Mechanistic Insights into Replication Across from Bulky DNA Adducts: A Mutant Polymerase I Allows an *N*-Acetyl-2-aminofluorene Adduct To Be Accommodated during DNA Synthesis[†]

Samer Lone and Louis J. Romano*

Department of Chemistry, Wayne State University, Detroit, Michigan 48202

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ABSTRACT: The molecular mechanism that allows a polymerase to incorporate a nucleotide opposite a DNA lesion is not well-understood. One way to study this process is to characterize the altered molecular interactions that occur between the polymerase and a damaged template. Prior studies have determined the polymerase−template dissociation constants and used kinetic analyses and a protease digestion assay to measure the effect of various DNA adducts positioned in the active site of Klenow fragment (KF). Here, a mutator polymerase was used in which the tyrosine at position 766 of the KF has been replaced with a serine. This position is located at the junction of the fingers and palm domain and is thought to be involved in maintaining the active site geometry. The primer-template was modified with *N*-acetyl-2-aminofluorene (AAF), a well-studied carcinogenic adduct. The mutant polymerase displayed a significant increase in the rate of incorporation of the correct nucleotide opposite the adduct but was much less prone to incorporate an incorrect nucleotide relative to the wild-type polymerase. Both the wild-type and the mutant polymerase bound much more tightly to the AAF-modified primer-template; however, unlike the wild-type polymerase, the binding strength of the mutant was influenced by the presence of a dNTP. Moreover, the mutant polymerase was able to undergo a dNTP-induced conformational change when the AAF adduct was positioned in the active site, while the wild-type enzyme could not. A model is proposed in which the looser active site of the mutant is able to better accommodate the AAF adduct.

The mechanism used by DNA polymerases to accurately incorporate a nucleotide during DNA replication has been the subject of intense study for the past four decades (*1*). Kinetics and crystallographic analyses have shown that this process proceeds through a number of sequential steps that involve at least two different conformations of the DNA polymerase (*2*−*4*). The conformational rearrangement from an open binary to a closed ternary complex that is induced by the binding of a dNTP results in a structure in which the dNTP is paired with the template base and is properly aligned for the nucleophilic attack by the 3′-hydroxyl of the primer. Apparently, polymerases are only able to reach a fully active catalytic configuration if the nucleotide base can adopt a Watson−Crick geometry with the template base, and it is thought that this process provides the much of the selectivity during nucleotide incorporation.

The similarity of the mechanistic details among a variety of polymerases is likely the result of the remarkable resemblance of the structures of these enzymes. The general structure of the polymerase has been compared to that of a human right-hand with three distinct subdomains designated as the palm, fingers, and thumb. In this model, the palm subdomain contains highly conserved catalytic residues that are involved in the phosphoryl transfer reaction, the fingers subdomain interacts with both the incoming nucleotide and the template base it will be paired with, and the thumb subdomain is involved in positioning the duplex DNA and has been shown to have roles in both processivity and translocation (reviewed in refs *4*−*6*).

The fingers subdomain contains several planer hydrophobic amino acid residues that participate in the correct positioning of the dNTP when the closed conformation is formed (*3*, *7*, *8*). One of the best studied of these residues is the tyrosine at position 766, which is located at the base of the O helix in the fingers subdomain near the junction with the palm (*5*, *9*). In both the Taq and the Bst polymerase open complexes, this tyrosine side chain is stacked above the terminal base pair of the primer-template. In the closed complex, the tyrosine has rotated away from the DNA, and the template base has moved into the position it had occupied allowing the formation of the base pair with the incoming dNTP (*3*, *10*). Studies with DNA polymerase I (Klenow fragment) (KF)[1] have shown that Y766S and Y766A mutants have mutator phenotypes (*11*), and analogous mutations in eukaryotic polymerase β also lead to higher levels of mutagenesis (*12*, *13*). It is likely that the reduced fidelity of these mutants is caused by a different active site conforma-

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* To whom correspondence should be addressed. Tel.: (313) 577-2584. Fax: (313) 577-8822. E-mail: LJR@chem.wayne.edu.

[1] Abbreviations: AAF, *N*-acetyl-2-aminofluorene; AF, 2-aminofluorene; KF, Klenow fragment; dG-C8-AAF, *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-C8-AF, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene; PAGE, polyacrylamide gel electrophoresis; K_M, Michaelis constant; V_{max}, the maximum rate of reaction; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; K_d, dissociation constant.

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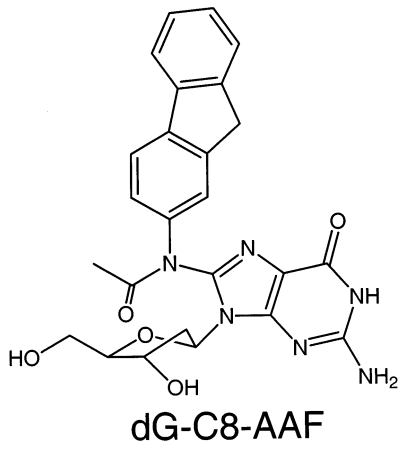


FIGURE 1: Structure of the *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF).

tion, possibly resulting from an alteration in the conversion from the open to the closed form.

