Interaction of *Escherichia coli* DNA Polymerase I (Klenow Fragment) with Primer-Templates Containing N-Acetyl-2-aminofluorene or N-2-Aminofluorene Adducts in the Active Site*

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DNA adducts formed by aromatic amines such as N-acetyl-2-aminofluorene (AAF) and N-2-aminofluorene (AF) are known to cause mutations by interfering with the process of DNA replication. To understand this phenomenon better, a gel retardation assay was used to measure the equilibrium dissociation constants for the binding of an exonuclease-deficient *Escherichia coli* DNA polymerase I (Klenow fragment) to DNA primer-templates modified with an AAF or AF adduct. The results indicate that the nature of the adduct as well as the presence and nature of an added dNTP have a significant influence on the strength of the binding of the polymerase to the DNA. More specifically, it was found that the binding is 5–10-fold stronger when an AAF adduct, but not an AF adduct, is positioned in the enzyme active site. In addition, the polymerase was found to bind the unmodified primer-template less strongly in the presence of a noncomplementary dNTP than in the presence of the correct nucleotide. The same trend holds true for the primer-template having an AF adduct, although the magnitude of this difference was lower. In the case of the AAF adduct, the interaction of the polymerase with the primer-template was stronger and almost independent of the nucleotide present.

It is well established that the presence of DNA adducts in the template strand can impede or block DNA synthesis at a replication fork. Although most bulky adducts inhibit DNA synthesis strongly, some can be bypassed readily *in vitro*. The well studied carcinogen N-acetyl-2-aminofluorene (AAF) can form both types of adducts in DNA; N-(deoxyguanosin-8-yl)-2-acetylaminofluorene adducts (dG-C8-AAF) are known to be strong blocks to DNA synthesis, whereas N-(deoxyguanosin-8-yl)-2-aminofluorene adducts (dG-C8-AF) can be bypassed by all polymerases tested (1). The mutagenic consequences of each adduct are also quite distinct. The dG-C8-AAF adduct results in mostly frameshift mutations in bacteria, whereas the other has the fluorene ring stacked within the helix (5). The ratio of these conformations seems to be dependent on the sequence within which the adduct lies (7). Although it has been assumed that the structural differences between the AAF and AF adducts are responsible for the observed in the biological effects, the molecular mechanism that is operating is not known in any detail.

The Klenow fragment of *Escherichia coli* DNA polymerase I is a 68-kDa protein that carries a polymerase and 3′–5′-exonuclease activities on a single polypeptide chain. Because of its simple structure, this enzyme has served as a model enzyme for studying the mechanism of DNA synthesis for three decades. In efforts to understand this process better, several crystal structures of the Klenow fragment and other polymerases have been solved (8–13), and mutations in conserved positions have indicated sites that are responsible for polymerization, proofreading, and DNA binding (for a review, see Ref. 8).

In addition, pre-steady-state kinetic experiments have provided a quantitative description of each step in the nucleotide insertion pathway by Klenow fragment (14–16). It is widely accepted that the process of incorporation of a dNTP into the nascent DNA chain involves a number of sequential steps that are generally described in terms of two different conformations of the DNA polymerase. It is thought that in the open conformation the polymerase can bind to the primer terminus after which it is converted to the closed conformation by the incoming dNTP (17, 18). Upon formation of a new phosphodiester bond, pyrophosphate is released, and the DNA polymerase returns to the open conformation, allowing for translocation of the enzyme to the new primer terminus. The nonchemical event that occurs between nucleotide binding and phosphodiester bond formation (presumably the conformational change) has been shown to limit the rate of incorporation of a correct nucleotide (14), whereas the chemical step itself limits the rate of a nucleotide misincorporation (15). These results, together with the fact that the energy difference between right (Watson-Crick) versus wrong (non-Watson-Crick) base pairing cannot account for the extraordinary fidelity of DNA replication (10⁻⁶–10⁻⁹ even in the absence of proofreading), imply a selection mechanism based on the geometry of the base pair and the protein active site. In this model, a wrong nucleotide cannot be positioned properly for the nucleophilic attack in the active site of the polymerase, and this slows down the rate of the bond

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1 The abbreviations used are: AAF, N-acetyl-2-aminofluorene; AF, N-2-aminofluorene; dG-C8-AAF, N-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-C8-AF, N-(2′-deoxyguanosin-8-yl)-2-aminofluorene; HPLC, high performance liquid chromatography.
FIG. 1. Oligonucleotide primers and templates. Primers shown contained either a 3'-OH or were terminated with a dideoxy sugar at the 3'-end. The 28-mer that was used as a template was either unmodified or modified at the C8 position of G6 by an AF or AAF adduct as indicated.

formation significantly. The major role of the base pair geometry in replication has been confirmed recently by Kool and co-workers (19, 20), who showed that a thymidine triphosphate shape analog lacking Watson-Crick pairing ability is replicated with high sequence selectivity.

In the present study we have determined the equilibrium dissociation constant for the interaction of Klenow fragment with AF- and AAF-modified primer-templates and compared them with the values for unmodified primer-templates in the presence or absence of dNTPs. Our results demonstrate that the enzyme-DNA complex is stabilized by the presence of the next correct nucleotide, but binding is weaker when a non-complementary nucleotide is present. Binding of the polymerase to AAF-modified DNA was stronger and almost independent of the nature of the nucleotide present. These data provide additional insight into the mechanisms of replication and mutagenesis.

EXPERIMENTAL PROCEDURES

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 plasmid, which results in about a 100-fold reduction of endogenous 3'-5' exonuclease activity (11). T7 DNA polymerase (exo-), T4 polymerase kinase, and T4 DNA ligase were also purchased from Amersham Pharmacia Biotech.

Oligonucleotides were obtained from Midland Certified, Inc. Site-specifically modified 12-mer (GTGATGCTGGGATCTGCTCTAA) was synthesized and purified as described previously (21). All dNTPs and dideoxy-NTPs were ordered from Amersham Pharmacia Biotech. [γ-32P]ATP was from ICN Biomedicals.

Methods—The sequences of oligonucleotides that were used in this study to create model primer-templates are shown in Fig. 1. The 28-mer template oligonucleotide was modified with an AF or AAF adduct at G6, which positions the adduct on the junction of the single strand and template oligonucleotide was modified with an AF or AAF adduct at G6, respectively, having a 3'-OH. Lanes 4–6 are the same oligonucleotides after primer extension. Lanes 7 and 8 are 21- and 22-mers with 3'H. Lanes 9 and 10 are the same oligonucleotides after primer extension.

**Primer Extension Reactions**—To show the absence of a 3'-OH on the primer terminus of the oligonucleotides, primer extension reactions were carried out using these oligonucleotides as primers. Thus, primer-templates consisting of an unmodified 28-mer and the 32P-labeled primer (approximately 1 μM each) were incubated with a 0.4 mM concentration of each of the four dNTPs and 2 units of the Klenow fragment in 20 μl of 50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, and 50 mM NaCl. The corresponding dideoxy-NTP (0.4 mM) and Sequanase 2.0 (2 μl) were added to the reaction mixture. The resulting 21-mer and 22-mer were purified by electrophoresis in 20% polyacrylamide gel with 8 M urea.

**Determination of the Equilibrium Dissociation Constant**—To determine the equilibrium dissociation constants for the polymerase-primer templates was carried out by a method similar to that described by Astatke et al. (23). This method has been employed successfully to measure the binding constants for DNA polymerase I and polymerase I mutants (23). The DNA binding reactions were performed in 50 mM Tris-HCl, pH 7, containing 10 mM MgCl₂, 1 mM dithiothreitol, and 0.05 mg/ml bovine serum albumin. 2-μl aliquots of the reaction mixture were taken, and the reaction was stopped with 10 μl of gel loading buffer containing 90% formamide and 5 mg/ml bromophen blue and xylene cyanol. The samples were analyzed on 20% denaturing polyacrylamide gel electrophoresis (Fig. 2). An identical procedure was used for the primer extension studies on the AAF- and AF-modified templates (Fig. 3).

**Gel Retardation Assay**—Determination of the equilibrium dissociation constants for the polymerase-primer templates was carried out by a method similar to that described by Astatke et al. (23). This method has been employed successfully to measure the binding constants for DNA polymerase I and polymerase I mutants (23). The DNA binding reactions were performed in 50 mM Tris-HCl, pH 7, containing 10 mM MgCl₂, 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 1-μl reaction containing 10–100 pm labeled duplex DNA, increasing amounts of Klenow exonuclease (typically 0.05–81 μM), and 36 μM dNTP (if present). The reaction mixtures were loaded onto a native 7% polyacrylamide gel pre-equilibrated with 0.4 × TB buffer (0.04 M Tris borate, pH 8.3). Gels were fixed with 7% acetic acid, dried, and scanned using a PhosphorImager. The amount of complex formed at equilibrium was estimated as the difference in the band intensities of free primer-template and the intensity of this band without the polymerase addition.

**Data Analysis**—The dissociation constant (Kₜ) of a protein-DNA complex measured by gel retardation assay is equal to the protein concentration at which half of the DNA is bound to the protein, providing that protein concentration used is much higher than the DNA concentration. The protein-bound and free oligonucleotides separated by gel electrophoresis were quantified by scanning the gels in a Molecular Dynamics PhosphorImager SF. To obtain the Kₜ, the fraction of the DNA bound to the protein was plotted against the 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. The values for the Kₜ ± S.E. were obtained from the resulting fit of this equation.

**RESULTS**

**Primer Extensions Using AAF- and AF-modified Templates**—The dG-C8-AAF adduct is generally known to block DNA replication much more strongly than the dG-C8-AF ad-
extension was carried out using the Klenow fragment (exo 5′ → 3′). The 21-mer primer was annealed to either the unmodified 28-mer or the AAF- or AF-containing template. Primer extension was carried out using the Klenow fragment (exo 5′ → 3′), and the reaction was terminated after the period of time indicated under each lane as described under “Experimental Procedures.”

Determination of the Equilibrium Dissociation Constants—To determine the dissociation constants for the binding of the Klenow fragment to DNA, 32P-labeled primers were annealed to the templates, and these duplexes were incubated with increasing amounts of polymerase. These mixtures were then immediately loaded and run on a nondenaturing polyacrylamide gel producing a band that was the result of the formation of the polymerase-DNA complex (Fig. 4A). The small amount of free primer is caused by the dissociation of the primer-template which is unavoidable at the low DNA concentrations used for these experiments. The polymerase does not form any detectable complexes with the single-stranded primer even at protein concentrations much higher than that used in this study (Fig. 4A, lane 9), nor does it bind to the modified or unmodified templates (not shown). The amount of complex formed at equilibrium was estimated from the ratio of band intensities of free primer-template to the intensity of this band without the addition of polymerase. This analysis allows measurement of the amount of complex formed in solution before it was loaded on the gel and the neglect of the slight amount of dissociation of DNA-protein complexes which occurs in the gel. The data obtained this way were then fitted to an equation for single-site ligand binding (Fig. 4B), and apparent $K_d$ values were calculated. A slight variation of this method was used successfully in prior studies to determine the dissociation constants for polymerase I binding to primer-templates (25).

Absence of a 3′-OH on the Primer Does Not Change the Dissociation Constant—To determine the effect of the presence of dNTPs on the dissociation constant, the binding experiments need to be carried out under the conditions where nucleotides cannot be incorporated. To prevent this reaction, a primer-template having a deoxynucleotide on the 3′-end of the primer was prepared and used to form the primer-template. In the absence of a dNTP, the dissociation constant determined for interaction of the protein with the 3′-dideoxy terminated primer-template was found to be identical to that for interactions with normal primer-templates (Table I). Consistent with the available structural information (8), this result implies that there is no direct interaction of the DNA polymerase active site carboxylates with the 3′-OH of the primer terminus in the ground state. Also, as shown below, binding was the same in the presence and absence of a 3′-OH in the primer for the modified templates.

Binding to AAF- and AF-modified Templates—To gain an understanding of which step of a nucleotide incorporation is influenced by the DNA adduct, we first determined the dissociation constants in the absence of dNTPs for the Klenow fragment on primer-templates containing AAF and AF adducts (Table I). These data indicate that when the AAF adduct is positioned so that it is the next position for incorporation, the polymerase binding is about 10-fold stronger than to the unmodified primer template. Surprisingly, binding to an identical template but containing an AF adduct gave dissociation constants identical to the unmodified template. The absence of a 3′-OH had no effect on these results (Table I). If the adduct is positioned at the +1 or +2 position in line for incorporation, placing them in the single-stranded region of the template,
TABLE I

Dissociation constants of the Klenow fragment-DNA complexes in the absence of dNTPs in which the adducts are positioned in the polymerase active site

| Template          | Kd of 22-mer primer | 3'-OH | 3'-H |
|-------------------|----------------------|-------|------|
| Unmodified        | 0.46 ± 0.20          | 0.40 ± 0.20 | nM  |
| AAF-modified      | 0.07 ± 0.03          | 0.05 ± 0.02 | nM  |
| AF-modified       | 0.41 ± 0.22          | 0.38 ± 0.06 | nM  |

Klenow Fragment Binding in the Presence of dNTPs—It has been shown that E. coli DNA polymerase I shows little discrimination against nonpairing dNTPs at the stage of nucleotide binding (24). Thus the dissociation constants for the four nucleotides from the polymerase bound to the primer-template junction are approximately the same. However, the dissociation constants for the polymerase in the presence of each nucleotide have never been measured. To make these measurements, we prepared primer-templates where the primer contained a 3'-dideoxy-terminated nucleotide and measured the dissociation constants of the polymerase in the presence of each dNTP. Interestingly, unlike the data measured for dNTP dissociation, the binding of the polymerase to DNA is much stronger in the presence of the correctly paired nucleotide than in the presence of an incorrect nucleotide (Table III). Indeed, the complex is about 100 times more stable when the next correct nucleotide (dCTP in this case) is present than in the presence of a dATP or dGTP and about 15 times more stable in the presence of another pyrimidine, dTTP. Virtually identical results were obtained for an unmodified template using a 3'-dideoxy-terminated 21-mer primer; in this case the next correct nucleotide (dTTP) caused the strongest binding, dATP and dGTP caused 100-fold higher dissociation constants, and dCTP was about 10-fold higher (data not shown). Thus the differences in binding are not the result of a special interaction with one of the nucleotides but are clearly dependent on having the correct versus incorrect nucleotide bound in the active site.

When the binding studies were carried out on the primer-template that positions an AAF adduct in the active site of the polymerase, the presence or identity of the dNTP had no effect on the dissociation constants; in all cases the Kd was about 0.1 nM (Table III), very close to the value in the absence of the nucleotide for the AAF-modified primer-template (Table I). When an AF adduct was present in the active site of the protein, the Kd in each case was intermediate between the value determined for binding to the unmodified and AAF-modified primer-template (Table III). The only exception was dCTP, the next correct nucleotide, where the binding strength was lower than that determined for the unmodified and AAF-modified templates.

DISCUSSION

Acetylaminofluorene adducts have been studied intensively as model DNA lesions to understand the mechanism of mutagenesis and carcinogenesis. Despite a great deal of structural, mutational, and kinetic data available regarding these DNA lesions, the exact mechanism of the mutagenicity and the reason for the different behavior of AF and AAF adducts during DNA replication are not clear. We have begun to address these questions by attempting to understand the molecular interactions that occur between DNA polymerases and primer templates that position these adducts within the enzyme active site. In the experiments described here, we have measured the dissociation constants of the Klenow fragment from AAF- and AF-modified primer-template complexes where the adducts are positioned in or near the active site and in the presence or absence of each of the dNTPs.

Measurement of equilibrium binding constants of DNA polymerases with DNA has been used extensively to study the mechanism of DNA polymerase action and the function of polymerase mutants. These constants are typically measured using single-turnover kinetics experiments (14, 25–27), DNase I footprinting methods (28), or gel mobility shift assays (23). The values that have been measured for wild-type Klenow fragment range from 0.2 to 8 nM, and prior measurements using the gel mobility assay in the absence of dNTP gave a value of 0.3 nM (23). The present study, which used a slightly different DNA construct, gave a comparable value of 0.4 nM under otherwise similar conditions.

No prior studies have measured dissociation constants in the presence of incorrect dNTPs because the kinetic method normally used to measure dissociation constants needs to have the correct nucleotide present. In the present study, measurements in the presence of each of the dNTPs was accomplished by the use of a primer-template containing a nonreactive 3'-dideoxynucleotide primer terminus. Under these experimental conditions, it was impossible for the dNTP to be incorporated, and the only possible fate of the complex was dissociation presumably because the rate of dissociation of the Klenow-DNA complex is much faster than the rate of shifting of the primer terminus to the exonuclease domain (15, 29, 30). Also the absence of a 3'-OH did not effect the binding strength; in the absence of a dNTP the dissociation constants were identical.
for templates containing or lacking a 3'-OH (Table I). This result provides further evidence that the 3'-OH is not involved directly in the ground state DNA binding of the polymerase, consistent with the published crystal structures of polymerase-DNA complexes (8), (10). Although Mg$^{2+}$-mediated interactions between one of the active site carboxylates and the 3'-OH of the primer have been proposed, these must be too weak to be detected in this system or might be formed after the conversion to the closed conformation (see below).

A great deal is known regarding the interactions of the polymerase with the primer-template (17). It is fairly well established that most DNA polymerases can adopt two different conformations during the process of nucleotide incorporation. These enzymes apparently bind to DNA in a so-called "open" conformation and then undergo a conformational change upon binding of a nucleotide (closed complex). This conformational rearrangement is thought to help bring the incoming nucleotide to the polymerase active site and align it properly for the nucleophilic attack by the 3'-hydroxyl of the primer. Unlike many other polymerases, DNA polymerase I apparently does not discriminate between the correct and incorrect dNTP at the time of nucleotide binding (24). Instead, checking for proper pairing is thought to take place during or after the conformational change that occurs when the polymerase adopts a closed structure. However, apparently all polymerases are able to reach a fully active catalytic configuration if the nucleotide can adopt a Watson-Crick geometry, and it is thought that this so-called induced fit mechanism provides the selectivity during nucleotide incorporation. Moreover, it has been shown for both the HIV reverse transcriptase (26) and the T7 DNA polymerase (17) that the rate of dissociation of the polymerase decreases when the polymerase is bound to a nucleotide and in the closed conformation and increases when the polymerase is bound to a nucleotide in the open conformation.

Consistent with these, we find that positioning of a nonpairing nucleotide in the Klenow active site decreases the stability of the complex with DNA, whereas a correct nucleotide increases the stability (Table III). It is possible that the geometry of an incorrect dNTP bound to the active site does not allow the protein to complete the conformational change to the closed structure or that if the conformational change occurs, the structure is perturbed in the presence of a noncomplementary base. In either case the interactions between protein and the DNA are disturbed thereby decreasing the stability of the complex.

The dissociation constants obtained when an AAF or AF adduct is positioned in the polymerase active site clearly indicate that their presence affect the interaction of the polymerase with both the template and incoming nucleotide. In the absence of a dNTP, the dissociation constant is about 5-fold lower when an AAF adduct is positioned in the active site, whereas an AF adduct has no effect on the binding (Table I). The enhanced binding was somewhat unexpected because the AAF adduct is known to cause a substantial distortion in the structure in the DNA helix (2) and has been shown to inhibit strongly the incorporation across from the adduct by most polymerases (1). Thus, it is possible that the AAF adduct may drastically alter the structure of the primer-template within the active site which results in a complex having vastly different binding properties possibly involving specific interactions between the AAF moiety and amino acid side chains in the active site. In agreement with this model, a recently published footprinting analysis of the complex of T7 polymerase with AAF-modified primer-template (31) demonstrates that the presence of an AAF adduct reduces the hypersensitive sites seen in the footprint of unmodified DNA. It is important to note that a kinetic analysis has shown that the presence of an AAF adduct does not affect the dissociation rate for the T7 DNA polymerase (32), which may mean that our results are specific to polymerase I.

A second important observation from this study is that this enhanced AAF-induced binding is not affected by the presence of a complementary or noncomplementary dNTP, whereas on an unmodified template the Watson-Crick nucleotide enhances binding, and the other nucleotides greatly reduce it (Table III).

A recent molecular modeling study of DNA polymerase β bound to a primer-template modified with the bulky polycyclic aromatic hydrocarbon, benzo[a]pyrene, showed that the adduct may interact with the same amino acids that are involved into the nucleotide binding (33). It is tempting to propose that the AAF adduct can also interfere with the nucleotide binding either sterically or by hydrogen bonding with one of the active site amino acids. Although no structural information has yet been published for an AAF adduct positioned at a primer-template junction, it has been shown that in some sequence contexts the dG-C8-AP adduct structure can resemble that determined for a dG-benzo[a]pyrene adduct (34). Thus, it is possible that the AAF adduct can resemble a benzo[a]pyrene adduct at a primer-template junction where the benzo[a]pyrene adduct has been suggested to displace the modified guanine into the major groove and can stack over the junctional base pair (35). Alternatively, it may be possible that the presence of the AAF adduct inhibits the conformational change that occurs upon nucleotide binding and removes any possible contribution that a nucleotide would have on the dissociation constants.

Consistent with its ability to adopt two different conformations, the AF adduct produced a set of dissociation constants that had intermediate values between the unmodified and AAF-modified primer-template when a noncomplementary nucleotide was present (Table III). One possible explanation for this observation is that what was being measured is the average of two sets of data, one with the adducted guanine in the normal anti conformation and the other having the more distorted AAF-like syn conformation. We also note that the ternary complex is most stable in the presence of dCTP, which is in agreement with incorporation data showing that dCTP is most often incorporated across from an AP adduct (36).

Finally, no differences in the dissociation constants compared with the unmodified case are observed when either adduct is positioned at the +1 or +2 position on the primer-template (Table II). This result is consistent with the data from a recent study by Miller and Grollman (37) who demonstrated that kinetic parameters for nucleotide incorporation are not influenced by an adduct positioned away from the primer-template junction (37). Thus, whatever interactions are causing the AAF-induced enhanced binding they are not simply a nonspecific interaction of the adduct with the hydrophobic amino acids and are most probably related to the mechanism of polymerization in the active site. This is supported further by the differences observed between the AAF and AF adduct, which are chemically very similar but lead to very different behaviors when positioned in the active site. A full understanding of these interactions awaits a crystal structure of a DNA polymerase complexed with a primer-template containing an AAF and AF adduct.

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