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Identification and Amino Acid Sequence of the Deoxynucleoside Triphosphate Binding Site in *Escherichia coli* DNA Polymerase I†

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ABSTRACT: We have labeled the large fragment of *Escherichia coli* DNA polymerase I (Pol I) with pyridoxal 5′-phosphate, a substrate binding site directed reagent for DNA polymerases [Modak, M. J. (1976) *Biochemistry 15*, 3620–3626]. A covalent attachment of pyridoxal phosphate to Pol I results in the loss of substrate binding as well as the polymerase activity. The inactivation was found to be strictly dependent on the presence of a divalent metal ion. Four moles of pyridoxal phosphate was found to react per mole of the enzyme, while in the presence of substrate deoxynucleoside triphosphate only 3 mol of pyridoxal phosphate was bound. To identify the substrate-protected site on the enzyme, tryptic peptides from enzyme labeled with pyridoxal phosphate and tritiated borohydride, in the presence and absence of substrate, were resolved on a C-18 reverse-phase column. A single peptide containing the substrate-protected site was identified and further purified. The amino acid composition and sequence analysis of this peptide revealed it to span residues 756–775 in the primary acid sequence of Pol I. Lys-758 of this sequence was found to be the site of the pyridoxal phosphate reaction. It is therefore concluded that Lys-758 is the site of binding for the metal chelate form of nucleotide substrates in *E. coli* DNA polymerase I.

The general mechanism of the enzymatic synthesis of DNA has been elucidated, but the features of the molecular details of template-directed base selection and the basis for fidelity have not been clarified. Recent developments in the cloning of the enzymatically active large fragment (Klenow fragment) of *Escherichia coli* DNA polymerase I (Pol I)[1] (Joyce & Grindley, 1983), together with the knowledge of the primary amino acid sequence of entire Pol I (Joyce et al., 1982), have made the study of the structure–function relationship of this enzyme attainable. With the demonstration of high-resoluton

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; PLP, pyridoxal 5′-phosphate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; dNTP, deoxynucleoside triphosphate; $NaBH_4$, sodium borohydride; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

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crystal structure of the Klenow fragment of Pol I (Ollis et al., 1985), such studies could be meaningfully interpreted and have provided a model system for DNA replication enzymes in general.

Toward the general goal of defining the structure–function relationship in DNA polymerases, we had used a number of enzyme inhibitors and determined the mechanism of their action. These studies permitted the correlation between the loss of a specific step in catalysis to the site of inhibitor action. The identification and definition of the sites of reactivity of various inhibitors have direct utility in the identification of important structural domains in DNA polymerases. For example, pyridoxal 5′-phosphate (PLP) specifically reacts at the substrate triphosphate binding site (Modak, 1976a; Modak & Dumaswala, 1981), while phenylglyoxal is a reagent which inhibits the template-primer binding ability of various DNA polymerase (Srivastava & Modak, 1980), On the basis of our original observation that PLP is a competitive inhibitor of various DNA polymerases with respect to substrate dNTPs (Modak, 1976a), we have now labeled and identified the pyridoxal phosphate reactive lysine-758 in the substrate dNTP binding domain of the Klenow fragment of *E. coli* DNA polymerase I.

Materials and Methods

Calf thymus DNA and all nonradioactive nucleoside triphosphates were obtained from P-L Biochemicals. Pyridoxal 5′-phosphate and trifluoroacetic acid were from Sigma. Trypsin (TPCK treated) was obtained from Worthington. Acetonitrile and HPLC-grade water were products of Fisher Scientific Co. [α-^{32}P]TTP (400 Ci/mmol) was from Amersham Inc. Tritiated $NaBH_4$ was obtained from ICN. All other chemicals were of analytical grade. DNA polymerase I (Klenow fragment) was purified from a clone (Joyce & Grindley, 1983) generously given by Dr. Catherine Joyce of Yale University.

DNA Polymerase Assay. DNA polymerase assay conditions were essentially similar to those described by Srivastava and Modak (1980).

Treatment of DNA Polymerase with Pyridoxal Phosphate. DNA Pol I (adjusted to 200 μg/mL) was incubated with pyridoxal phosphate in the dark in a solution containing 50 mM Hepes, 7.8, 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 20% (v/v) glycerol, and 1 mM pyridoxal phosphate, at 37 °C for 10 min. The reaction mixture was immediately cooled to 0 °C, and freshly prepared chilled solution of either unlabeled $NaBH_4$ or NaB^3H_4 in 5 mM NaOH was added to give a final concentration of 10 mM. The mixture was allowed to stand on ice for 15 min or until the yellow color of the reaction mixture completely disappeared. Small aliquots (2–4 μg) were immediately withdrawn and used for substrate binding and template-primer binding studies as described later. To check for DNA polymerase activity, an aliquot of PLP-treated enzyme was diluted in 0.1% BSA solution to a final concentration of 1 μg/mL, and 5 μL of diluted enzyme was used for polymerase assay. For protection experiments, the above-described incubation mixture was supplemented with the desired concentration of either substrate triphosphates or activated DNA from calf thymus. To measure the extent of PLP incorporation into enzyme protein, the pyridoxylated enzyme was treated with NaB^3H_4 as above and left standing for 15 min over ice. The protein was precipitated by 10% trichloroacetic acid (TCA) with 100 μg of bovine serum albumin as a carrier protein. Precipitates were collected on a nitrocellulose filter and washed extensively with 5% TCA and finally with water. The filters were dried and counted for radioactivity. The specific activity of NaB^3H_4 was 1.4 Ci/mmol, and the number of PLP residues incorporated was calculated by assuming one tritium atom incorporated per mole of PLP.

For peptide analysis, 4–5 nmol of the enzyme was treated with PLP and $NaBH_4$ as described above, and the protein was precipitated by the addition of TCA to a final concentration of 10%. After standing on ice for 30 min, the precipitate was collected by centrifugation in microfuge tube and washed repeatedly with 5% TCA and finally with ether to remove TCA.

UV-Mediated Cross-Linking of Substrate dNTP to Pol I and Template-Primer Binding Assays. The protocol for UV-mediated cross-linking of substrate dNTP to Pol I and the nitrocellulose filter binding assay to determine the template-primer binding ability of the enzyme have been described earlier (Abraham & Modak, 1984; Srivastava & Modak, 1983).

Protein Chemistry Studies. The TCA-precipitated pyridoxylated enzyme protein was washed and dried as described above and was suspended in 100 μL of 50 mM ammonium bicarbonate, pH 8.0. Trypsin (TPCK treated) was added at a protein:enzyme ratio of 50:1, and the mixture was incubated at 37 °C. After 2 h, a second aliquot of trypsin (50:1 protein to enzyme ratio) was added, and the incubation was continued for 24 h. The digests were mixed with trifluoroacetic acid (final concentration 0.1%) and directly injected onto a Synchropak C-18 reverse-phase column equilibrated with 0.1% trifluoroacetic acid solution, pH 2.0. Peptides were eluted by increasing the concentration of 70% acetonitrile and 0.05% TFA (solution B) as follows: 0–40% B (0–90 min), 40–70% B (90–130 min), and 70–100% B (130–150 min). The elution rate was 0.7 mL/min. All the HPLC analyses were done by using Varian Vista 5500 liquid chromatography system equipped with a polychrome 9060 diode array detector. Peptides were monitored simultaneously at 215 and 280 nm.

Fractions containing pyridoxylated peptides were monitored by three different criteria: (i) a small aliquot of each fraction was directly counted in a scintillation counter for tritium incorporation; (ii) the spectrum of each peptide peak was scanned between 220 and 365 nm; and finally (iii) each fraction was analyzed by spectrophotometric excitation at 325 nm and emission recorded at 410 nm. Those peptides requiring further purification were dried in vacuo, redissolved in 0.1% TFA solution, and then separated on a C-4 reverse-phase column equilibrated in the same buffer. The acetonitrile gradient used for elution is described in the legend to Figure 3.

Amino Acid and Peptide Sequence Analysis. Aliquots of the peptides purified on a C-4 reverse-phase column were hydrolyzed in 6 N HCl containing 0.2% phenol for 16 h at 115 °C, converted to their phenylthiohydantoin derivatives, and analyzed on a Beckman 121 amino acid analyzer. For amino acid sequence analyses, the peptide samples (volume ranging from 0.2 to 0.5 mL) were loaded directly onto an applied Biosystems Model 470 gas-phase sequencer. The resulting phenylthiohydantoin derivatives were analyzed by HPLC as described earlier (Merril et al., 1984). Both the composition and sequence analyses were performed at the core facility for protein chemistry at Yale University supervised by Dr. K. R. Williams.

Results

Inactivation of DNA Polymerase I (Klenow Fragment) by Pyridoxal 5′-Phosphate. Figure 1 shows the effect of PLP incubation with Klenow fragment followed by reduction with

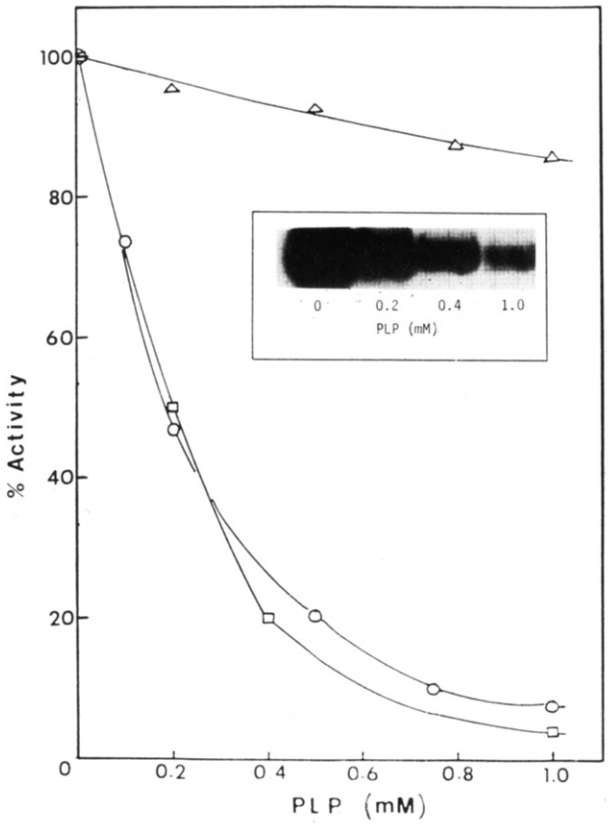

FIGURE 1: Effect of pyridoxal 5′-phosphate on the polymerase, substrate, and template binding activities of *E. coli* DNA polymerase I. Thirty five micrograms of *E. coli* DNA Pol I (Klenow fragment) was treated with the indicated concentration of PLP followed by reduction of the PLP–enzyme complex with a 10-fold excess of $NaBH_4$. Aliquots of modified enzyme, after 1:200 dilution, were used for DNA polymerase assays (○) while undiluted aliquots were used in substrate dNTP (□) and template binding (△) assays. The extent of substrate binding abilities of differentially modified enzyme was determined by UV-mediated cross-linking of dTTP to enzyme protein (see below) while the ability to bind to template-primer was quantitated by the nitrocellulose filter binding assay (see Materials and Methods. The inset is an autoradiogram of an SDS–polyacrylamide gel showing results of UV-mediated cross-linking of [^{32}P]dTTP to PLP-modified enzyme. For quantitation of cross-linking of dTTP to enzyme, appropriate radioactive bands were excised from the gel, and radioactivity was counted in a scintillation counter. These numbers are then used as a measure of substrate binding activity (□) in this figure.

sodium borohydride on the various activities of Pol I as a function of pyridoxal phosphate concentration. As can be seen from Figure 1, there is a progressive and linear decrease in the ability of the enzyme to bind to substrate dNTP (as judged by the extent of UV crosslinking of substrate dNTP to the enzyme) with an increase in PLP concentration. This pattern of inactivation of substrate binding ability was found to be very similar to the shape of the DNA polymerase inhibition curve. However, the DNA binding ability of the enzyme (as judged by the nitrocellulose filter binding assay) under the above conditions is not affected significantly. The inactivation of Pol I by PLP was found to be strictly dependent on the presence of Mg^{2+} (Table I). The presence of natural (e.g., activated DNA from calf thymus) or synthetic [e.g., poly-(dA)·$(dT)_{12}$; data not shown] DNA in the PLP modification mixture had no significant effect on the inactivation (Table I). However, a partial recovery of DNA polymerase activity was observed when substrate dNTP was included in the reaction mixture. This protection was found to be independent of the presence of template-primer. The extent of incorporation of PLP per mole of the enzyme, both in the presence and in the absence of substrate triphosphates, was measured

Table I: Effects of Substrate, Template, and Divalent Cation on Inactivation of Pol I by Pyridoxal 5′-Phosphate[a]

addition	pmol of TMP incorporated/15 min	% activity
control (no PLP)	50.0	100
PLP	2.0	4
PLP (minus Mg^{2+})	45.0	90
PLP + 2.5 mM TTP	13.0	27
PLP + 5 mM TTP	19.0	37
PLP + 5 μg of DNA	3.5	7
PLP + 10 μg of DNA	4.0	8

[a] Thirty-five micrograms of large fragment of *E. coli* DNA polymerase I was incubated for 5 min in a solution containing 50 mM Hepes–KOH, pH 7.8, 10 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 20% (v/v) glycerol, and 1 mM PLP. Other components were added to this reaction mixture at the indicated concentrations, and effects of these additions on enzyme inactivation were determined after reducing the enzyme–PLP complex with borohydride. For the determination of DNA polymerase activity, modified enzyme was diluted 1:200 in 0.1% bovine serum albumin, and a 5-μL aliquot was used in the standard DNA polymerase assay (see Materials and Methods).

Table II: Effects of Thymidine 5′-Phosphate in *E. coli* DNA Polymerase I[a]

addition	DNA polymerase % activity	mol of PLP/mol of enzyme[b]
control[c]	100	
PLP	20	3.6 ± 0.2
PLP + 5 mM TTP[d]	50	2.6 ● 0.3

[a] Seventy micrograms (about 1 nmol) of large fragment of *E. coli* DNA polymerase I was treated with 1 mM PLP as described in the legend to Table I. The enzyme–PLP complex, however, was reduced by using tritiated $NaBH_4$, and the incorporation of tritium into protein was quantitated by acid precipitation of the complex. The molar ratio of PLP incorporation into enzyme protein was calculated from the specific activity of borohydride. For determination of enzyme activity, modified and control enzyme solutions were diluted 1:200 in 0.1% BSA, and a 5-μL aliquot was used in the standard DNA polymerase assay. One hundred percent activity represents incorporation of 45 pmol of dTTP/15 min. [b] Values represented are an average of three experiments. [c] Enzyme treated with tritiated borohydride (without PLP) served as a control. [d] In this experiment, 5 mM dTTP was added along with PLP.

by determining the incorporation of tritiated sodium borohydride into pyridoxylated enzyme (Table II). At 80% inactivation, which is the upper limit of the linear range of PLP-mediated inactivation (Figure 1), a stoichiometry of about 4 mol of PLP/mol of enzyme was observed. However, the presence of TTP reduced the number of reacting PLP to 3, strongly suggesting that the TTP-protected site is the true dNTP binding site.

Comparative Tryptic Maps of the Native, PLP-Treated, and dNTP-Protected Pol I (Klenow Fragment). Earlier studies by us using Rauscher leukemia virus DNA polymerase (Modak & Dumaswala, 1981) and with Pol I (Hazra et al., 1984) have shown that PLP exclusively modifies the lysine residues in these enzymes. In order to determine the target lysine residue which is protected from pyridoxylation by substrate dNTP and thereby map the region where dNTPs bind, we resorted to tryptic peptide mapping using reverse-phase HPLC. Figure 2A,B illustrates the tryptic peptide maps of the native and PLP-inactivated enzyme. From the comparative tryptic peptide maps, it is evident that only three peptides (Figure 2A, panel II) are pyridoxylated as judged by the absorbance at 215 and 280 nm and by the distribution of radioactivity. The presence of reduced pyridoxal phosphate was also monitored by the absorbance at 325 nm (Figure 3, inset) and by the fluorescence emission at 410 nm. To determine which of these three peptides are involved in the

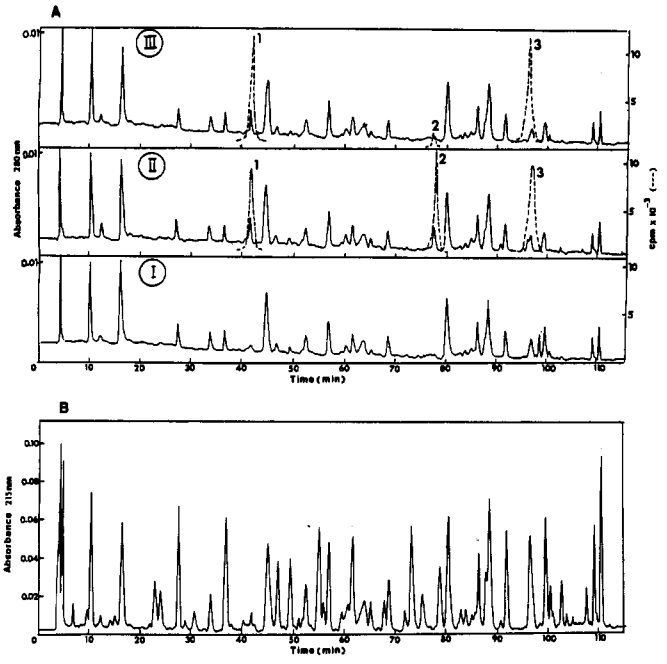

FIGURE 2: Reverse-phase HPLC separation of tryptic peptides of Pol I (Klenow fragment). The tryptic peptides derived from 1 nmol of Pol I were resolved on a Vydac C-18 column (The Separations Group) equilibrated with 0.1% trifluoroacetic acid. The elution conditions are described under Materials and Methods. The absorbance of peptide at 280 nm is shown in panel A. The three plots shown correspond to tryptic peptides obtained from untreated control enzyme (I), PLP-treated enzyme (II), and PLP treated in the presence of dNTP (III). Panel B represents a peptide separation pattern of unmodified enzyme monitored at 215 nm.

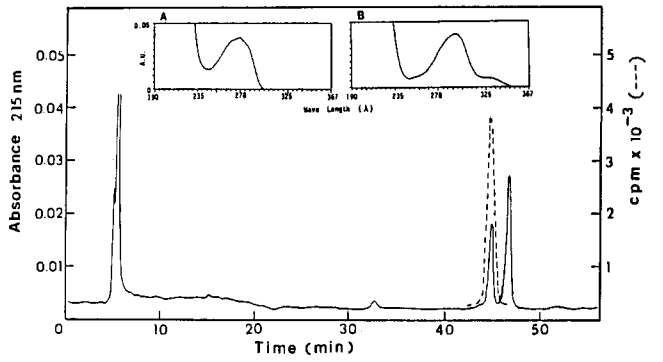

FIGURE 3: Purification of peptide 2 on a C-4 reverse-phase column. The peak fraction eluting at 78 min (Figure 2A, panel II) from a preparative run was lyophilized, redissolved in 0.1% TFA, loaded onto a Vydac C-4 column equilibrated with 0.05% TFA, and then eluted at a flow rate of 0.7 mL/min with increasing concentration of buffer B (0.05% TFA in 70% acetonitrile solution): 0–10 min (0–20% B), 10–90 min (20–40% B). The inset shows the comparison of the UV spectrum of peptides containing aromatic amino acids (A) and those containing pyridoxylated lysine residues (B).

process of dNTP binding, peptide patterns of the PLP-modified enzyme in the presence and absence of dNTP were compared (Figure 2). It is clear from Figure 2 that only peptide 2 was protected from modification by PLP in the presence of dNTP, as judged by the total disappearance of the peak eluting at 78 min in both 215- and 280-nm scans. In addition, there was more than 90% reduction in the radioactivity eluting at 78 min. From the scans, it can be seen that there is no significant protection for either peptide 1 or peptide 3. Thus, it is evident that the pyridoxylated peptide eluting at 78 min (Figure 2A, panel II) is directly responsible for the loss in the ability of enzyme to binding substrate dNTP and hence the loss in polymerase activity.

Table III: Amino Acid Composition of Peptide 2[a]

amino acid residue	residues/mol of peptide: found	residues/mol of peptide: expected from sequence 756–775[b]
Cys		0
Asp	0.9	1
Thr	0.3	0
Ser	1.7	2
Glu	0.4	0
Pro	0.2	0
Gly	3.4	3
Ala	2.3[c]	4
Val	0.3	0
Met	0.9	1
Ile	2.2	2
Leu	1.7	2
Tyr	1.0	1
Phe	1.2[d]	2
His	ND[f]	0
Lys	0.2[e]	1
Arg	0.4[c]	1
Trp	0.4[c]	0

[a] See Figure 2A (panel II) and Figure 3. [b] Represents amino acid residues 756–775 in the primary sequence of *E. coli* DNA polymerase I (Joyce et al., 1982). This sequence would normally yield two tryptic peptides; however, pyridoxylation of Lys-758 makes it insensitive to trypsin. [c] Not corrected for incomplete hydrolysis. [d] Not corrected for losses during hydrolysis. [e] Absence of lysine in this peptide is attributed to the modification of that residue with PLP. Pyridoxylation of lysine residue has been shown to result in disappearance of the lysine peak during amino acid analysis (Benesch et al., 1982). [f] ND, not determined.

A careful comparison of the tryptic peptide maps of the digests from the native and pyridoxylated enzymes did not clearly show the disappearance of specific peptide peaks from the native enzyme digest (Figure 2). Thus, it was not possible to determine the location of peptides generated from the unmodified enzyme which correspond to the pyridoxylated peptides (Figure 2, panel II). Since the pyridoxylated peptides were easily identified and purified, we made no further attempts to identify the corresponding peptides from the digest of native enzyme.

Amino Acid Composition and Sequence of Pyridoxylated Peptide 2. Peptide 2 from the PLP-treated enzyme was isolated and further purified by chromatography on a Synchropak C-4 reverse-phase (5-μm particle size, 25 × 0.4 cm) column (Figure 3). The amino acid composition of purified PLP peptide 2, which contained the dNTP binding domain of *E. coli* DNA polymerase I, is shown in Table III. The amino acid sequence analysis of this peptide revealed the following sequence: Ser-Ala-Lys-Ala-Ile-Asn-Phe-Gly-Leu-Ile-Tyr-Gly-Met-Ser-Ala-Phe-Gly-Leu-Ala-Arg. This sequence corresponds to amino acid residues 756–775 in the primary amino acid sequence *E. coli* DNA Pol I (Joyce et al., 1982). The amino acid composition of this peptide (Table III) is in excellent agreement with the sequence analysis. The results of the sequence analysis up to cycle 13 are shown in Table IV. The lysine residue, which is the site of pyridoxylation, was conspicuously missing both from the composition analysis (Table III) and from the third cycle in the sequence analysis (Figure 4 and Table IV), which is consistent with the behavior of pyridoxylated lysine Benesch et al., 1982).

The precise sequence and location of the pyridoxylated peptides (not protected by dNTP) eluting at 42 and 97 min were not determined. However, preliminary sequence studies of peptide 3 (eluting at 97 min) revealed that it represents amino acid residues 638–651 (results not shown). This sequence lies in helix I of the Klenow fragment (Ollis et al., 1985).

Table IV: Amino Acid Sequence Analysis of Peptide 2[a]

cycle	residue[b]	identification	yield (pmol)
1	756	Ser	14.2
2	757	Ala	40.0
3	758	Lys	*c*
4	759	Ala	39.0
5	760	Ile	24.5
6	761	Asn	19.6
7	762	Phe	24.1
8	763	Gly	24.5
9	764	Leu	22.1
10	765	Ile	20.3
11	766	Tyr	19.9
12	767	Gly	15.5
13	768	Met	6.0

[a] See Figure 2A (panel II) and Figure 3. [b] The amino acid numbers correspond to the primary sequence of residues in *E. coli* DNA Pol I. [c] No Lys peak was observed at cycle 3 (see Figure 4), indicating modification of this residue (see text).

Discussion

We have earlier shown that PLP is a competitive inhibitor of DNA polymerases with respect to the substrate dNTPs (Modak, 1976a) and that it had no effect on the nucleolytic activity associated with some of these enzymes (Modak, 1976b). Since PLP-mediated inhibition is strictly dependent on the presence of both the phosphate and aldehyde groups of PLP (Modak, 1976a), and covalent bonding between PLP and reacting lysine residues can be achieved by borohydride reduction, specific labeling of the substrate binding site in various DNA polymerases could be obtained. Earlier, Hazra et al. (1984) had used this approach to label the dNTP binding site in the Klenow fragment of Pol I and had shown the presence of a lysine residue at this site. We have extended these studies to determine the location of the lysine residue involved in dNTP binding in Pol I. The characterization of PLP-inactivated Klenow fragment clearly showed that PLP affected the process of dNTP binding and not the template-primer binding. We then used PLP and [^{3}H]borohydride to label Klenow fragment in the presence and absence of substrate dNTP and found that only three tryptic peptides contained PLP. Of these, only one peptide is specifically protected by dNTP from pyridoxylation (Figure 2A). As shown under Results, the amino acid composition and sequence analysis of this peptide showed that it consists of residues 756–775 in the primary amino acid sequence of *E. coli* DNA Pol I. We have further identified lysine-758 to be the site of PLP reactivity and hence the amino acid involved in the binding of dNTP.

The molecular mechanism of substrate binding in DNA polymerases is complex since enzymes must recognize three structural components of dNTP, namely, the sugar residue (deoxy form), the purine or pyrimidine ring, and the triphosphate moiety which is in its metal chelate form. An additional constraint in the selection of complementary dNTP may be exercised by the template-primer bound to the enzyme molecule. Recent knowledge of the crystal structure of the Klenow fragment of Pol I (Ollis et al., 1985) has provided some clues towards the molecular orientation of different regions of the enzyme. The Lys-758, which we have identified in the present study as being the site of dNTP binding, is present in a domain spanning residues 756–775 of the "O" helix which forms a part of the active-site cleft (Ollis et al., 1985). Recently, