Photoaffinity Labeling of the Klenow Fragment with 8-Azido-dATP*

(Received for publication, July 12, 1989)

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The photoaffinity compound 8-azido-dATP was used as a probe for the deoxyribonucleoside triphosphate-binding site of the large fragment of DNA polymerase I. Azido-dATP specifically modified a saturable binding site within the Klenow fragment, and each of the four natural deoxyribonucleoside triphosphate substrates competed with labeling at this site in proportion to its binding constant, as previously defined by equilibrium dialysis. Analysis of tryptic peptides after azido-dATP modification revealed five major cross-linking products, which apparently arose from five distinct photoproducts formed near Tyr-766.

DNA polymerase I of *Escherichia coli* fulfills its biological roles by coordinating three associated activities: polymerase, editing (3'-to-5') exonuclease, and nick translating (5'-to-3') exonuclease (1). Its activities can be partitioned by limited proteolysis, which produces two fragments (2, 3). The larger of these, the Klenow fragment, is a useful model for structural and mechanistic studies. Although smaller than most DNA polymerases, the Klenow fragment, is a useful model for structural studies. The structure of the Klenow fragment has been determined by x-ray crystallography as a complex with dTMP (4), an end-product inhibitor of its exonuclease activity (5). The Klenow fragment consists of two domains (Fig. 1). dTMP binds to the small domain with its deoxyribose-phosphate buried in the protein, an orientation compatible with the action of an exonuclease similarly bound at this site (6). Other studies place the exonuclease active site nearer to the small domain, remote from the dTMP-binding site (7).

To determine the location of the deoxyribonucleoside triphosphate (dNTP) binding site, we chose to cross-link the photoaffinity label 8-azido-dATP (Na,dATP) to the Klenow fragment. With this compound, cross-link formation is not constrained by the reactivity of amino acid side chains because the azido group decomposes to a highly reactive nitrene upon ultraviolet irradiation (8). Therefore, Na,dATP should modify residues near the polymerase active site regardless of their inherent reactivity, complementing the footprinting experiments described above.

MATERIALS AND METHODS

Preparation of the Klenow Fragment—Two plasmids, provided by Catherine Joyce (Yale University), were used for overproducing the Klenow fragment in *E. coli*. The plasmid pCJ122, was used as described in Ref. 14. Strain AR120, a cryptic-X N99 derivative that is cI+, was transformed with pCJ122, and overexpressed Klenow fragment was purified according to the method of Joyce and Grindley (14).

Preparation of [γ-32P] 8-N&dATP—Non-radioactive Na,dATP was the generous gift of Bob Haley (University of Kentucky). The γ-phosphate of Na,dATP was made radioactive by an enzymatic exchange reaction developed by Glynn and Chappell (16), modified for use with azido-nucleoside triphosphate compounds (17). A typical exchange reaction used 4 μCi of 32P, and 0.5 μmol of Na,dATP and provided [γ-32P] 8-N&dATP at a specific activity of 3-6.5 μCi/μmol.

Analytical and Preparative Photoaffinity Labeling of the Klenow Fragment with 8-N&dATP—Analytical cross-linking experiments were performed at room temperature in 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.1 mM β-mercaptoethanol, 50 mM NaCl (buffer D), using Klenow fragment which had been dialyzed against buffer D lacking MgCl₂. Irradiation mixtures were constructed as 50-100-μl droplets on a flat sheet of parafilm. All components were premixed, and aqueous Na,dATP was mixed thoroughly and carefully into the droplets with a micropipette. The droplets were irradiated with an ultraviolet lamp (Mineral Light, UVG-54) held at a fixed distance of 13 cm from the droplets by a stand specifically designed to hold the lamp (UV Products, J-129). The flux of ultraviolet light under these conditions was measured as 271 microwatts/cm² with a dosimeter (S.I. Schamberger).

Preparative scale cross-linking was performed similarly, except that paraffin-lined microtiter plate lids were used to contain the correspondingly larger volumes, usually about 10 ml, and all components of the irradiation mixture including Na,dATP were premixed.

Footprinting experiments described above.

1. The abbreviations used are: dNTP, deoxyribonucleoside triphosphate; Na,dATP, 8-azido-dATP; FTH, phenylthiohydantoin; HPLC, high performance liquid chromatography.

2. C. Joyce, unpublished observations.
The final concentrations of Klenow fragment and NsdATP during preparative irradiation were 2.7 mg/ml and 41 nM, respectively.

**Quantitation of the Extent of Cross-linking—**The extent of NsdATP modification was measured by separating the Klenow fragment and non-cross-linked NsdATP with open gel filtration columns, essentially according to Penefsky (18). Sephadex G-50 superfine in 50 mM Tris-HCl, pH 7.5, was spun in a 1.5-ml disposable microcolumn to a large and small domains are joined by the loop between helices F and G. dTMP binds to the small domain, near the carboxyl terminus of strand 2. Reprinted by permission of Thomas A. Steitz and of Nature, Vol. 313, p. 762, copyright 1985, Macmillan Magazines Limited.

The amount of cross-linked Klenow fragment was calculated from the amount of radioactivity in the effluent and the specific activity of [γ-32P]NsdATP with spun gel filtration columns, essentially according to Penefsky (18). Sephadex G-50 superfine in 50 mM Tris-HCl, pH 7.5, was spun in a 1.5-ml disposable microcolumn to a packed volume of approximately 1 ml. Before loading columns, analytical cross-linking mixtures were treated with 1 μl of β-mercaptoethanol/25 μl of irradiation mixture (19), followed by addition of two volumes of bovine serum albumin (20 mg/ml in buffer D), which acted as a carrier during separation.

The amount of cross-linked Klenow fragment was calculated from the amount of radioactivity in the effluent and the specific activity of [γ-32P]NsdATP. This value, divided by the total amount of Klenow fragment applied to the column, gives the cross-linking efficiency, the fraction of protein molecules modified with NsdATP assuming a stoichiometry of modification of one. Control experiments showed that the recovery of Klenow fragment exceeded 95% and that this procedure efficiently separated the Klenow fragment and unincorporated NsdATP, so that without irradiation less than 0.2% of the applied radioactivity was recovered in the effluent. In addition, a large number of samples could be processed rapidly by a procedure that made very few assumptions about photoadduct stability.

**Preparative Trypsin Digestion and Primary Reverse phase Separations—**An irradiated cross-linking mixture containing 29.5 mg or 433 nmol of Klenow fragment was removed to a centrifuge tube, and the Klenow fragment was precipitated by adding cold trichloroacetic acid to a final concentration of 10% (v/v). The suspension was left on ice for 10 min before centrifugation for 10 min at 4 °C. The pellet was washed with cold acetone to remove residual trichloroacetic acid and was allowed to dry before resuspension in 3 ml of 8 M urea, 50 mM NH₄HCO₃. This suspension was diluted 5-fold with 50 mM NH₄HCO₃, the pH was checked with paper to ensure that it was near 8.0, and trypsin was added to a 1:50 weight ratio to protein. After 2 h at 37 °C, the digest was injected sequentially as 12 consecutive 1-ml aliquots onto a Vydac C₂₃ reverse-phase column (1.0 × 25 cm, 218TP1010; The Separations Group) thoroughly equilibrated with a mixture (1% B) of the starting (A, 20 mM Na₂HPO₄, pH 6.8) and the developing (B, 70% aqueous acetonitrile) solvents. The column was developed at a flow rate of 3.3 ml/min with the following gradient: 1% B isocratic wash for 10 min, 1-35% B in 45 min, 35-55% B in 80 min, and 55-99% B in 22 min. Fractions (2.3 ml), collected throughout sample application and the gradient, were assayed for 32P radioactivity.

**Rechromatography of Bound Radioactive Peaks—**Rechromatography of the radioactive peaks was purified further by rechromatography on Vydac C₂₃ reverse-phase columns (0.46 × 25 cm, 214TP54; The Separations Group). The starting solvent (A) was 10 mM KH₂PO₄, pH 2.55, and the developing solvent (B) was 70% aqueous acetonitrile. Fractions for rechromatography were diluted with an equal volume of solvent A and were then injected as 1 ml aliquots onto a column that was thoroughly equilibrated with 1% B. The column was developed at a flow rate of 0.7 ml/min with the following gradient: 1% B isocratic wash for 10 min, 0-35% B in 10 min, 35-75% B in 10 min, and 75-99% B in 10 min. Fractions (0.35 ml) were monitored for radioactivity by Cerenkov counting and were stored at 4 °C until protein chemical analysis.

**Analytical Cross-linking of NsdATP to the Klenow Fragment—**To determine the optimal conditions for preparative photocross-linking, we first evaluated how the level of modification varied with Klenow fragment concentration, irradiation time, and NsdATP concentration. We found that NsdATP modified approximately 50% of the Klenow fragment (Fig. 2A) and that cross-linking was a hyperbolic function of irradiation time (Fig. 2B). Titration of the cross-linking reaction with NsdATP showed that it modifies a saturable site within the Klenow fragment (Fig. 2C), with half-saturation occurring at a concentration of about 35 nM. This value is similar to the Kₐ for unmodified dATP (33 nM) observed by equilibrium dialysis of DNA polymerase I in the absence of substrate DNA (21). This suggests that the saturable site within the Klenow fragment modified by NsdATP is the dNTP-binding site, as defined by equilibrium dialysis.

**Preparative Cross-linking and Peptide Purification—**A preparative scale cross-linking mixture was irradiated, and the Klenow fragment was separated from unincorporated NsdATP by trichloroacetic acid precipitation, then digested with trypsin. Before precipitation, the level of modification was evaluated as 48% with spin gel filtration columns. Amino acid analysis and scintillation counting after digestion provided a similar measure of cross-linking efficiency (92%). Equally important, this second method of quantitation demonstrated that photoadducts were not degraded by brief exposure to trichloroacetic acid, since the expected amount of...
Affinity Labeling of the Klenow Fragment with 8-Azido-dATP

I. Universal concentration, irradiation time, and N-ATP concentration. A, effect of varying Klenow fragment concentration. The Klenow fragment concentration was varied from 0.059 to 0.950 mg/ml as the irradiation time and N-ATP concentration were held at 5 min and 50 µM, respectively. Incorporation (indicated by circles) remained constant at about 50%. B, effect of varying irradiation time. Incorporation was measured over a 5-min interval as the concentrations of N-ATP and the Klenow fragment were held constant at 50 µM and 0.950 mg/ml, respectively. C, effect of varying N-ATP concentration, with and without competition by dATP. The irradiation time and the Klenow fragment concentration were held at 5 min and 0.850 mg/ml, respectively, as the concentration of N-ATP was varied from 10 to 90 µM. Titrations were performed in the absence (circles) and presence (squares) of 1 mM dATP.

FIG. 2. Incorporation of N-ATP as a function of Klenow fragment concentration, irradiation time, and N-ATP concentration. A, effect of varying Klenow fragment concentration. The Klenow fragment concentration was varied from 0.059 to 0.950 mg/ml as the irradiation time and N-ATP concentration were held at 5 min and 50 µM, respectively. Incorporation (indicated by circles) increased in proportion to the Klenow fragment concentration, with a slope of approximately 2, while the cross-linking efficiency (squares) remained constant at about 50%. B, effect of varying irradiation time. Incorporation was measured over a 5-min interval as the concentrations of N-ATP and the Klenow fragment were held constant at 50 µM and 0.950 mg/ml, respectively. C, effect of varying N-ATP concentration, with and without competition by dATP. Incorporation (indicated by circles) remained constant at about 50%.

Radioactivity precipitated with the Klenow fragment.

Tryptic peptides were fractionated in two steps, the first reverse-phase HPLC at neutral pH. This produced a profile of radioactivity containing 12 peaks of unbound material, generated by 12 sequential injections of the digest, and five major retained peaks, labeled I through V in Fig. 4. These five radioactive peaks were in a region where little absorbance material eluted, and the bulk of absorbance peaks was not associated with significant levels of radioactivity.

Several observations suggest that the unbound radioactive material is an undefined degradation product and that all major cross-linked peptides are represented by peaks I through V. First, unbound material failed to bind to re-equilibrated columns, as would be expected of low molecular weight compounds. Second, the percentage of unbound radioactivity varied with each digest, generally accounting for 30-70% of the recovered radioactivity. However, variations were not encountered with aliquots of the same digest and were not reflected in the level of modification, which did not vary more than 5%. Third and most important, variations did not result in the appearance of new retained peaks. Finally, correcting the amount of peptide in peaks I through V for recovery (1/0.72) and degradation (1/0.31) (Table I) gives a cross-linking efficiency of 42%, in good agreement with the values cited above. This suggests strongly that the radioactivity in the 12 unbound peaks was once associated with protein.

