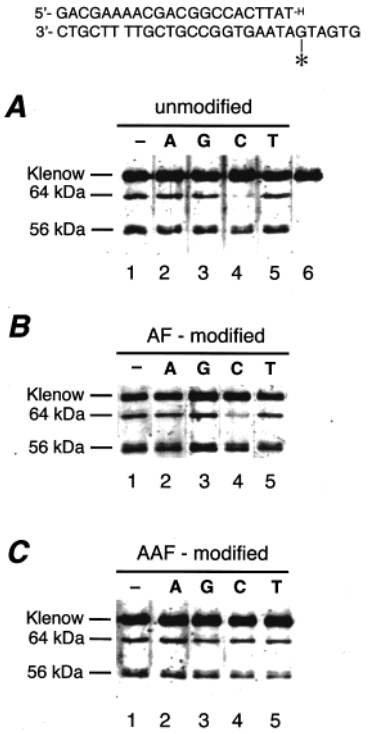

FIGURE 3: Tryptic digestion of KF bound to primer-templates and dNTPs where the adducts are positioned in the active site. KF (0.3 μM) was incubated with the 22/28-mer primer-template (0.6 μM) and dNTPs (10 mM, if present) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Lanes 1−5: tryptic digestion in the absence of dNTP (lane 1) and in the presence of dATP, dGTP, dCTP, and dTTP (lanes 2−5, respectively). Panel A, unmodified primer-template; panel B, AF-modified primer-template; panel C, AAF-modified primer-template. Lane 6 in panel A represents KF not treated with trypsin.

either an AAF or AF an adduct. We have recently shown that the protease digestion analysis can be used to distinguish between "open" binary and "closed" ternary complexes formed by KF on DNA (*13*).

When this analysis was carried out on the binary complex of KF with unmodified primer-template, two major cleavage fragments of approximate molecular masses of 56 and 64 kDa were detected (Figure 3A, lane 1). In the presence of dCTP, the nucleotide complementary to the template base in the active site, the trypsin cleavage site that generates the 64 kDa band was no longer as accessible, thus producing a very weak band (Figure 3A, lane 4). This effect presumably is indicative of the global conformational change that occurs when the ternary closed complex is formed. When this same experiment was carried out on a template that positioned an AAF adduct in the active site of the polymerase (Figure 3C), the polymerase was not able to undergo this conformational change in the presence of the complementary (Figure 3C, lane 4) or any other nucleotide (Figure 3C, lanes 2, 3, 5). This was not simply due to an inhibition of binding to this template since prior studies have shown that binding is actually stronger in the presence of the AAF adduct (*11*), but instead indicates that the AAF adduct is inhibiting the formation of a stable closed complex. Interestingly, when the more easily bypassed AF adduct was positioned in the protein active site, inhibition of the 64 kDa band formation in the presence of dCTP was now observed, suggesting that

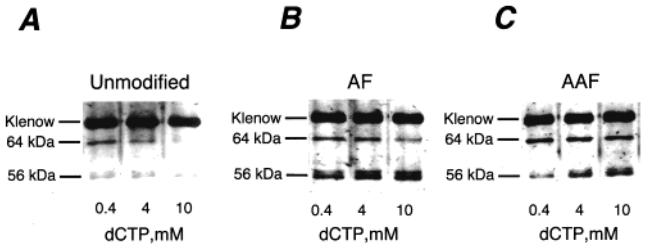

FIGURE 4: Tryptic digestion of KF bound to the 22/28-mer primer-templates in the presence of increasing concentrations of the next correct nucleotide, dCTP. KF (0.3 μM) was incubated with the primer-template (0.6 μM) and dCTP (0.4−10 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Panel A, unmodified primer-template; panel B, AF-modified primer-template; panel C, AAF-modified primer-template.

in the presence of this adduct the conformational change occurs and the closed complex forms.

Relative Ability of the AAF and AF Adducts To Inhibit the Conformational Change. To estimate the relative abilities of the unmodified and the AF- and AAF-modified primer-templates to allow formation of the closed complexes in the presence of a complementary dNTP, the tryptic digestion experiments were carried out over a range of dCTP concentrations for each of these templates. As shown in Figure 4, the closed complex was formed to an appreciable extent on an unmodified template at 4 mM dCTP (Figure 4A), while on an AF-modified template no change was observed at this level. For this template, the inhibition of trypsin cleavage occurs only at higher concentrations (10 mM) of dCTP (Figure 4B). Levels of dNTP higher than 10 mM could not be used because of the nonspecific inhibition of trypsin that occurred. Finally, increasing concentrations of dCTP had no effect on KF proteolysis using the AAF-modified template (Figure 4C): at all concentrations, equivalent amounts of the 64 kDa fragment were formed.

Effect of an Adduct in the Single-Stranded Region of the Primer-Template. To ensure that the strong inhibition of the polymerase conformational change detected in the presence of the AAF adduct is the result of placing the adduct in the active site at the next position for nucleotide addition, the tryptic digestion experiments were carried out on complexes consisting of KF bound to 21-mer/28-mer primer-templates in which the adducts were placed in the single-stranded region of the template, one position before the active site (Figure 5, top). At this position, dTTP is the next correct nucleotide for incorporation opposite the A in the template. Using this template, the presence and nature of the adduct had no influence on the ability of the polymerase to undergo the conformational change in the presence of dTTP (Figure 5). Although not shown, the polymerase was unable to form the closed complex on any of these templates in the presence of the nonpairing dNTPs.

Binding of KF to Primer-Templates. We and others have previously shown that the formation of the closed ternary complex (consisting of the polymerase bound to primer-template and the next correct dNTP) from the binary polymerase−DNA complex is accompanied by an increase in the complex stability (*11*, *20*, *21*). Although an AAF or AF adduct affects the stability of these complexes if they are in the active site, the equilibrium dissociation constants

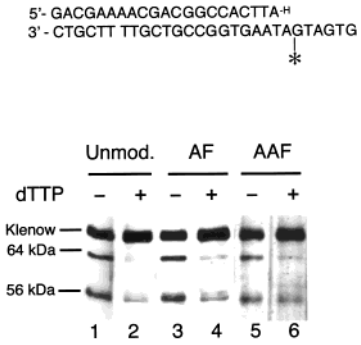

FIGURE 5: Tryptic digestion of KF bound to primer-templates and dNTPs where the adducts are positioned one base before the active site. KF (0.3 μM) was incubated with the 21/28-mer primer-template (0.6 μM) and 10 mM of the complementary nucleotide, dTTP (if present). The conformation of the resulting complex was probed by limited tryptic digestion as described under Materials and Methods. Lanes 1 and 2, unmodified primer-template; lanes 3 and 4, AF-modified primer-template; lanes 5 and 6, AAF-modified primer-template.

Table 1: Dissociation Constants (K_d) for the Complexes of KF with the Primer-Template in the Presence or Absence of the Next Correct Nucleotide[a]

	K_d (nM)	
template	–	dTTP
unmodified	0.26 ± 0.09	0.03 ± 0.02
AAF-modified	0.23 ± 0.10	0.08 ± 0.05
AF-modified	0.28 ± 0.10	0.04 ± 0.01

[a] Dissociation constants were determined using the gel-retardation assay as described under Materials and Methods using the 21/28-mer primer-templates in which the adducts are positioned one nucleotide before the polymerase active site (Figure 5).

(K_d) for the binary complexes are not affected if either adduct is positioned in the template one nucleotide before the active site (*11*). To further characterize the proposed conformational change that can occur when the adducts are located at this position in the template (Figure 5, top), we have measured the K_d values for KF complexes on this template in the presence or absence of the dNTP complementary to the template base (Table 1). The results of this experiment indicate that binding in the presence of dTTP is about 3−9-fold stronger than in the absence of nucleotide on both the unmodified and modified templates. This enhanced binding is indicative of the formation of the closed ternary complex and is consistent with the results of the tryptic digestion experiments, which showed that the presence of either adduct one position before the polymerase active site does not significantly affect the ability of the polymerase to undergo a conformational change to the closed ternary complex.

DISCUSSION

It has been demonstrated that the biological consequences of damaging DNA with either an AF and AAF adduct are very different (*1*). The AAF adducts are much more toxic for the cell and have been shown to trigger the SOS response in bacteria more efficiently. Also AF and AAF lesions are known to have remarkably different mutagenic properties. While AF adducts mostly induce G to T transversions in bacteria, the AAF adducts have been found to cause mostly frameshift mutations (*4*−*6*). The mechanism of frameshift mutagenesis by aromatic amines in repetitive sequences has been proposed to begin with retardation of the DNA polymerase by the bulky adduct, formation and stabilization of a slipped mutagenic intermediate, and subsequent chain elongation from the slipped structure (*22*, *23*). Retardation of the DNA polymerase by the adducts also may lead to frameshift mutations in random sequences, although in this case misinsertion of bases opposite the lesion has been suggested to precede the primer-template misalignment (*19*). Overall, the AAF adduct has been shown to block DNA synthesis much more strongly than the AF adduct. Results of steady-state kinetics measurements indicate that the insertion fidelity of dCTP is 0.36 for AF and only 6.6×10^{-3} for AAF relative to incorporation opposite an unmodified guanine (*24*). As measured by single-turnover kinetics, the rate of dCTP incorporation by T7 polymerase across the dG-C8-AAF adduct is 10^6-fold slower than across unmodified guanine and 100 times slower than across the dG-C8-AF adduct (*25*). Using the specific experimental system (primer-templates, polymerase, and buffers) employed in the binding and conformational change studies described here, we also found that an AF adduct is bypassed much more efficiently than an AAF adduct (*11*).

It believed that the strikingly different replication properties of these adducts that have such similar chemical structures are explained by the different conformations that they tend to adopt in duplex DNA and presumably at the single-stranded−double-stranded junction of the primer template (*7*, *8*). On the molecular level, a reasonable model for how these adducts interrupt DNA synthesis predicts that the structurally altered DNA molecule is a less suitable substrate for a DNA polymerase, presumably because the protein−DNA interactions and conformational transitions required for insertion of a nucleotide are obstructed. In the experiments described in this work, we have detected structural differences between the complexes formed between KF and modified or unmodified DNA using nuclease and protease digestion approaches. The same experiments in the presence of dNTPs allowed us to implicate the inhibition of a conformational change that leads to formation of the catalytically active closed ternary complex as one factor that results in the different replication properties.

DNase I footprinting analysis of KF−DNA complex has previously been used to determine the length of DNA contacted by this enzyme (*18*). In this previous study, it was demonstrated that the 11 base pair region contains the majority of the important enzyme−DNA contacts 5′ of the primer terminus. A later crystal structure revealing an editing complex of KF bound to an 11 base pair duplex DNA was consistent with this result (*26*). However, as pointed out by Joyce and Steitz (*27*), various footprinting, fluorescence, and photo-cross-linking experiments indicate that only 5−8 base pairs of duplex DNA are covered by Klenow fragment when the primer terminus is in the polymerase site. These authors suggest that this range of values reflects requirements for access of the footprinting agents and uncertainties in precise orientation of the probes attached to DNA. The protected region in the duplex part of the primer-template determined in Figure 2 is consistent with these previous studies. More significantly, when the dNTP complementary to the base in the active site is present, better protection is observed, a result indicative of stronger binding. Conversely, in the presence

of a noncomplementary nucleotide, the protection becomes weaker. These results are consistent with our previous studies of KF−DNA interactions (*11*) and suggest that there may be a difference in the structure of the protein−DNA complexes in the absence or presence of dNTPs. This structural difference was attributed to the formation of a stable ternary KF−DNA−dNTP complex that only occurs in the presence of the complementary nucleotide (*13*). Based on the induced fit model for DNA polymerase fidelity, we also suggested that the attempt to accommodate an incorrect nucleotide in the active site might lead to the formation of an unstable ternary complex and therefore the weaker KF−DNA binding is observed under these conditions.

It is now clear that the presence of either an AF or an AAF adduct in the polymerase active site alters the interaction of a polymerase with both the primer-template and the incoming dNTP. DNase I footprinting of T7 DNA polymerase bound to an AAF-modified primer-template demonstrated that the adduct did not change the protection pattern, but did inhibit cleavage at hypersensitive sites (*28*). This suggested that T7 polymerase occupies the same position on unmodified and AAF-modified primer-templates, but that the overall conformation of the protein−DNA complex is modified by the presence of the adduct. Consistent with these prior results, the presence of either an AAF or an AF adduct in the present study has no effect on the KF binding site size or cleavage pattern. However, the binding of KF to the primer-template positioning an AAF adduct in the active site is stronger *(11*) and independent of the presence of a dNTP (*11* and Figure 2), suggesting that there is a structural difference between complexes of KF with unmodified vs AAF-modified DNA. Prior measurements of the strength of KF−primer-template interactions in the presence of dNTPs suggested that the AAF adduct in the polymerase active site may inhibit the nucleotide-induced conformational change from an open to closed complex and that the less distorting AF adduct is less effective at preventing this change *(11*).

This idea that the AAF adduct in the DNA polymerase active site inhibits the conformational change in the structure of the enzyme that is necessary for insertion of a nucleotide is supported by the results of tryptic digestion experiments. This method allows the detection of the formation of a stable ternary closed complex of the KF with a primer-template and the complementary dNTP by the inhibition of the formation of a 64 kDa band in the digestion pattern produced by limiting amounts of trypsin (*13*). While the placement of an AAF adduct in the protein active site completely inhibits this conformational change, an AF adduct, which is less distorting and is easily bypassed by KF, inhibits the conformational rearrangement to a much lesser extent. Also consistent with previously published kinetic data (*12*), the effects of the adducts on the polymerase−DNA dissociation constants (ref *11*, and this work) and the conformational change (this work) are detected only when they are positioned in the primer-template junction and are not observed if the adduct is one nucleotide before the active site position.

The results described here do not alone provide enough information to allow the determination of which particular step in the nucleotide insertion process is inhibited by the AAF adduct. Generally, the same protease cleavage pattern might be produced if any one of the following occurred: (i) nucleotide binding is blocked by the adduct, thus inhibiting the conformational change; (ii) the nucleotide cannot induce the conformational change because of the contacts made by the adduct in the active site, or because the structure of DNA is altered by the adduct; or (iii) the conformational change is induced, but does not lead to formation of the stable, catalytically competent closed complex because the structure of the active site is perturbed by the adduct.

The latter situation would resemble the one that takes place when the DNA polymerase I attempts to incorporate a noncomplementary nucleotide. Indeed, DNA polymerase I does not efficiently distinguish between correct and incorrect dNTPs in ground state binding, and thus its fidelity relies mostly on a geometric selection mechanism of the correct nucleotide in the closed complex (*29, 30*). Kinetic studies have shown that the chemical step becomes rate limiting for nucleotide misincorporation by DNA polymerase I, suggesting that, even though the change is induced, an incorrect nucleotide cannot be positioned in the active site in the proper geometry for nucleophilic attack (*31*). The results of measurements of KF−DNA dissociation constants in the presence of all possible dNTPs suggest that an attempt to accommodate an incorrect dNTP in the ternary complex leads to incorrect active site geometry and a destabilization the KF−DNA complex (*11*). It was also shown *(11*) that when an AAF adduct is positioned in the protein active site, the binding becomes almost independent of the nature of the dNTP present, suggesting that the effect of the adduct is manifested by the inhibition of the initiation of the conformational change and not in the destabilization of the ternary complex.

There are not enough data accumulated in the literature to distinguish between the first two possibilities. Steady-state kinetic experiments have shown that the K_m for incorporation of a dCTP across the dG-C8-AAF adduct is about 50 times higher than opposite an unmodified guanine (*19*). But the K_m is an equilibrium parameter indicating how long the dNTP remains bound to the enzyme−DNA complex, so a high K_m can represent both lower dNTP binding affinity and more time spent in the initial open stage (*32*) caused by a slow rate of conformational change. Since DNA polymerase I shows little discrimination against an incorrect dNTP at the stage of nucleotide binding, it is reasonable to suggest that a perfect match of the structure of the active site and the substrate geometry are not very important for this polymerase at this initial stage. Therefore, it might be expected that the adduct in the polymerase active site does not interfere significantly with nucleotide binding. It must be noted, however, that a recent molecular modeling study of DNA polymerase β bound to a primer-template containing a benzo[*a*]pyrene adduct showed that this adduct may interact with the same amino acids that are involved in the binding of the incoming dNTP (*33*). Determination of the K_d for nucleotide binding by KF bound to AAF-modified DNA might further clarify the mechanism of inhibition of the conformational change in this system.

In summary, the most important observation made from this study is that the AAF adduct inhibits the conformational change in the structure of the DNA polymerase and that it does it more strongly than the AF adduct. A wealth of scientific data have been accumulated showing that the deacetylated AF adduct is structurally different from the AAF adduct and is much more easily bypassed by the replicative

enzymes. In the absence of available structural data on complexes of polymerases with AF- or AAF-modified DNA, to our knowledge the results described here provide the first mechanistic explanation for this phenomenon.

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