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Inactivation of DNA Polymerase I (Klenow Fragment) by Adenosine 2′,3′-Epoxide 5′-Triphosphate: Evidence for the Formation of a Tight-Binding Inhibitor†

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ABSTRACT: The suicidal inactivation of *Escherichia coli* DNA polymerase I by epoxy-ATP has been previously reported (Abboud et al., 1978). We have examined in detail the mechanism of this inactivation utilizing a synthetic DNA template-primer of defined sequence. Epoxy-ATP inactivates the large fragment of DNA polymerase I (the Klenow fragment) in a time- and concentration-dependent manner (K_I = 21 μM; k_{inact} = 0.021 s^{-1}). Concomitant with inactivation is the incorporation of epoxy-AMP into the primer strand. The elongated DNA duplex directly inhibits the polymerase activity of the enzyme (no time dependence) and is resistant to degradation by the 3′ → 5′ exonuclease and pyrophosphorylase activities of the enzyme. Inactivation of the enzyme results from slow (4×10^{-4} s^{-1}) dissociation of the intact epoxy-terminated template-primer from the enzyme and is thus characterized as a tight-binding inhibition. Surprisingly, while the polymerase activity of the enzyme is completely suppressed by epoxy-ATP, the 3′ → 5′ exonuclease activity remains intact. The data presented demonstrate that even though the polymerase site is occupied with duplex DNA, the enzyme can bind a second DNA duplex and carry out exonucleolytic cleavage.

DNA polymerase I of *Escherichia coli* (Pol I)[1] is a multifunctional enzyme involved in the replication and repair of DNA in vivo (Kornberg, 1980). Replication proceeds through the 5′ → 3′ polymerization of dNTP's onto a primer strand directed by a DNA template. The fidelity of this duplication is ensured by an associated 3′ → 5′ exonuclease activity that removes errors introduced by the polymerase. The enzyme also possesses a distinct 5′ → 3′ exonuclease activity that is involved in nick translation during DNA repair (Setlow & Kornberg, 1972). Limited proteolytic digestion of Pol I (103 kDa) yields a large fragment, the Klenow fragment (68 kDa), which contains polymerase and 3′ → 5′ exonuclease activities but lacks the 5′ → 3′ exonuclease activity (Klenow & Henningsen, 1970; Brutlag et al., 1969).

It has long been appreciated that the polymerase and 3′ → 5′ exonuclease activities constitute discrete catalytic sites. This was first suggested by the selective inhibition of polymerase (Que et al., 1979) and 3′ → 5′ exonuclease (Que et al., 1978)

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activities and is supported by the recent 3.3-Å crystal structure of the Klenow fragment with dTMP bound at the exonuclease site (Ollis et al., 1985).

A DNA duplex has been model-built into the protein structure in a domain characterized by a large, positively charged cleft (Warwicker et al., 1985). The construction of Klenow mutants deficient in DNA binding (Joyce et al., 1985, 1986) and chemical modification studies (Mohan et al., 1988; Pandey & Modak, 1988) support this model-built structure. The DNA primer terminus in this complex resides approximately 25 Å from the dTMP bound at the exonuclease site of the enzyme. That there is physical separation of polymerase and exonuclease sites is further evidenced by the recent cloning of the C-terminal domain of the Klenow fragment. While the cloned protein possesses polymerase activity, the peptide lacks the smaller dTMP binding domain and, as expected, has no detectable exonuclease activity (Freemont et al., 1986).

Epoxy-ATP is an analogue of dATP containing an epoxide ring across the 2′ and 3′ carbons of the ribofuranose ring. It has previously been demonstrated that this nucleotide is a suicide inactivator of Pol I (Abboud et al., 1978). The authors suggested that epoxy-ATP is incorporated into DNA and that the epoxide ring is opened by an active-site nucleophile forming a covalent protein–DNA complex. Given that the site of polymerase activity has been determined only from circumstantial evidence, we were quite interested in locating the site of the putative enzyme–DNA cross-link.

We have therefore reexamined the inactivation of the Klenow fragment by epoxy-ATP using a synthetic DNA template-primer of defined sequence. No evidence for a covalently modified protein was obtained. Instead, we provide evidence for the formation of a tight-binding inhibitor of the Klenow fragment in the presence of DNA and epoxy-ATP. Furthermore, detailed analysis has revealed that while the polymerase activity of the enzyme is totally abolished by epoxy-ATP, the 3′ → 5′ exonuclease activity remains intact.

Experimental Procedures

Materials. Poly(dA), oligo(dT)$_{10}$, and unlabeled nucleotides were purchased from Pharmacia. ^{32}P-Radiolabeled nucleotides were purchased from New England Nuclear. Polynucleotide kinase was purchased from U.S. Biochemical Corp. Arabino-ATP, arabino-AMP, nucleotide pyrophosphatase, yeast inorganic pyrophosphatase, and Na_4PP_i were purchased from Sigma. The PP_i was purified and quantitated as previously described (Kuchta et al., 1987). DNase I was purchased from Cooper Biochemicals. Epoxy-AMP and epoxy-ATP, generously provided by Dr. M. Cowart, were synthesized essentially as described by Abboud et al. (1978). Klenow fragment was purified as previously described (Joyce & Grindley, 1983) from *E. coli* strains kindly provided by Dr. C. Joyce. Overproducing strains for both wild-type Klenow fragment (Joyce & Grindley, 1983) and a 3′ → 5′ exonuclease deficient mutant (D355A, E357A; Derbyshire et al., 1988) have been described. Protein concentrations of both enzymes were determined spectrally with $\epsilon_{278} = 6.32 \times 10^4$ M^{-1} cm^{-1} (Setlow et al., 1972). All other materials were of the highest quality commercially available.

Chart I: Oligonucleotides Utilized in This Study

9/20mer:	5′-TCGCAGCCG-3′ 3′-AGCGTCGGCAGGTTCCCAAA-5′
13/20mer:	5′-TCGCAGCCGTCCA-3′ 3′-AGCGTCGGCAGGTTCCCAAA-5′
11^{AZ}/20mer:	5′-TCGCAGCCGTC-3′ 3′-AGCGTCGGCAGGTTCCCAAA-5′

T= Azido-labeled T[a]

[a] Gibson & Benkovic, 1987.

Oligonucleotides. DNA oligomers were synthesized with an Applied Biosystems 380A DNA synthesizer. Duplex formation and quantitation were accomplished essentially as previously described (Kuchta et al., 1987) except the duplexes were formed at room temperature. No difference was observed between duplexes formed at room temperature and those formed by heating to 65 °C and slowly cooling. Incorporation of radioactivity was determined by DE81 (Whatman) filter binding assay (Bryant et al., 1983) using either a Beckman LS8100 or a Beckman LS6800 scintillation counter. 5′-^{32}P labeling of the DNA oligomers has also been described (Mizrahi et al., 1986). The DNA template-primer systems utilized are shown in Chart I. Poly(dA)–oligo(dT)$_{10}$ was prepared by mixing poly(dA) and oligo(dT)$_{10}$ in a 20:1 base ratio. Base concentrations were determined spectrally, and the concentration of oligo(dT)$_{10}$ 3′ ends was calculated on the basis of a chain length of 10.

HPLC and FPLC. Both HPLC and FPLC chromatographic separations were performed on a Waters 990 chromatography workstation interfaced to a NEC powermate II computer for data capture. Reverse-phase HPLC separations utilized a Waters μBondapak (0.39 × 30 cm) reverse-phase column equilibrated with 100 mM TEAA (pH 7.0). The compounds of interest were eluted with an acetonitrile gradient to 25% over 45 min. Anion-exchange HPLC was performed on a Whatman Partisil 10 SAX (0.46 × 25 cm) column eluting with 10 mM ammonium phosphates (pH 6.8) (isocratic). A Pharmacia Mono-Q (5 × 50 mm) column was used when FPLC separations were desired. The flow rate in all cases was 1 mL/min.

Enzyme Inactivation by Epoxy-ATP. Unless otherwise indicated, inactivation of the Klenow fragment was accomplished by incubation of 0.4 μM enzyme with 0.6 μM 13/20-mer DNA and 40 μM epoxy-ATP in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ at room temperature for 15 min. When necessary, reactions were stopped with 20 mM EDTA.

Polymerization Assay. The standard polymerization assay mixture contained 1 μM (3′ ends) poly(dA)–oligo(dT)$_{10}$, 2 μM each dATP, dGTP, and dCTP, and 5 μM [α-^{32}P]dTTP (approximately 5000–10 000 cpm/pmol) in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. (When EDTA was present in the reaction mixture, magnesium was added to yield an excess of 5 mM.) The reaction was initiated with the addition of enzyme (control or epoxy-ATP inactivated, vide supra) to a final concentration of 4 nM and incorporation of [^{32}P]dTMP into poly(dA)–oligo(dT)$_{10}$ monitored by filter binding assay. This typically resulted in the simultaneous

[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; epoxy-AMP, adenosine 2′,3′-epoxide 5′-monophosphate; epoxy-ATP, adenosine 2′,3′-epoxide 5′-triphosphate; dNTP, deoxynucleoside 5′-triphosphate; arabino-AMP, 9-β-D-arabinofuranosyladenine 5′-monophosphate; arabino-ATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; ATP, adenosine 5′-triphosphate; poly(dA), poly(deoxyriboadenylate); oligo(dT)10, decakis(deoxyribothymidylate); 14^e/20-mer, elongation product of 13/20-mer template-primer (Chart I) and epoxy-ATP; 14^d/20-mer, elongation product of 13/20-mer template-primer and dATP; 12/20-mer, *n* − 1 exonuclease product of 13/20-mer template-primer; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecyl sulfate (denaturing)–polyacrylamide gel electrophoresis; kDa, kilodalton(s); PP_i, inorganic pyrophosphate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEAA, triethylammonium acetate.

addition of 13/20-mer duplex and epoxy-ATP to final concentrations of 6 nM and 0.4 μM, respectively. Control experiments demonstrated that neither 13/20-mer duplex nor epoxy-ATP at these concentrations had any significant effect on the polymerization assay.

Electrophoresis and Sequencing Gel Assay of Oligonucleotide Products. Proteins were analyzed on a 12.5% polyacrylamide gel by the method of Laemmli (1970). 5′-^{32}P-Labeled oligonucleotides were fractionated by electrophoresis on a denaturing 15% polyacrylamide sequencing gel and the bands visualized by autoradiography (Mizrahi et al., 1986). Quantitation was accomplished by excision of the radioactive bands and scintillation counting in 3 mL of Scintiverse II cocktail (Fisher).

FPLC Analysis of Epoxy-ATP-Inactivated Klenow Fragment and Isolation of 14^e/20-mer. A mixture of 2 μM 13/20-mer, 40 μM epoxy-ATP, and 2.2 μM Klenow fragment (1-mL total volume) was incubated at room temperature for 15 min. The entire mixture was then injected onto a Mono-Q FPLC column equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and the oligonucleotide eluted with a KCl gradient to 0.6 M over 60 min, followed by a 10-min gradient to 1 M KCl. The flow rate was 1 mL/min. The DNA was collected, desalted on a Sep Pak, and taken to dryness (Savant Speed Vac) and the residue taken into 500 μL of 50 mM Tris-HCl buffer (pH 7.5). The final DNA concentration was determined spectrally with ϵ_{260} = 370 mM^{-1} cm^{-1}. This extinction coefficient was derived by summation of the extinction coefficients for each of the individual bases without correction for duplex-induced hypochromicity.

Nature of the Elongated 14^e/20-mer Product. The 14^e/20-mer was prepared and isolated as described above starting with 5 nmol of 13/20-mer and 5.4 nmol of Klenow fragment. The purified DNA (2.7 nmol) was taken into 1.7 mL of 10 mM Tris-HCl (pH 7.5) buffer containing 10 mM $MgCl_2$, 10 mM KCl, and 5 mM $CaCl_2$. DNase I (450 μg) was added and the mixture heated to 37 °C. Nucleotide pyrophosphatase was added at 45 and 65 min (180 and 270 μg, respectively) and the incubation continued for a total of 90 min. The entire sample was then analyzed by reverse-phase HPLC. The appropriate peaks were collected, taken to dryness (Savant Speed Vac), and rechromatographed by anion-exchange HPLC.

Off Rate of 14^e/20-mer DNA from the Klenow Fragment. A mutant Klenow fragment devoid of exonuclease activity was utilized for these studies. Control experiments confirmed that the rate of incorporation of epoxy-ATP into 13/20-mer DNA and the rate of inactivation by epoxy-ATP were identical for both wild-type and mutant Klenow fragments. The 14^e/20-mer (0.8 μM, isolated by FPLC) and 0.4 μM enzyme were preincubated for 60 s in 50 mM Tris-HCl (pH 7.5) buffer containing 5 mM $MgCl_2$. This was then diluted 1:50 into the same buffer containing 1 μM 5′-^{32}P-labeled 13/20-mer DNA and 40 μM epoxy-ATP. Aliquots were removed at the indicated times, and the reaction was quenched into sequencing gel load buffer. Elongation of the radiolabeled 13-mer primer to the epoxy-terminated 14^e-mer was assayed by the gel assay described above. The experiment was repeated with wild-type (exo$^+$) Klenow fragment except the 14^e/20-mer was formed in situ from 0.41 μM Klenow fragment, 0.7 μM 13/20-mer, and 40 μM epoxy-ATP and preincubation was for 15 min.

Exonuclease Activity on 14^e/20-mer DNA by the Klenow Fragment. A 4-fold excess of Klenow fragment was added to 0.1 μM 5′-^{32}P-labeled 14^e/20-mer DNA (isolated by FPLC) in the standard buffer. Aliquots were removed at various times and the DNA products analyzed by gel assay.

Exonuclease Activity of Epoxy-ATP-Inactivated Klenow Fragment. The Klenow fragment was inactivated according to standard conditions described above. This was then diluted 1:20 into a solution containing 5′-^{32}P-labeled 13/20-mer and epoxy-ATP. Final concentrations were 0.02 μM Klenow fragment, 1 μM DNA, and 40 μM epoxy-ATP. Aliquots were removed at various times and the oligonucleotide products analyzed by gel assay. The control incubation was treated similarly except epoxy-ATP was excluded from the incubation mixture.

Search for a Ternary Enzyme–DNA–PP$_i$ Complex. The Klenow fragment (1.1 nmol) was inactivated with 1 equiv of 13/20-mer DNA and 40 μM epoxy-ATP in 100 μL of standard reaction buffer. After 15 min, the protein was isolated by spun column chromatography using a 1-mL column of Sephadex G-50 equilibrated with 50 mM Tris-HCl, pH 7.5 (Maniatis, 1982). A control incubation was treated similarly except that epoxy-ATP was excluded from the incubation mixture and the Klenow fragment was added immediately prior to spun column chromatography. After heat denaturation, yeast inorganic pyrophosphatase (1 unit) and $MgCl_2$ (5 mM) were added to the column eluent, and the mixture was kept at room temperature for 25 min. Inorganic phosphate was then analyzed by the Lanzetta phosphate assay (1979).

Data Analysis and Computer Simulation. The GraphPAD program (ISI Software, Philadelphia, PA) was utilized for both linear and exponential curve fits. Computer simulations were performed by using a modified version of the program SIMUL (Barshop et al., 1983; Anderson et al., 1988). Kinetic constants directly measured in this study (K_I and k_{inact} for epoxy-ATP, k_{off} and k_{exo} for the 14^e/20-mer) were held constant. The association rate of enzyme and 14^e/20-mer DNA (k_{on}) was assumed to be equal to that of the 13/20-mer duplex (12 μM^{-1} s^{-1}; Kuchta et al., 1987) and was also held constant. Similarly, the K_D for all natural (not epoxy terminated) DNA species was assumed to be equal to that of the 13/20-mer (5 nM; Kuchta et al., 1987). It was further assumed that this K_D was unaffected by enzyme inactivation with epoxy-ATP. The data were fit to the model outlined in Scheme IV by varying only the 3′ → 5′ exonuclease rate ($k_{exo\,1}$,$k_{exo\,2}$), yielding a final value of 4.5 × 10^{-3} s^{-1}. This value agrees well with rates observed from a variety of related template-primer systems (Kuchta et al., 1988) and also agrees with the experimentally determined rate[2] (vide infra).

Results

Inactivation of the Klenow Fragment by Epoxy-ATP. Figure 1A shows that, in the presence of 13/20-mer DNA, epoxy-ATP completely inactivates the Klenow fragment in a time-dependent manner. The 13-mer primer is elongated by one nucleotide under these conditions (Figure 1B) with an observed rate constant (5.6 × 10^{-3} s^{-1}) roughly equal to that of enzyme inactivation (7.7 × 10^{-3} s^{-1}). Once epoxy-AMP has been added to the primer strand, further elongation upon addition of dGTP was not observed even up to 90-min incubation periods (data not shown).

Table I shows that both epoxy-ATP and 13/20-mer are strictly required for inactivation. That incorporation of ep-

[2] The best-fit 3′ → 5′ exonuclease rates differ by a factor of 2 due to differences in experimental conditions utilized to generate the data. Briefly, the enzyme concentration used to generate the experimental data for the computer simulation was significantly higher than that used to directly measure the 3′ → 5′ exonuclease rate (400 and 20 nM, respectively). The exact conditions for each experiment are outlined under Experimental Procedures.

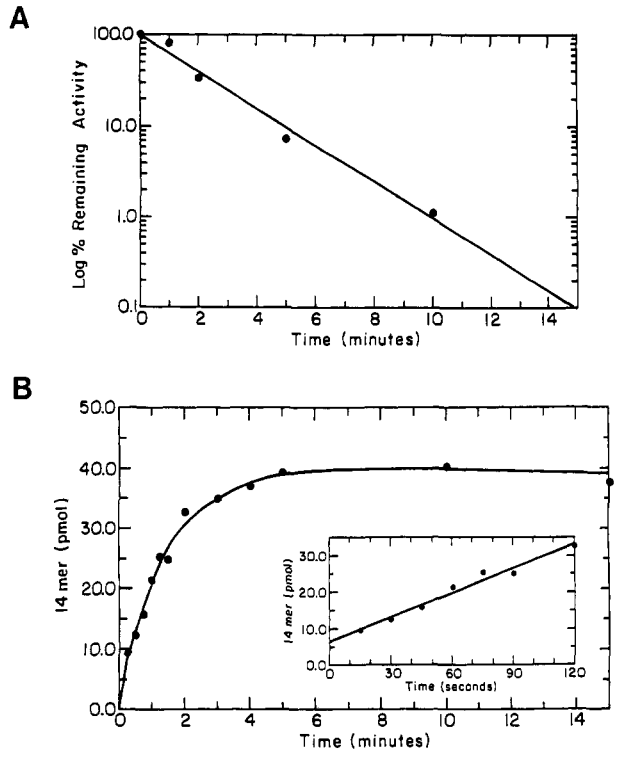


FIGURE 1: Enzyme inactivation and DNA elongation as a function of time. (A) Loss of polymerase activity with time in the presence of epoxy-ATP and 13/20-mer DNA. Inactivation and polymerization assay was performed as described under Experimental Procedures. (B) Elongation of the 13/20-mer primer strand in the presence of epoxy-ATP and the Klenow fragment. Conditions were as in (A). Incorporation of epoxy-AMP was measured by gel assay as described under Experimental Procedures. The inset shows the time course for the first 2 min of the reaction.

Table I: Inactivation of the Klenow Fragment

incubation	% activity[a]
control	100
+epoxy-ATP	109
+epoxy-ATP + 9/20-mer	125
+epoxy-ATP + 13/20-mer	ND[b]

[a] Enzyme inactivation determined by polymerization assay. Control contained only the Klenow fragment. [b] ND, not detectable.

Table II: Protection of the Klenow Fragment by dATP

[deoxy-ATP] (μM)	% activity[a]	[deoxy-ATP] (μM)	% activity[a]
0.0	2.2	5.0	78.3
0.5	2.2	10.0	93.5
2.0	4.3	control	100.0

[a] The enzyme was inactivated by using standard conditions (Experimental Procedures). Enzyme activity was determined by polymerization assay. Control incubation lacked epoxy-ATP.

oxy-ATP into the nascent DNA chain is a prerequisite for inactivation was demonstrated by the impotence of the 9/20-mer as a template-primer in the inactivation mixture (Table I). Correct Watson–Crick base pairing between epoxy-ATP and 9/20-mer DNA is not possible so that epoxy-ATP is not incorporated into the primer strand.

Table II reveals that dATP protected the enzyme from inactivation by epoxy-ATP. Depletion of dATP by the Klenow fragment (idling turnover) again gave epoxy-ATP-dependent inactivation.

Kinetics and Stoichiometry of Inactivation. Increasing the epoxy-ATP concentration resulted in an increase in the rate of enzyme inactivation (data not shown). A plot of $[k_{obs}]^{-1}$ vs $[\text{epoxy-ATP}]^{-1}$ gave a K_I and k_{inact} of 21 μM and 2.1×10^{-2} s^{-1}, respectively. This K_I is very close to the K_D for dATP (5 μM) obtained when the 13/20-mer duplex was used (Kuchta et al., 1987). The rate of epoxy-ATP incorporation, however, is greater than 3 orders of magnitude slower than that of the natural nucleotide substrate (50 s^{-1}; Kuchta et al., 1987).

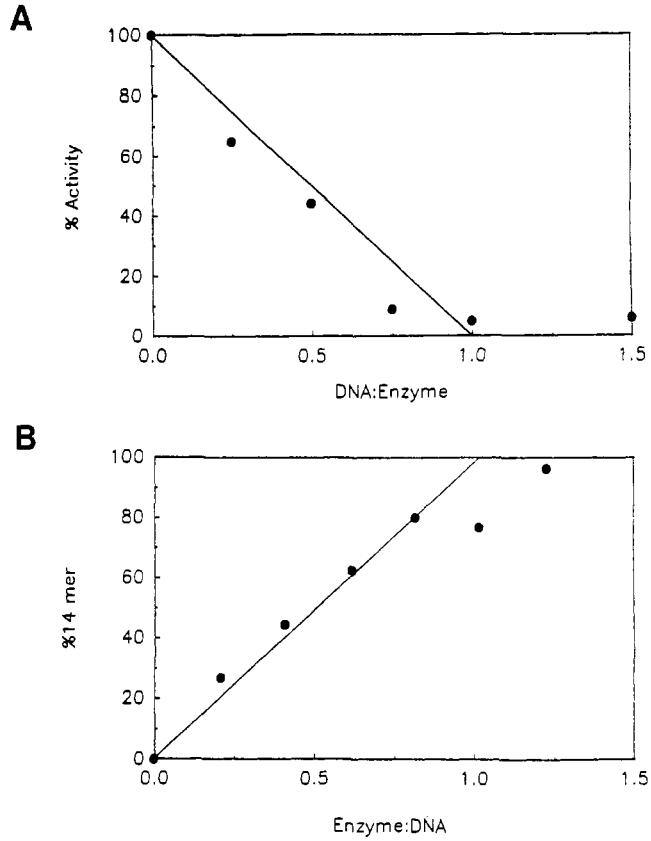


FIGURE 2: Stoichiometry of the inactivation reaction. (A) Inactivation of polymerase activity as a function of DNA:enzyme ratio. The solid line corresponds to stoichiometric inactivation by DNA. (B) Elongation of 13/20-mer DNA as a function of enzyme:DNA ratio. The solid line corresponds to stoichiometric elongation by the enzyme. Conditions are described under Experimental Procedures.

Figure 2A reveals that, within experimental error, 1 equiv of Klenow fragment is inactivated per mole of DNA included in the inactivation mixture. Conversely, incubation of the Klenow fragment with excess DNA resulted in the formation of 1 equiv of elongated product per mole of enzyme (Figure 2B). This suggests that the addition of epoxy-AMP to the primer terminus is not only necessary but sufficient for inactivation and corresponds to a partition ratio (catalytic events:inactivation events) of 1.

Absence of a Stable Protein–DNA Adduct. Photolabeling of the Klenow fragment with a 5′-^{32}P-labeled azido-DNA photoprobe formed a covalent protein–DNA complex readily detectable by SDS–PAGE (Gibson & Benkovic, 1987). Similar analysis of the Klenow fragment inactivated with 5′-^{32}P-labeled 13/20-mer and epoxy-ATP did not result in DNA comigrating with the enzyme (data not shown). Abboud et al. (1978) similarly reported difficulty in detecting a covalent protein–DNA adduct by SDS–PAGE.

Figure 3 shows an FPLC chromatogram of the Klenow fragment inactivated with epoxy-ATP and 13/20-mer DNA (lower trace). No covalent protein–DNA complex was detected under these conditions. The covalent protein–DNA complex resulting from photolabeling of the Klenow fragment with the azido-DNA photoprobe is shown in Figure 3 (upper trace) for comparison. Whereas the epoxy-ATP-inactivated

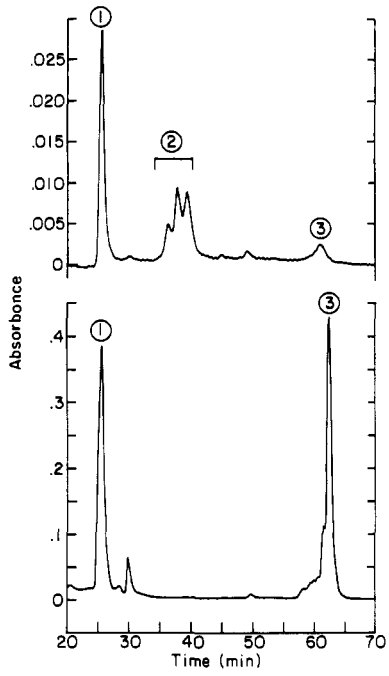
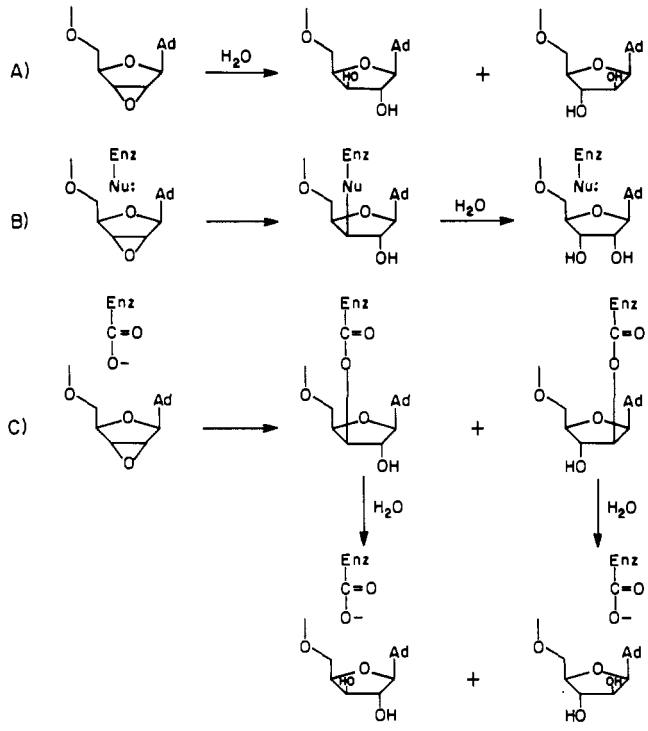


FIGURE 3: Absence of a stable covalent protein–DNA complex. FPLC chromatogram of the Klenow fragment treated with 11^{AZ}/20-mer and UV light (upper trace) or 13/20-mer and epoxy-ATP (lower trace). Epoxy-ATP and the Klenow fragment elute at 25 min (250 mM NaCl, peak 1), and the oligonucleotides elute at 62 min (620 mM NaCl, peak 3). The three closely spaced peaks eluting between 34 and 37 min (upper trace, peak 2) have been identified as DNA-labeled enzyme. Details of this photolysis will be published separately. The small peaks eluting at 28 and 30 min (lower trace) are present in control incubations lacking enzyme.

Scheme I



enzyme retained only 3.5% residual activity, protein recovered from the FPLC column was fully (98%) active compared to control.

The DNA isolated from the inactivation mixture was completely elongated to the 14^e/20-mer under the conditions utilized. Addition of the 14^e/20-mer duplex to Klenow gave

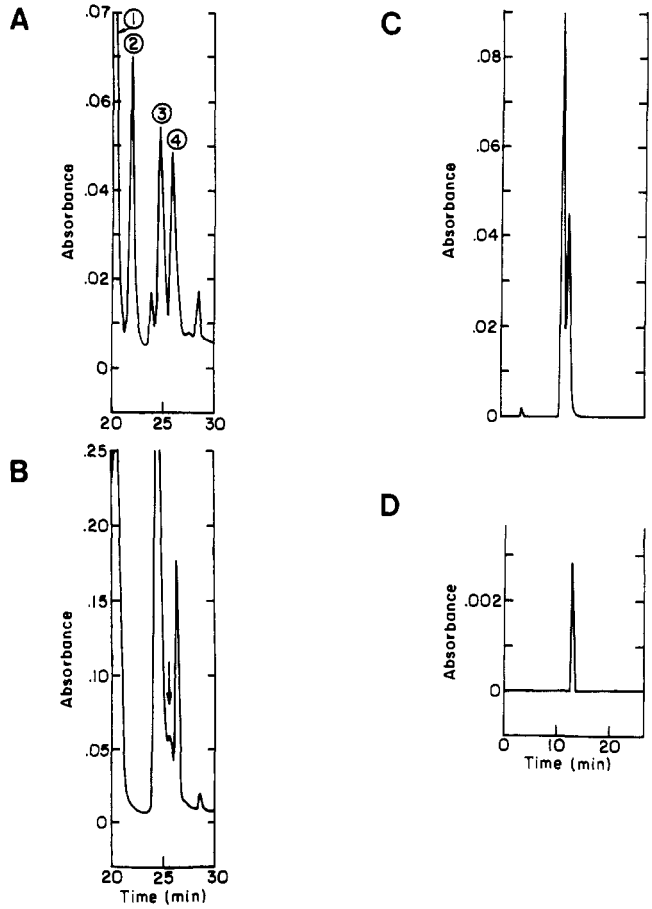


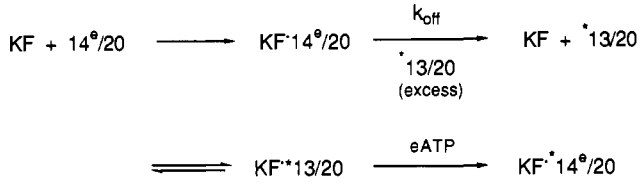
FIGURE 4: Identification of the 14^e/20-mer primer terminus as epoxy-AMP. (A) Reverse-phase HPLC chromatogram (260 nm) of the nucleotide standards: (1) AMP and dGMP (20.2 min), (2) arabino-AMP (21.8 min), (3) dAMP (24.7 min), and (4) epoxy-AMP (25.8 min). (B) Reverse-phase HPLC chromatogram (260 nm) of the enzymatic digest of purified 14^e/20-mer. The arrow (25.6 min) corresponds to the peak collected. (C) Anion-exchange HPLC chromatogram (260 nm) of dAMP (11.7 min) and epoxy-AMP (12.8 min) standards. (D) Anion-exchange HPLC chromatogram (260 nm) of the peak isolated in (B). The elution time was 13.0 min. Details of the procedures are given under Experimental Procedures.

greater than 99% inactivation without the time dependence observed in the epoxy-ATP/DNA system. Addition of dGTP to the 14^e/20-mer-treated enzyme did not lead to elongation of the primer strand (data not shown).

Nature of the Elongated 14^e/20-mer Product. The possibility exists that once incorporated into DNA the epoxy-AMP is ring opened via either water or a nucleophile at the active site of the enzyme. Simple hydrolysis should yield equal amounts of both the arabino and xylo sugar derivatives (Scheme IA). Enzyme-catalyzed ring opening would most likely yield the ribo sugar upon disruption of the protein–DNA complex (Scheme IB). A final possibility, however, is the nucleophilic attack on the epoxide ring by a glutamate or aspartate residue, followed by hydrolysis at the acyl carbon to yield either the arabino or the xylo sugar moieities (Scheme IC).

The FPLC-purified 14^e/20-mer (2.7 nmol) was digested through the combined action of DNase I and nucleotide pyrophosphatase as described under Experimental Procedures. Reverse-phase HPLC analysis of the digestion products revealed that all of the DNA was degraded to nucleotide 5′-monophosphates. A small peak not present in the control digest was observed as a shoulder on the dAMP peak (Figure 4B) and was collected. The peak height and retention time of this sample were coincident with that of 2.7 nmol of au-

Scheme II: Determination of the 14^e/20-mer Dissociation Rate (k_{off}) from the Klenow Fragment[a]



[a] *13/20 = 5′-^{32}P-labeled 13/20-mer DNA.

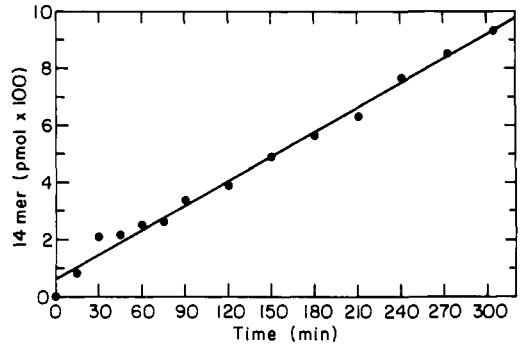


FIGURE 5: Dissociation rate of the 14^e/20-mer from the enzyme. Details of the measurement are given under Experimental Procedures and in the text.

thentic epoxy-AMP. The sample was further analyzed by anion-exchange HPLC. A single peak was evident with a retention time (Figure 4D) and electronic spectrum (not shown) matching those of authentic epoxy-AMP. These data strongly suggest that the primer terminus of the 14^e/20-mer remains largely intact as the epoxide-bearing sugar.

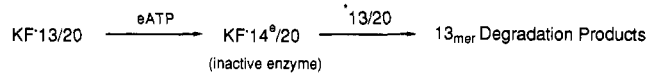
Off Rate for the 14^e/20-mer. The method utilized to determine the rate of DNA dissociation from the enzyme (k_{off}) is outlined in Scheme II. The formation of radiolabeled 14^e/20-mer in this system is limited by DNA dissociation and therefore represents the k_{off} directly. Figure 5 shows the rate of 5′-^{32}P-labeled 14^e-mer formation by the mutant (exo$^-$) Klenow fragment with time. Linear regression analysis yielded an observed off rate of 4×10^{-4} s^{-1}. Similar analysis using wild-type (exo$^+$) Klenow fragment gave an off rate of 5×10^{-4} s^{-1} (data not shown).

Lack of 3′ → 5′ Exonuclease and Pyrophosphorolysis Activities on the 14^e/20-mer. The rate of 3′ → 5′ hydrolysis and pyrophosphorolysis for 14^d/20-mer DNA by the Klenow fragment is 2×10^{-3} s^{-1} and 0.12 s^{-1}, respectively (Kuchta et al., 1987, 1988). Incubation of the 14^e/20-mer (isolated by FPLC) with excess Klenow fragment did not result in any detectable degradation of the 14-mer primer during the 90-min assay period. Addition of pyrophosphate up to 1.4 mM similarly had no effect. There is, therefore, no detectable 3′ → 5′ exonuclease or pyrophosphorylase activity by the Klenow fragment on the 14^e/20-mer.

3′ → 5′ Exonuclease Activity of Epoxy-ATP-Inactivated Klenow Fragment. Sequencing gel analysis of DNA products derived from incubation of the Klenow fragment with epoxy-ATP and 13/20-mer DNA suggested that although there was no detectable degradation of the epoxy-terminated primer strand, the 3′ → 5′ exonuclease activity of the enzyme remained intact even after the polymerase activity was completely abolished. This was investigated further by measuring the exonuclease rate of epoxy-ATP-inactivated Klenow fragment as outlined in Scheme III.

The polymerase activity of the Klenow fragment was completely inactivated with epoxy-ATP and 13/20-mer DNA. The 3′ → 5′ exonuclease activity of the inactivated enzyme

Scheme III: Determination of the 3′ → 5′ Exonuclease Rate of Epoxy-ATP-Inactivated Klenow Fragment[a]



[a] *13/20 = 5′-^{32}P-labeled 13/20-mer DNA.

on exogenous radiolabeled 13/20-mer was then measured by gel assay as described under Experimental Procedures. A control incubation was treated similarly except epoxy-ATP was omitted from the incubation mixtures. The observed 3′ → 5′ exonuclease rate of epoxy-ATP-inactivated Klenow fragment (2.4×10^{-3} s^{-1}) was not significantly different from that of the nontreated control (3.4×10^{-3} s^{-1}).

Absence of a Ternary Enzyme–DNA–PP$_i$ Complex. The properties of the epoxy-ATP-inactivated Klenow fragment suggest that the enzyme might be locked into an intermediate state of catalysis (vide infra). The possibility exists, therefore, that pyrophosphate release might be slow enough to trap a ternary enzyme–DNA–PP$_i$ complex. The inactivated enzyme was isolated by spun column chromatography as outlined under Experimental Procedures. Phosphate content was then measured after protein denaturation and inorganic pyrophosphatase treatment. Recovery of the Klenow fragment from a spun column was approximately 50% under the conditions utilized. If a stable ternary enzyme–DNA–PP$_i$ complex existed, 1 nmol of phosphate (easily detectable by the Lanzetta phosphate assay) should have been recovered in the spun column eluent. The analysis relative to control did not reveal significant amounts of pyrophosphate eluting with epoxy-ATP-inactivated enzyme.

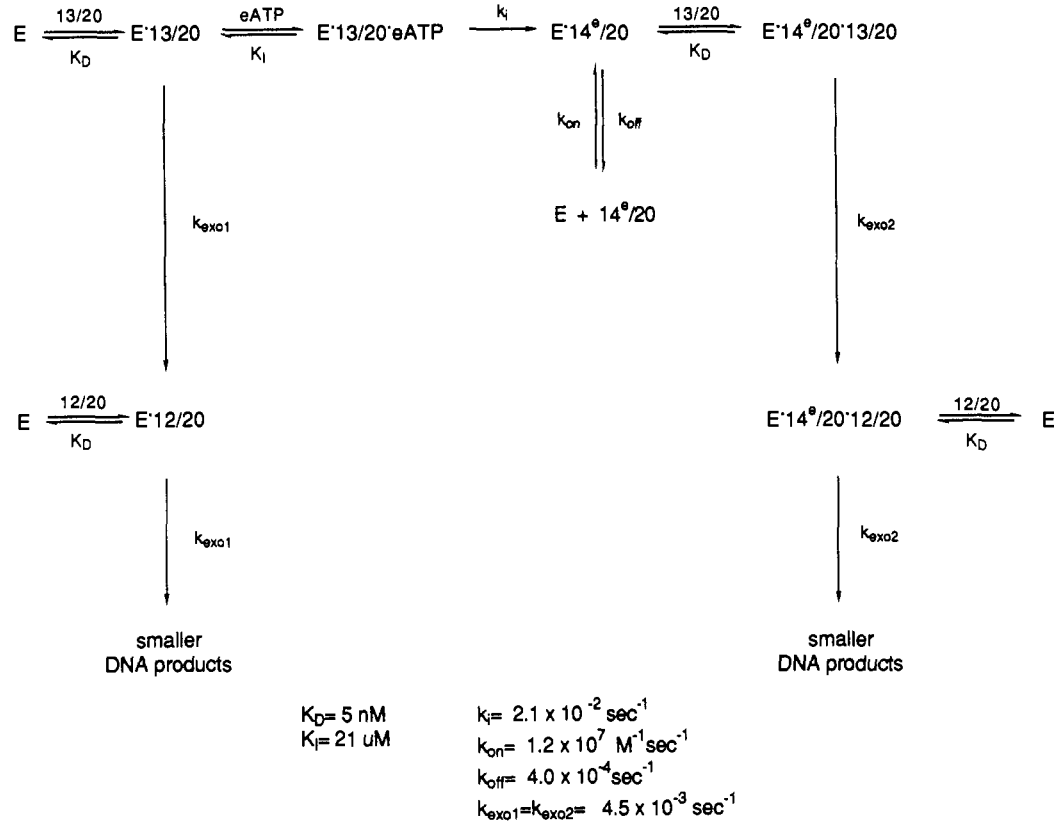
Discussion

Epoxy-ATP inactivates the Klenow fragment in a time- (Figure 1A) and concentration-dependent manner, and this inactivation can be suppressed by the natural substrate dATP (Table II). The inactivation is strictly dependent on the presence of both epoxy-ATP and a complementary DNA template-primer (Table I). Concomitant with this inactivation is the incorporation of one nucleotide into the primer strand (Figure 1B). That the elongated DNA product represents the inactivating species is supported by several experimental facts: (1) the rate of enzyme inactivation is equal to the rate of epoxy-AMP incorporation into DNA (Figure 1), (2) 1 mol of enzyme is inactivated per mole of DNA (Figure 2A), and (3) 14^e/20-mer isolated by FPLC inactivates the enzyme without the time dependence required for inactivation by epoxy-ATP and 13/20-mer.

Previous studies have suggested that incorporation of epoxy-ATP into DNA by Pol I ultimately gave a covalent enzyme–DNA adduct. We have been unable to detect a stable complex by SDS–PAGE or by anion-exchange FPLC. A Klenow–DNA complex is readily detectable under identical conditions when the protein was photolabeled with an azido-DNA photoprobe (Figure 3). Furthermore, disruption of the enzyme–14^e/20-mer DNA complex by FPLC resulted in complete recovery of enzymatic activity.

That the epoxide ring remains intact after separation of DNA from the protein is supported by the following data. Arabino-ATP is incorporated into the 13/20-mer by the Klenow fragment, and subsequent addition of dGTP results in primer extension of the 17-mer (data not shown). Kornberg has similarly shown that ribonucleotide primers are elongated by the Klenow fragment in the presence of dNTP's (Kornberg, 1980). Addition of dGTP to the enzyme–14^e/20-mer complex, whether formed in situ from epoxy-ATP and 13/20-mer or

Scheme IV: Proposed Mechanism for Epoxy-ATP Inactivation of the Klenow Fragment



formed by addition of FPLC-purified 14^e/20-mer to the enzyme, does not result in primer elongation. These data rule out both arabino and ribo sugar derivatives and identify the primer terminus as either intact epoxy-AMP or the ring-opened xylo sugar derivative (Scheme I). Complete digestion of the 14^e/20-mer with DNase I and nucleotide pyrophosphatase followed by HPLC analysis of the products yielded epoxy-AMP in appropriate amounts. It is difficult to reconcile these data with the formation of a covalent enzyme–DNA adduct, and we do not believe that such a complex exists.

The proposal that a covalent protein–DNA adduct is formed by treatment of Pol I with epoxy-ATP is based on gel filtration analysis in which high salt concentrations (0.5 M NaCl) were used to dissociate noncovalent enzyme–DNA complexes (Abboud et al., 1978). Under these conditions, the protein comigrates with DNA only upon prior treatment with epoxy-ATP. The data presented here suggest that while 0.5 M NaCl is sufficient to disrupt a normal enzyme–DNA complex, the unusually high affinity of epoxy-terminated DNA for the enzyme precludes dissociation under the experimental conditions utilized.

How then does epoxy-ATP inactivate the Klenow fragment? Figure 2B reveals that only 1 mol of 14^e/20-mer is formed per mole of enzyme. This suggests that the release of DNA from the enzyme is dramatically impaired. The rate-limiting step in nonprocessive DNA synthesis by the Klenow fragment is dissociation of DNA from the enzyme (0.06 s^{-1} for the 14^d/20-mer; Kuchta et al., 1987). Incorporation of epoxy-AMP results in a 150-fold decrease (4×10^{-4} s^{-1}; Figure 5) in the rate of DNA dissociation from the enzyme. Once incorporated, the epoxy-terminated primer is resistant to hydrolysis by both the 3′ → 5′ exonuclease and pyrophosphorylase activities of the enzyme.

A proposed mechanism for enzyme inactivation by epoxy-ATP consistent with all the data is outlined in Scheme IV. DNA and epoxy-ATP bind to the enzyme normally and catalysis ensues, elongating the primer strand by one base. This generates the inactivating species, the 14^e/20-mer, which is resistant to degradation by the 3′ → 5′ exonuclease activity of the enzyme. The lack of a 3′-hydroxyl group at the primer terminus makes further elongation impossible, and the enzyme is poisoned by the slow release of bound DNA.

Inactivation of the Klenow fragment by epoxy-ATP meets all the criteria demanded of a mechanism-based inhibitor (Walsh, 1984). It is important to note, however, that this suicide inactivation does not yield a covalently modified protein, but rather the catalytic pathway generates a tight-binding inhibitor that prevents further functioning of the enzyme. The inactivation of γ-glutamylcysteine synthetase by buthionine sulfoxime (Griffith, 1982) and the oxidation of 22-thiacholesterol to the tight-binding 22-sulfoxide by cytochrome P-450$_{SCC}$ (Walsh, 1984) provide precedent for this unusual type of mechanism-based inhibition.

Of some significance is the fact that the partition ratio for inactivation of the Klenow fragment by epoxy-ATP is 1. That is, for every chemical event (incorporation of epoxy-AMP into DNA) there is an inactivation event (dead enzyme). Walsh has argued (1984) that the partition ratio is a key indicator of a compound's utility as an enzyme inactivator in vivo. The specificity of inactivation increases as the partition ratio decreases since few reactive molecules escape the enzyme. This minimizes the nonspecific modification of other cellular components, which may result in side effects and toxicity. Thus, epoxy-ATP joins a select group of "perfect" inhibitors including gabaculine (inactivates GABA transaminase, partition ratio = 1; Burnett et al., 1980), vinylglycine (inactivates L-aspartate transaminase, partition ratio = 1; Rando, 1974), and isatoic anhydride (inactivates chymotrypsin, partition ratio = 1; Moorman & Abeles, 1982).

An unexpected finding was that while epoxy-ATP is able to completely suppress the polymerase activity of the Klenow

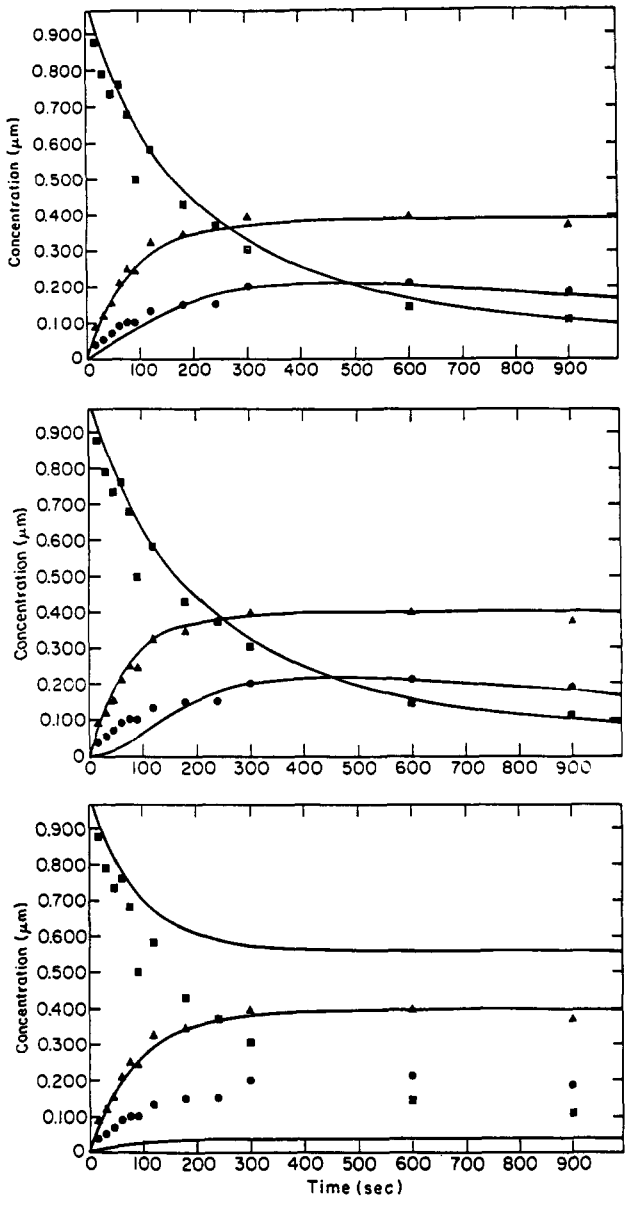


FIGURE 6: Computer simulation of the model depicted in Scheme IV. Experimental data were obtained by incubation of 0.4 μM enzyme, 1 μM 5′-^{32}P-end-labeled 13/20-mer, and 40 μM epoxy-ATP in the standard reaction buffer. Aliquots were removed at the indicated times, and the DNA products were analyzed by gel assay: (▲) 14-mer, (■) 13-mer, and (●) 12-mer DNA primers. Upper trace, complete simulation; middle trace, simulation setting $k_{exo\,1} = 0$; lower trace, simulation setting $k_{exo\,2} = 0$. Details of the simulation are given under Experimental Procedures.

fragment, the enzyme retains 3′ → 5′ exonuclease activity. A mechanism is proposed for the inactivation of the Klenow fragment by epoxy-ATP and DNA (Scheme IV). Note that there are two species capable of binding and degrading DNA in this mechanism: free enzyme (E) and the Klenow–14^e/20-mer complex (E–14^e/20). Computer simulation of the mechanism outlined in Scheme IV produces a reasonable fit to the experimental data (Figure 6). Neglecting the exonuclease activity associated with free enzyme ($k_{exo\,1} = 0$, Scheme IV) has little effect on the simulation; however, similar removal of exonuclease activity associated with the Klenow–14^e/20-mer complex ($k_{exo\,2} = 0$, Scheme IV) clearly results in a poor fit to the experimental data.

This simulation suggests that the epoxy-ATP-inactivated enzyme (E–14^e/20) is the major species responsible for DNA degradation. That the epoxy-bearing primer terminus resides exclusively at the polymer binding site is supported by the selective suppression of polymerase activity by 14^e/20-mer DNA and the lack of hydrolysis of the epoxy-terminated primer by the exonucleolytic activity of the enzyme. The simulation also suggests that the enzyme can bind DNA at the exonuclease binding site in a kinetically competent manner even while the polymerase site is occupied with duplex DNA.

The Klenow fragment has been cocrystallized with duplex DNA as well as with single-stranded DNA (Steitz et al., 1987). Difference electron density maps between both of the DNA complexes and the native protein show three to four nucleotides of single-stranded DNA bound at the exonuclease site and extending toward the 3′ end of the model-built duplex. It has been postulated that the binding of the 3′ primer terminus to the exonuclease site of the enzyme requires melting of three to four bases of duplex DNA and sliding of the primer strand into a single-stranded binding region of the protein (Steitz et al., 1987). The template strand is not visible in the difference maps but is presumably still bound in the polymerase (duplex) binding site of the enzyme.

The 3′ → 5′ hydrolysis of radiolabeled DNA by the Klenow fragment is little affected by prior inactivation of the polymerase activity with epoxy-ATP. It is unlikely that the observed degradation is actually due to hydrolysis of single-stranded DNA. The duplex DNA substrate used in this experiment was radiolabeled only in the primer strand and contained a slight (3%) excess of the template. It is possible that single-stranded DNA is present in solution in equilibrium with the duplex template-primer. This cannot account for the observed exonuclease rate, however, since the K_D for single-stranded DNA is substantially higher than that of the duplex (R. Kuchta, unpublished results). These data suggest that it is the intact duplex that is a substrate for hydrolysis by the exonuclease activity of the epoxy-ATP-treated enzyme.

It is surprising that the presence of 14^e/20-mer bound at the polymerase site has little effect on the observed activity at the exonuclease site. One would expect either the K_D for exogenous duplex DNA to increase or the $k_{exo\,2}$ to decrease for the E–14^e/20-mer complex since the duplex binding site is presumably occupied. Fixing the epoxy-bearing primer terminus tightly at the polymerase site may sufficiently expose the exonuclease binding site, thus allowing the approach of a second DNA substrate. It is likely that the rate-limiting step in DNA hydrolysis is an enzyme-induced melting of the DNA duplex to accommodate interaction with the exonuclease (single-stranded) binding site (R. Kuchta, unpublished results). The data presented here suggest that the enzyme can effect DNA melting without prior binding of the duplex to the polymerase binding site of the enzyme.

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Characterization of Antibodies to Dihydrothymine, a Radiolysis Product of DNA†

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ABSTRACT: Antibodies to dihydrothymine were elicited by immunizing rabbits with dihydrothymidine monophosphate conjugated by carbodiimide to BSA. By use of an ELISA assay, the antibodies produced were found to be specific for dihydrothymine. Hapten inhibition studies showed that dihydrothymidine monophosphate was 3 orders of magnitude more effective as an inhibitor than thymidine monophosphate and 4 orders of magnitude more effective than thymidine glycol monophosphate. With DNA containing dihydrothymine, antibody reactivity was observed at 20 fmol of dihydrothymine, which is approximately 0.1 dihydrothymine per 10000 bases. Thus, the assay is very sensitive. The antibody reacted with denatured DNA containing dihydrothymine but not with native DNA containing this lesion. The antibody was used for measurement of in vivo incorporation of dihydrothymidine in wild-type *Escherichia coli* or mutants defective in their ability to remove dihydrothymine from DNA or in the de novo synthesis of thymidylate. Lastly, antibodies to dihydrothymine were used to quantitate the formation of dihydrothymine in DNA X-irradiated under N_2. Production of dihydrothymine in irradiated DNA correlated with the level of reducing species produced by X-rays, and dihydrothymine was produced preferentially in irradiated single-stranded or denatured DNA as compared to irradiated duplex DNA.

Ionizing radiation produces a broad spectrum of DNA base modifications. Many of these have been identified in X-irradiated solutions of purines, pyrimidines, and nucleotides, as well as in DNA [for review, see Teoule (1987), von Sonntag and Schuchmann (1986), and Hutchinson (1985)]. Although damage to DNA bases constitutes the largest class of lesions produced by ionizing radiation, individual damages are produced in low yields, making their detection and quantitation difficult.

The spectrum of base damage produced in DNA by ionizing radiation can differ depending on the irradiation conditions. In irradiated aerated solutions of DNA, products are formed not only from attack by radicals produced from ionized water

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