The amount of cross-linked peptide in peaks I through V, calculated from the radioactivity associated with each peak, ranged from 5.6 to 10.8 nmol (Table I). The anticipated background of non-cross-linked peptides was 270 nmol. Because the radioactivity peaks could not be assigned to specific absorbance peaks, they were rechromatographed separately at pH 2.55, to ensure unambiguous protein chemical analysis (Fig. 5). Lowering the pH for chromatography protonated all peptide carboxylates and, for cross-linked peptides, the 2-amino group of adenine and the triphosphate, resulting in a two-dimensional peptide fractionation. All modified peptides (except IV and V) eluted at different positions during rechromatography as they had during the primary separation, indicative of undefined physical differences.

Recoveries of the five cross-linked peptides ranged from 40 to 68% (Table I), typical for reverse-phase HPLC of peptides. Therefore, peptides I through V represent the major products of N-ATP modification. More than 90% of the recovered radioactivity was retained during rechromatography, in contrast to the apparent degradation observed during the primary separation, suggesting that photoproduc degradation occurs at an early stage in the preparation of peptides.

After rechromatography, broad radioactive peaks could be correlated with similarly broad peaks in the absorbance chromatogram, an indication that the modified peptides were sufficiently pure for protein chemical characterization. These peptides eluted in larger volumes (1.75-3.5 ml) than non-
FIG. 4. Elution profiles obtained during the first peptide purification step. A tryptic digest of Klenow fragment (180 nmol) modified with N3dATP was fractionated by reverse-phase HPLC at neutral pH. The column effluent was monitored for A250 (upper tracing). The profile of radioactivity (shaded) obtained from fractions collected at 0.7 min intervals is overlaid. Labels I–V refer to the major radioactive peaks.

TABLE I
Quantitation of cross-linked peptide recoveries

<table>
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<tr>
<th>Step</th>
<th>Description</th>
<th>Recovery</th>
<th>UF%</th>
<th>UB%</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>M</th>
</tr>
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<td>48</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Preparative tryptic digestion</td>
<td></td>
<td>87</td>
<td>180</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>Primary HPLC separation</td>
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<td></td>
<td>80.7</td>
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<td>4B</td>
<td>Peak II rechromatography</td>
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<td>68</td>
<td>3.2</td>
<td>4.0</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>3.0</td>
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<tr>
<td>4E</td>
<td>Peak V rechromatography</td>
<td></td>
<td>51</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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</tbody>
</table>

a Unfractionated protein or peptides.
b Unbound material.
c Minor peaks and side fractions.
d Starting with 143 nmol of unmodified Klenow fragment.
e Cross-linking efficiency, as measured with spun columns.
f See Fig. 4.
g See Fig. 5.

cross-linked peptides (0.2–0.4 ml) and were often spiked with the more sharply co-eluting non-cross-linked peptides. This elution behavior, also observed during the primary separation, must be considered when evaluating purity; the difference in elution volumes causes modified peptides to product peaks 10-fold shorter than equivalent amounts of non-cross-linked peptides.

Protein Chemical Characterization of Peptides Cross-linked to 8-N3dATP—The amino acid sequences and compositions of peptides I through V showed that (i) these peptides had the same sequence, (ii) all modifications occurred near Tyr-766, (iii) some cross-links were at the polypeptide backbone, and (iv) cross-linking may have been confined to adjacent chemical groups.

A single tryptic peptide, spanning residues 759–775 of the Klenow fragment sequence, accounted for the five major products of N3dATP modification (Table II). Curiously, peptide I could not be sequenced beyond Leu-764 and peptide II beyond Ile-765, even though they were produced by trypsin digestion. Peptides III through V, in contrast, could be sequenced to Arg-770, but did not produce a PTH derivative at cycle 770, which would interfere with the chemistry of the Edman reaction, or insertion of an aromatic nitrene at an a-carbon, which would sterically block cyclization (22, 23). Therefore, Tyr-766 was the most likely candidate for a cross-linked amino acid because it was not found in cycle 8 of peptides III, IV, and V. In this scheme, peptides III, IV, and V separated during HPLC because of different sites of modification at Tyr-766.