Although a great deal is known regarding the mechanism of synthesis by a polymerase using a normal DNA template, very little is known about how a bulky DNA lesion affects this process. An understanding of how a polymerase interacts with this class of DNA damage is particularly important because of the discovery of the bypass or Y-family polymerases, whose role is to carry out DNA synthesis past DNA damage that otherwise blocks replication (*14*, *15*). There have been a few indirect studies that have attempted to explain the effects of bulky lesions on DNA replication by correlating the structures of these adducts in double-stranded DNA or at a primer-template junction with the mutagenic event that a particular structure induces (*16*, *17*). We have developed several methods to study directly the interactions between a polymerase and a primer-template and have used them to measure how the presence of bulky adducts in the active site affects these interactions. In this regard, we have used a gel retardation assay (*18*, *19*) and a limited trypsin digestion analysis (*20*) to show that bulky lesions, such as benzo[*a*]pyrene, 2-aminofluorene (AF), and *N*-acetyl-2-aminofluorene (AAF) adducts can be well-accommodated within the active site of the polymerase in the open binary complex but that these adducts can interfere with the conformational change of the polymerase to the closed catalytically active ternary complex. Presumably, these bulky adducts alter the structure and stability of the ternary complex, thus affecting an important step that is crucial in determining the fidelity of the nucleotide incorporation step (*1*).

One of the best studied bulky DNA adduct is one formed by treatment of cells with the potent model chemical carcinogen, AAF. Two major adducts result from this exposure: the *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF) (Figure 1) and its deacetylated derivative, the *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene adduct (dG-C8-AF). Although the AF adduct has been shown to be easily bypassed during in vitro DNA synthesis, the AAF adduct represents a strong block to synthesis by all replicative polymerases studied (*21*, *22*). These differences are thought to be related to the structure that each adduct presents to the DNA polymerase. Most spectral, enzymatic, and theoretical studies suggest that the AF structure produces much less distortion in the DNA helix than the AAF adduct (*23*−*27*). Multidimensional NMR experiments have shown that the guanine bearing the C8-AAF adduct rotates from anti to syn conformation in double-stranded DNA helix with the fluorene ring inserted into the helix (base displacement model, ref *28*).

In the present study, we report our initial efforts aimed at understanding how specific amino acid residues in the polymerase active site affect the interactions with a modified DNA template. We find that substitution of the tyrosine at position 766 of KF with a serine substantially alters the interactions and mechanism of action of the polymerase on both unmodified and AAF-modified DNA. Not only does this mutation affect the rate of nucleotide incorporation opposite the adduct, but it also alters the effect of dNTPs on both the binding to the primer-template and the conformational change to the closed ternary complex.

MATERIALS AND METHODS

Materials. Wild-type and Y766S Klenow fragment (exo-) clones were generously provided by Dr. Catherine Joyce of Yale University. Both WT and Y766S Proteins were overexpressed and purified as described (*29*) and contained the D424A mutation, which eliminates the 3′-5′ exonuclease activity. The specific activity of the protein was determined as described (*30*). Protein concentrations were determined colorimetrically by the Bradford assay (*31*) using Bio-Rad laboratory reagents. T4 polynucleotide kinase was purchased from Amersham Pharmacia Biotech. Trypsin and terminal deoxynucleotide transferase came from Boehringer Mannheim. Oligonucleotides were obtained from Midland Certified Inc. dNTPs were purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from ICN Biomedicals.

Synthesis and Purification of Oligonucleotides. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Site-specifically modified 28-mer templates were synthesized, purified, and characterized as described (*18*). The primers lacking 3′-OH were obtained by extension of corresponding oligonucleotides with ddNMPs using terminal deoxynucleotide transferase as described (*20*).

Primer Extension Analysis. ^{32}P-labeled 12-mer primer (1 nM) was annealed to a 2-fold excess of the AAF-modified 28mer template (2 nM). The reactions were started with dNTPs (100 μM) and 10 nM KF in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. At the indicated time points after the addition of the polymerase (0−60 min), 5 μL aliquots of the reaction mixture were taken, and the reaction was stopped by addition to 10 μL of gel loading buffer containing 90% formamide and 5 mg/mL bromophenol blue and xylene cyanol. The samples were analyzed on a 20% denaturing polyacrylamide gel. Product formation was measured by phosphoimager analysis and quantified using Molecular Dynamics ImageQuant. Total bypass synthesis was determined by dividing the total radioactivity across from and extended past the adduct at each time point by the total radioactivity in each lane. Full extension was determined by dividing the total radioactivity of the 28-mer product by the total radioactivity in each lane.

Steady-State Kinetics. Steady-state kinetics using standing-start single nucleotide insertions and extensions were carried

out similarly to those described (*32*). Typical reactions were carried out in 10 μL volumes in the presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 0.05 mg/mL BSA. 0.1 μM primer-templates were annealed by heating to 90° and slow cooling. Reaction mixtures containing 0.001−0.03 units of DNA polymerase were incubated at room temperature for 1−12 min. Both polymerase concentrations and times were varied for each nucleotide examined so that less than 20% incorporation occurred. The extent of each reaction was determined by running quenched reaction samples (95% formamide, 20 mM EDTA, 0.05% xylene cylanol, and bromophenol blue) on a 20% denaturing polyacrylamide gel to separate unreacted primer from insertion products. As described previously (*33*), relative velocities were calculated as the extent of the reaction divided by the reaction time and normalized for the varying enzyme concentrations used. The Michaelis constant (K_m) and maximum rate of the reaction (V_{max}) were obtained from Hanes−Wolf plots of the kinetic data. Insertion (F_{ins}) and extension (F_{ext}) frequencies were determined relative to dC:dG and dA:dT, respectively, according to equations developed by Mendelman et al. (*34*, *35*). The frequency of insertion and extension are defined as $F = (V_{max}/K_m)$[wrong pair]/(V_{max}/K_m)[right pair]. All reactions reported represent an average of at least three experiments and had standard deviations less than 20%.

Gel Retardation Assay. Equilibrium dissociation constants (K_d) for the polymerase−primer-template complexes were determined as described (*18*). 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 $MgCl_2$, 1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, 4% glycerol, and 0.4 mM dNTP (if present). The reaction mixtures were analyzed on a native 7% polyacrylamide gel preequilibrated with 36 mM Tris borate buffer, pH 8.3. Quantification was performed using Molecular Dynamics PhosphorImager 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 was 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 Unmodified and AAF-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 KF (exo-), and 0−16 mM dNTP. Two microliters of trypsin solution in water (15 ug/mL final) 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 (*36*). Gels were fixed and stained using the Silver Stain Plus Kit (BIO-RAD) according to the manufacturer's protocol. At 16 mM dNTP concentration, the relative levels of cleavage were determined by scanning the gels and using NIH Image to quantify the intensity of each band. Each determination is representative of two to three individual experiments.

RESULTS

Primer Extensions using the AAF-Modified Templates. As a first step to determine the effect of the replacement of tyrosine with serine at position 766 of KF on the replication of AAF-modified DNA, DNA synthesis was carried out using the primer-template shown in Figure 2. Using wild-type KF, most of the DNA synthesis on this template was stalled at a position one nucleotide before the AAF adduct in the template. Over the 60 min time course, no more than 20% of the synthesis occurred across from the adduct or extended past the adduct position (Figure 2A,B). With the Y766S mutant polymerase, synthesis was also blocked one nucleotide before the adduct position, but in this case a much higher percentage of synthesis was able to occur across from the adduct (Figure 2A): after 60 min, approximately 40% of the product extended to the position across from the AAF adduct (Figure 2B). Interestingly, a much smaller percentage of full extension occurred, using this mutant (Figure 2C), whereas the wild-type KF was able to give 6% full extension, and the mutant only produced about 1%. Thus, while the mutant was better able to incorporate across from the adduct, it was less able to extend from this structure.

Single Nucleotide Incorporation Kinetics. To more fully explore the differences between the wild-type and the Y766S polymerases, a single nucleotide steady-state kinetic analysis was carried out using the templates shown at the top of Table 1. Using the unmodified primer-template, both the wild-type KF and the Y766S mutant had a strong preference for the incorporation of dC across from the template dG, with the V_{max}/K_m for Y766S about 1/3 that of the wild-type KF.

Thus, the F_{ins} for the incorporation of dG, dA, and dT across from a dG was 3.6, 12, and 72 times greater, respectively, for the mutant than was found for wild-type KF (Table 1), although the differences in the absolute values of V_{max}/K_m for the two polymerases for the incorporation of the incorrect nucleotides were smaller (Table 1). Prior studies compared the steady-state rate of misinsertion of dTTP by this mutant across from an unmodified dG (*37*). Both this study and the data shown in Table 1 indicate that the mutant enzyme is able to incorporate dTTP across from a dG about 50-fold faster.

When these experiments were repeated with the AAF-modified primer-templates, the V_{max}/K_m for the incorporation of dC across from the modified G was 16 times greater for the mutant polymerase (Table 1), confirming the enhanced ability of Y766S to incorporate a nucleotide opposite the adduct as shown in Figure 2. Interestingly, the reduced ability of the mutant to discriminate between the correct and the incorrect nucleotide that was observed in the case of the unmodified template was not observed using an AAF-modified template. Although the absolute rate of misincorporation by the mutant is equal to or higher than that observed for the wild-type polymerase (compare V_{max}/K_m values in Table 1), the F_{ins} is lower for the Y766S mutant for the incorporation of each of the incorrect nucleotides.

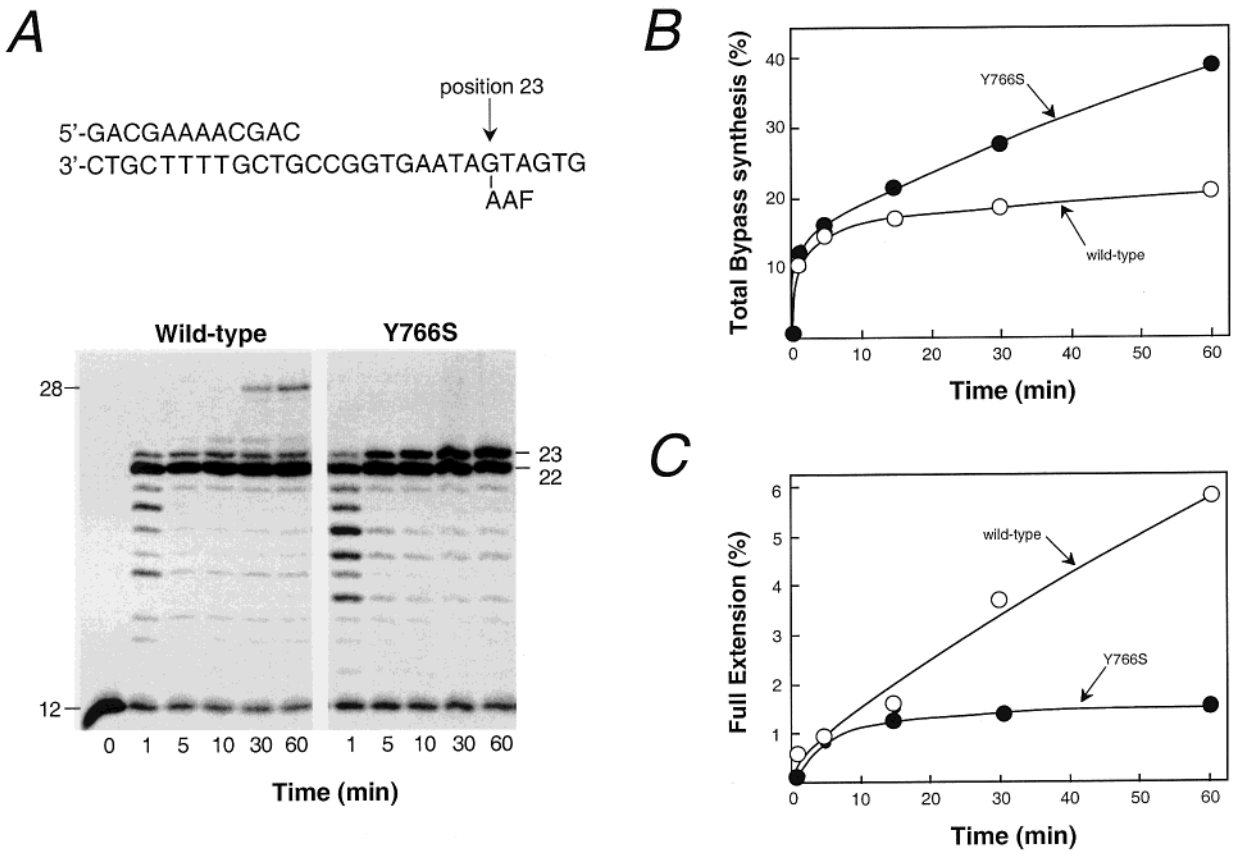


FIGURE 2: (A) Running-start primer extension analysis of AAF-modified primer-templates. 12-mer primer was annealed to the 28-mer template, containing an adduct at the position indicated. For both WT and Y766S Klenow fragments (exo-) all four dNTPs were added, and the reaction was terminated after the period of time indicated under each lane. (B) The insertion and extension products were quantified as explained in Materials and Methods and were plotted as a function of time.

Table 1: Kinetic Parameters for Insertion by Wild-Type and Y766S Klenow Fragments[a]

dNTP
5'-GACGAAAACGACGGCCACTTAT
3'-CTGCTTTTGCTGCCGGTGAATAXTAGTG

	WT		Y766S		
dNTP:X[b]	V_{max}/K_m (% min^{-1} μM^{-1})	F^c_{ins}	V_{max}/K_m (% min^{-1} μM^{-1})	F_{ins}	F_{ins} (Y766s)/ F_{ins} (WT)
dCTP:dG	22.6	1	6.6	1	1
dGTP:dG	3.0×10^{-2}	1.3×10^{-3}	3.1×10^{-2}	4.7×10^{-3}	3.6
dATP:dG	1.3×10^{-2}	5.8×10^{-4}	4.5×10^{-2}	6.8×10^{-3}	12
dTTP:dG	8.2×10^{-3}	3.6×10^{-4}	1.7×10^{-1}	2.6×10^{-2}	72
dCTP:dG-AAF	4.4×10^{-2}	1	7.3×10^{-1}	1	1
dGTP:dG-AAF	3.5×10^{-4}	8.0×10^{-3}	4.3×10^{-4}	5.9×10^{-4}	0.074
dATP:dG-AAF	6.9×10^{-3}	1.6×10^{-1}	6.7×10^{-3}	9.2×10^{-3}	0.058
dTTP:dG-AAF	1.6×10^{-3}	3.6×10^{-2}	4.7×10^{-3}	6.4×10^{-3}	0.18

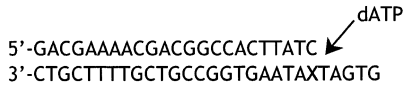
[a] Kinetics of insertion were determined as described under Materials and Methods using the primer-template shown above. All values represent the mean of at least three experiments and have standard deviations <20%. [b] X = unmodified or AAF-modified guanine. [c] Frequencies of nucleotide insertion were determined using the equation $F_{ins} = (V_{max}/K_m)$[wrong pair]/(V_{max}/K_m)[right pair = dC:dX].