The amino acid compositions of peptides I, II, and III were indistinguishable (Table III), even though peptide III produced a sequence 11 residues longer than peptide I and 10 residues longer than peptide II. Therefore, peptides I and II stopped sequencing prematurely because they were modified by N3dATP in a way that prevented further sequencing. At least two kinds of cross-link could block sequencing internally: modification of an a-carbonyl group, which would interfere with the chemistry of the Edman reaction, or insertion of an aromatic nitrene at an a-carbon, which would sterically block cyclization (22, 23). Therefore, the inability to sequence peptide I beyond Leu-764 could stem from a cross-link at the a-carbon or the carbonyl of Ile-765. Similar modifications at Tyr-766 would account for peptide II.

This protein chemical characterization is compatible with significant amounts of radioactivity were not extracted with PTH derivatives during sequencing. Presumably, the high charge density of N3dATP prevented extraction of cross-linked derivatives into the organic solvents used for sequencing. Furthermore, assignment of a residue to a particular cycle argued strongly that the residue was unmodified. Therefore, Tyr-766 was the most likely candidate for a cross-linked amino acid because it was not found in cycle 8 of peptides III, IV, and V. In this scheme, peptides III, IV, and V separated during HPLC because of different sites of modification at Tyr-766.
FIG. 5. Rechromatography of peaks I–V. The major peaks from the primary separation were purified further by reverse-phase HPLC at pH 2.55. The profile of radioactivity (shaded), from 0.5 min fractions, and the profile of absorbance are superimposed for peaks I (A), II (B), III (C), IV (D), and V (E). Points of inflection in the gradient slope (bold line) correspond to 1 and 37.5% solvent B.

Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>I</td>
<td>759 A 1 N F G L end 764</td>
</tr>
<tr>
<td>II</td>
<td>759 A 1 N F G L I end 765</td>
</tr>
<tr>
<td>III</td>
<td>759 A 1 N F G L I (Y) G M S A F G L A R end 775</td>
</tr>
<tr>
<td>IV</td>
<td>759 A 1 N F G L I (Y) G M S A F G L A R end 775</td>
</tr>
<tr>
<td>V</td>
<td>759 A 1 N F G L I (Y) G M S A F G L A R end 775</td>
</tr>
</tbody>
</table>

Table II shows the amino acid sequences of peptides I through V. The positions within the DNA polymerase I sequence of the amino- and carboxyl-terminal residues are given in italics. Tyr-766 of peptides III, IV, and V is enclosed in parentheses because it was not detected during sequencing.

Discussion

Examination of the Klenow fragment structure shows that Tyr-766, the residue modified by N₈dATP, is located at the carboxyl terminus of helix O, with its side chain projecting downward toward the floor of the proposed DNA binding cleft (Fig. 7). This is an intriguing position for the dNTP-binding site because it is consistent with the position of the polymerase active site as inferred from crystallographic and footprinting studies. Modeling DNA into its putative binding site reveals a pocket that could be occupied by a dNTP substrate (4). This pocket is surrounded by the floor of the β-pleated sheet, a cluster of α-helices which includes helix O, and the phosphodiester backbone of DNA. In addition, the length of the bound primer strand, as estimated by footprinting, positions the terminal nucleotide near the floor of the cleft and the carboxyl terminus of helix O (7). Thus, crystallographic, footprinting, and photocross-linking studies all agree with a model that places the polymerization substrates near the Tyr-766 side chain.

This location is also consistent with NMR spectroscopy of the dNTP-binding site (24, 25). These studies show that dATP binds in an anti conformation near at least two hydrophobic amino acid residues, which are thought to be some combination of isoleucine, leucine, and valine, and 1 aromatic residue, probably tyrosine. Interestingly, the 2 residues amino-terminal to Tyr-766 are hydrophobic amino acids, Ile-765 and Leu-764.

In addition, Modak and his co-workers have used three other reagents to probe the dNTP-binding site (see Fig. 7). Pyridoxal phosphate, a reagent specific for amino groups,
Theoretical compositions for peptides spanning Ala-759-Ile-765 or residue more than expected. Note that peptides I, II, and III lack one tyrosine and contain instead 1 glutamic acid residue more than expected.

<table>
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<th>Residue</th>
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<tr>
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<tr>
<td>Arg</td>
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<td>0.7</td>
<td>0.8</td>
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nmol found: 0.96 1.12 0.88
nmol expected: 1.11 1.23 0.62

*Variable, non-stoichiometric recoveries of Leu, Ile, and Val are observed when they are adjacent (37), as are Leu-764 and Ile-765.

Peptides I and II were analyzed in duplicate, peptide III only once.

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**Fig. 6.** *Proposed sites of NsdATP modification.* Ile-765 and Tyr-766, as they appear in the Klenow fragment structure, are viewed along three axes. Labels refer to the Ile-765 backbone carbonyl (1), the Tyr-766 backbone amide (2), and the Tyr-766 α, β, and γ-carbons (3-5). Panel A shows Ile-765 and Tyr-766 on the left and right, respectively. In panel B Tyr-766 is behind Ile-765, and in panel C both side chains project away from the viewer.

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reacts with 4 lysine residues of the Klenow fragment (26). However, the only one protected by primer-template or dTTP is Lys-768, in the middle of helix O. Unsubstituted dTTP, directly cross-linked to the Klenow fragment with ultraviolet light, forms an adduct with His-881 (27). Its side chain projects upward from the strand 12-strand 13 turn at the floor of the proposed DNA-binding cleft and is very near the Tyr-766 side chain. Although 5'-fluorosulfonylbenzoyl adenosine modifies two sites within the Klenow fragment, only its reaction with Arg-682 is competitive with primer-template or dNTP binding (28). Arg-682 is located in the random coil connecting helices J and K, on the side of the DNA-binding cleft opposite to Tyr-766. In summary, three of these four reagents place the dNTP-binding site near the carboxyl terminus of helix O and the strand 12-strand 13 turn. Intriguingly, all these residues, Tyr-766, Lys-768, His-881, and Arg-682, are strictly conserved among the Klenow fragment, the DNA polymerase I of *Thermus aquaticus* (29), the DNA polymerase I of *Streptococcus pneumoniae* (30), and T7 DNA polymerase (31). All but Arg-682 are conserved between the Klenow fragment and T5 DNA polymerase (32).

All affinity labeling experiments described in this paper and above omitted one substrate or cofactor required for polymerization, to prevent incorporation of the affinity labels into DNA. Therefore, these reagents might locate a binding site without precisely defining the one used during catalysis. In fact, rapid-quench experiments with DNA polymerase I show a partial rate-determining step that follows the binding of dNTP and DNA substrates but precedes phosphodiester bond formation (33). This rate-limiting step may arise from a conformational change of the enzyme-dNTP-DNA complex (33). Despite its limitations, affinity labeling usefully identifies candidate residues whose precise role in substrate binding and catalysis then can be addressed by directed mutagenesis.

Several lines of evidence (reviewed in Refs. 34 and 35) now support the view of separate sites for polymerization and editing activities in the Klenow fragment. This separation raises interesting questions about the mechanism of proof-reading. Although the active sites are about 30 Å apart, the Klenow fragment does not always dissociate from the primer-template when switching from polymerization to exonucleolytic degradation modes (36). Instead the same polymerase molecule that incorporates a mismatched nucleotide can also correct the error. If replication fidelity depends upon error checking after incorporation of each nucleotide and if polymerization errors are detected only at the exonuclease active site, then the enzyme must move the primer terminus the distance between the two sites after each round of polymerization. Alternatively, as yet undefined structural features in the polymerase domain might be responsible for error detection, which then switch the enzyme from a polymerization to exonucleolytic degradation mode (12). Thus, the recognition of a polymerization error and its active correction might correspond to two discrete mechanistic processes, each making important contributions to the maintenance of accurate DNA replication.

**Acknowledgments**—We thank Boyd E. Haley for his generous gifts of NsdATP, Brian Francia and Kendall L. Knight for advice during photocross-linking experiments, and Kenneth R. Williams, Kathryn L. Stone, and James Elliott for their assistance and advice during peptide purification and analysis. We are grateful to William E. Balch and Margaret M. Elliott for introducing us to their spun-column method and to Lorena S. Beese and Thomas A. Steitz for providing Figs. 1, 6, and 7.
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Photoaffinity labeling of the Klenow fragment with 8-azido-dATP.