Thus, compared with the wild-type polymerase, the Y766S mutant discriminates more efficiently against the misincorporation of dG, dA, and dT across from the AAF-modified base by approximately 14, 17, and six times, respectively.

Single Nucleotide Extension Kinetics. The primer extension analysis shown in Figure 2 suggested that the Y766S mutant was less able to extend from the primer that terminated across from the AAF-modified guanine. To further examine this result, a single nucleotide extension analysis was carried out using the primer-template shown in Table 2. In this case, the V_{max}/K_m for the wild-type polymerase using an AAF-modified template was about 66 times larger than for the Y766S mutant, confirming the implications of Figure 2.

Binding of KF and Y766S to Unmodified and AAF-Modified Primer-Templates. The role of the tyrosine residue at position 766 has been proposed to play a role in the geometric selection of correct nucleotide substrates (*5*, *9*). This residue is located at the junction of the fingers and palm domain and clearly moves as a result of the conformational change. It has been suggested that this residue might be

Table 2: Kinetic Parameters for Extension by Wild-Type and Y766S Klenow Fragments[a]



X[b]	wild-type V_{max}/K_m (% min^{-1} μM^{-1})	Y766S V_{max}/K_m (% min^{-1} μM^{-1})
dG	11.9	2.5
dG-AAF	2.9×10^{-4}	4.4×10^{-6}

[a] Kinetics of extension were determined as described under Materials and Methods using the primer-template shown above. All values represent the mean of at least three experiments and have standard deviations <20%. [b] X = unmodified or AAF-modified guanine.

responsible for guiding the template base into the active site during DNA translocation during the conversion to the closed complex (*38*). One method that has been used to study this conformational change is to measure the dissociation constants (K_ds) in the absence or presence of dNTPs. A significant decrease in the K_d upon the addition of the correctly pairing dNTP is taken as evidence of the conversion of the complex from the open binary to the closed ternary form (*18*).

The K_ds for the wild-type KF and the Y766S mutant polymerase were determined using the gel retardation method previously used to measure the stability of the complexes formed between the primer-template and either HIV-1 reverse transcriptase or KF (*18*, *39*, *40*) using either unmodified or adducted templates. In the present study, the K_ds for the binding of KF to 2′,3′-dideoxy-terminated primer templates were measured in the absence or presence of each of the four nucleotide bases using either the unmodified or the AAF-modified template shown in Table 1. Consistent with previous results, the addition of the correctly paired nucleotide resulted in an approximate 10-fold increase in the binding strength between the wild-type KF and an unmodified primer-template (Table 3). This enhanced binding provides evidence for the formation of the closed ternary complex. Moreover, the addition of an incorrect nucleotide resulted in a decrease in the stability of the complex (Table 3), which we have interpreted as an attempt by the polymerase to form a closed complex that results in a steric clash within the active site thus leading to a loss of stability of the complex (*19*). Alternatively, the decrease in stability of the closed complex could be attributed to a failure of the incorrect nucleotide to make the proper interactions within the polymerase active site that are normally formed when the correct nucleotide is present.

As with wild-type KF, the stability of the complex between the Y766S mutant and an unmodified primer-template is substantially enhanced in the presence of the correctly paired nucleotide, dCTP (Table 3), and is reduced in the presence of an incorrect dNTP. It is interesting that the increase in stability for the mutant is greater, and the decrease in stability is less than that observed with wild-type KF. This enhanced stability by the Y766S mutant in the presence of an incorrect nucleotide is consistent with the kinetic data shown in Table 1 and may be related to the lower fidelity of this mutant polymerase.

The binding of wild-type KF to an AAF-modified primer-template has previously been studied, and the presence of this adduct in the active site was shown to lead to a more stable complex (*18*). Moreover, it was found that the presence of any dNTP had no effect on the binding strength (Table 3) (*18*), presumably because the AAF adduct is interfering with the polymerase conformational change (*20*). In the absence of a dNTP, the Y766S mutant also binds more tightly to the AAF-modified primer-template than it does in the comparable unmodified case (Table 3). However, unlike what is found with the wild-type KF, the presence of the correctly paired dCTP leads to a substantial increase in the stability of the complex. In addition, the presence of each of the other dNTPs also leads to a more stable complex, a result that may be related to the decreased fidelity of this enzyme.

Relative Ability of Y766S to Undergo a Conformational Change. One method that has been used to confirm the conversion of the polymerase complex to a closed structure, as predicted by the K_d measurements, is a limited tryptic digestion analysis in the absence or presence of each of the four dNTPs (*41*). Trypsin cleavage of KF produces as the major product a fragment with a molecular mass of approximately 64 kDa (Figure 3). This cleavage position maps to a location situated in or near the polymerase active site (*41*). In the presence of the next correct dNTP, proteolysis at this site is inhibited, indicative of a conformational change presumably to the closed ternary complex (Figure 3, lane 5). Moreover, in the case of the wild-type KF, proteolysis is not inhibited in the presence of dTTP (Figure 3A), dATP, or dGTP (Table 4) (*41*).

Repeating these experiments with the Y766S mutant showed that the presence of dCTP also induced the conformational change that inhibited trypsin cleavage (Figure 3, lane 11). Interestingly, the presence of dTTP also resulted in a reduction in the ability of trypsin to cleave at this site, although this reduction was much less than that observed when the next correct nucleotide, dCTP, was present (cf. Figure 3, lanes 11 and 14, Table 4). The presence of dATP

Table 3: Dissociation Constants (K_d) for Complexes of Wild-Type and Y766S Klenow Fragments with Unmodified and AAF-Modified Primer-Templates[a]

	K_d (nM)				
complex	-	dCTP	dTTP	dATP	dGTP
wild-type					
unmodified	1.0 ± 0.4	0.07 ± 0.03	3.7 ± 1.5	8.9 ± 3.3	22.0 ± 11
AAF-modified	0.17 ± 0.08	0.36 ± 0.12	0.24 ± 0.15	0.3 ± 0.1	0.15 ± 0.06
Y766S					
unmodified	2.4 ± 1.3	0.1 ± 0.04	1.8 ± 0.7	7.9 ± 2.0	2.7 ± 1.2
AAF-modified	0.16 ± 0.04	0.02 ± 0.009	0.07 ± 0.03	0.04 ± 0.02	0.08 ± 0.05

[a] Dissociation constants were determined using the gel-retardation assay as described under Materials and Methods using template-primer shown in Table 1.

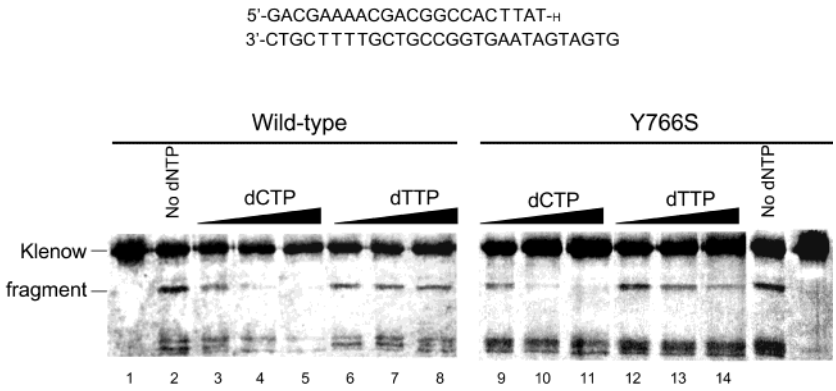


FIGURE 3: Tryptic digestion of WT and Y766S KF bound to the 22/28-mer primer-template in the presence of increasing concentrations of nucleotides. KF or Y766S (0.3 mM) was incubated with the primer-template (0.6 mM) and increasing dNTPs (1.5−16 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described in Materials and Methods. Panel A, WT (dCTP, dTTP); panel B, Y766S (dCTP, dTTP).

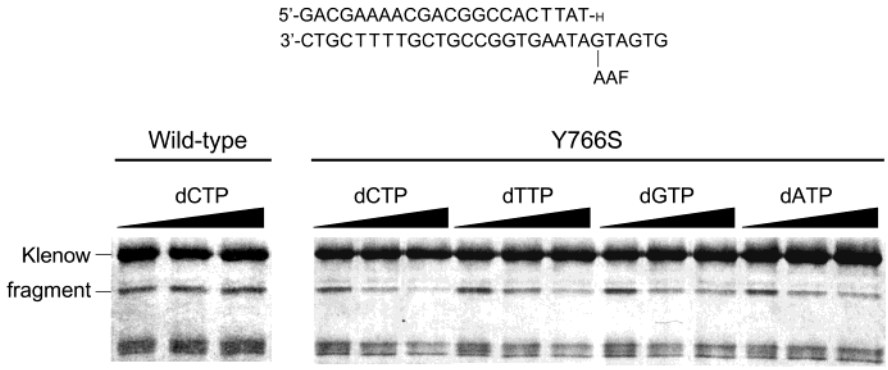


FIGURE 4: Tryptic digestion of WT and Y766S KF bound to AAF-modified 22/28-mer primer-templates in the presence of increasing concentrations of the next correct nucleotide, dNTP. KF (0.3 mM) was incubated with the primer-template (0.6 mM) and increasing dCTP (1.5−16 mM) as indicated. The conformation of the resulting complex was probed by limited tryptic digestion as described in Materials and Methods. Panel A, WT; panel B, Y766S.

Table 4: Relative Levels of Trypsin Cleavage[a]

polymerase	template	dCTP	dTTP	dATP	dGTP
wild-type	unmodified	0.10	0.96	1.0	0.93
Y766S	unmodified	0.20	0.45	0.92	0.87
wild-type	AAF	0.91	0.97	1.1	0.93
Y766S	AAF	0.16	0.31	0.42	0.43

[a] Relative levels of inhibition were determined as described in Materials and Methods by quantifying the polyacrylamide gels that were run using the template-primer shown in Table 1. Cleavage in the absence of dNTP was set to 1.0 for each experiment, and the levels of cleavage were determined relative to this value.

or dGTP did not result in a significant inhibition of trypsin cleavage (Table 4). Note that with the Y766S mutant, the V_{max}/K_m in the presence of dTTP is 1.7×10^{-1} (Table 1), a value significantly higher than that observed for the wild-type KF in the presence of dTTP (8.2×10^{-3}).

Prior studies have shown that the placement of an AAF adduct on the template G in the polymerase active site completely inhibits this conformational change for the wild-type KF (*20*). This result is shown in Figure 4 (left panel), where the presence of dCTP (or any other nucleotide (Table 4)) had no significant effect on the ability of trypsin to cleave the wild-type polymerase. However, when this experiment was repeated with the Y766S mutant (Figure 4, right panel), the addition of dCTP resulted in a clear inhibition of trypsin cleavage, suggesting that this mutant can undergo the conversion to a closed ternary complex even in the presence of an AAF adduct in the active site. The extent of cleavage inhibition for both the wild-type and the Y766S mutant in the presence of each of the four dNTPs is shown in Table 4, which indicates that there are intermediate inhibition levels for each of the noncomplementary dNTPs.

DISCUSSION

The selection of a properly paired nucleotide by a DNA polymerase is achieved through a multistep process in which binding to a dNTP induces a conversion in the structure of the polymerase from an open to closed form. This conformational rearrangement is thought to serve as a method for the polymerase to ensure the geometric shape of the template base and incoming nucleotide substrate fit into the polymerase active site, thereby promoting highly accurate DNA synthesis (*42*). Consistent with this hypothesis are structural studies of DNA polymerases that illustrate a stable conformational transition that is induced upon binding of the properly paired nucleotide (reviewed in refs *4* and *6*).

For the Pol A polymerase family, this structural rearrangement results in a sizable movement of the O-helix in the fingers domain to generate a binding pocket that encompasses the incoming dNTP and the complementary template base. Intimate contacts between the minor groove edge of the template base as well as the base, sugar, and phosphates of the incoming dNTP form the surface of the binding pocket. Amino acid substitutions that either modify the binding pocket geometry or the movements needed to initiate catalysis have been shown to have a compromised fidelity

(*1*). For example, polymerases with substitutions at the tyrosine at position 766 in KF, an integral part of the O-helix that has previously been shown to be important in preserving the active site geometry within the base-pair binding pocket, have been shown to have reduced fidelity (*11*, *37*, *43*). The structural information generated from the homologous Taq polymerase indicates that during the transition from the open to closed complex, the O-helix of the fingers domain rotates toward the active site causing this tyrosine to move from a stacked position on top of the terminal template base pair to a position in front of the incoming nucleotide (*44*).

In this study, we have measured DNA synthesis and polymerase-template interactions using a KF mutant in which the tyrosine at position 766 has been replaced by a serine. Both running-start primer extensions and single nucleotide insertion kinetics show that this mutant polymerase is able to insert the correct nucleotide faster than the wild-type enzyme at template positions modified with an AAF adduct. Also, the V_{max}/K_m for insertion of a C across from an AAF-modified G was 16 times greater for the mutant than the wild-type polymerase. Consistent with previous reports showing substitutions of either serine or alanine for the tyrosine at this position resulted in polymerases with mutator phenotypes (*11*, *37*); we found that the Y766S mutant displayed a decreased discrimination for the incorporation of a correct versus an incorrect nucleotide with an unmodified template. In agreement with these data, a prior steady-state analysis of the Y766S mutant also indicated that the Y766S mutant displayed a decreased fidelity on undamaged templates (*37*). Interestingly, in the present study this trend was reversed for incorporation across from an AAF-modified dG in which the mutant polymerase showed enhanced discrimination for dC.

The differences between the mutant and the wild-type polymerases are consistent with a model in which the altered fit that occurs in the active site of this mutant causes a different alignment for the pairing of the nascent base pair, thereby leading to higher levels of misincorporation. Substitution of a smaller serine for the tyrosine also leads to a more flexible active site that can presumably accommodate the bulky AAF adduct and allow for faster incorporation across from a G bearing this lesion. It is not clear why discrimination for a C is better for this mutant as compared with the wild-type polymerase, but it is possible that the adduct is positioned in such a way that it reduces the size of the active site leading to a tighter fit and a more stringent requirement for proper base pairing.

Others have shown that the Y766S mutant is inefficient at extension from a mispaired primer terminus (*11*, *37*). This, presumably, is due to the altered geometry of the active site that results in a structure that is a poor substrate for incorporation of the next nucleotide. Although insertion across from the AAF adduct was faster for the mutant relative to the wild-type polymerase, extension was significantly slower once a nucleotide was positioned opposite the AAF-modified dG. It is possible that this difference is also due to the altered geometry of the active site and that this structure may resemble the distortion created by a mispair at the primer-terminus.

In addition to differences in the rates of nucleotide incorporation and extension, the mutant polymerase also displayed differences in the interactions with the primer-template. Using a gel-shift analysis, we have previously shown that the presence of a correctly paired dNTP produces enhanced binding between the polymerase and the primer template, while a mispair leads to a reduction in binding strength (*18*). In addition, when the template is modified with an AAF adduct at the next position for incorporation, not only does the wild-type polymerase bind more tightly as compared with an unmodified template, but the binding strength is also independent of the presence of a dNTP (*18*). These results vary greatly from those observed for the Y766S mutant. First, mispairs on unmodified primer-templates led to more stable complexes as compared to those found for the wild-type enzyme. This is especially true in the case of a dG-dG mispair, which has a K_d of 22 nM with the wild-type KF and 2.7 nM with the mutant. The implication of these data is that the additional room in the active site can accommodate the mispair better than the wild-type polymerase, resulting in a smaller steric clash and a more stable complex. Second, using the AAF-modified template, the K_ds for binding to the primer-template in the absence of dNTP are approximately the same for the mutant and wild-type enzymes, suggesting that the interactions with the primer-template in the open binary conformation are similar for these two proteins. However, unlike that observed for the wild-type KF, the mutant displays a significant enhancement of binding strength upon the addition of a dNTP, suggesting that the mutant is capable of forming a stable closed complex while the wild-type enzyme cannot. Unlike what is observed using unmodified templates, this enhanced binding not only occurs when a correctly paired nucleotide is added but also when each of the other nucleotides are present. It is possible that the presumably looser active site present in the mutant polymerase is better able to accommodate the mispair leading to the formation of the stable ternary complex. It is unclear why this enhanced binding is only observed with the modified template.

Using a tryptic digestion analysis, we have been able to detect a conformational change in KF upon the addition of a properly paired dNTP that we have interpreted to indicate the formation of the closed ternary complex (*41*). As predicted by the stability of the K_d for the binding of wild-type KF to AAF-modified primer-templates upon the addition of dNTPs, this trypsin digestion analysis was unable to detect a conformational change when dNTPs were added (*20*). However, with the Y766S mutant a conformational change was observed not only when the correctly paired nucleotide was added but also when each of the other dNTPs was present. Because the 766 tyrosine residue is at the base of the O-helix, others have predicted that this position might be involved in the mechanism that leads to the conformational change that occurs when the closed complex forms (*5*, *9*). Although the mutant enzyme behaved similarly to the wild-type polymerase in both the absence of nucleotide and when the correctly paired nucleotide is added, unlike what is observed using the wild-type, the mutant gave partial trypsin cleavage for a T:G mispair. Moreover, using AAF-modified templates gave partial cleavage for each of the nucleotides with the Y766S mutant, while the wild-type polymerase was not resistant to cleavage under the same conditions. Taken together, these data support the model that the tyrosine at this position plays a role in the conformational change to the close complex.

The fact that additional room in the active site of polymerase I resulting from the Y766S mutation seems to allow accommodation of a bulky lesion during DNA synthesis may have implications regarding the mechanism by which bypass polymerases are able to incorporate nucleotides at template positions containing these types of adducts. This recently discovered class of polymerases, also referred to as Y-family polymerases, have been shown to traverse a variety of lesions in DNA that are blocks to synthesis by other polymerases. For example, pol η is able to efficiently replicate past a UV-induced thymine dimer with a similar fidelity as with undamaged templates (*45*, *46*). It can also use templates containing a variety of distorting DNA lesions, including an AAF adduct (*47*−*50*). Structural and kinetic studies indicate a similar shape and mechanism for synthesis by these polymerases; the major difference from replicative polymerases is that they have a more open active site and are lacking the O and O1 helices in the fingers domain (*51*, *52*). It has been suggested that the small fingers and thumb domains displayed by the Y-family polymerases may allow relaxed geometric constraints on base pairing that can better accommodate distorting lesions (*53*). It is interesting that, similar to the results presented here using the Y776S polymerase I mutant, many Y-family polymerases also have reduced fidelity as compared with replicative polymerases on undamaged templates but enhanced accuracy on damaged templates (*47*, *48*, *54*, *55*). Likewise, some Y-family polymerases are also less efficient at extending on a primer-template once a nucleotide is added across from the damage (*47*, *56*). It is possible that the similarity of the properties of the bypass polymerases and the Y766S polymerase I mutant studied here may have implications regarding the molecular mechanism of DNA replication on damaged DNA.

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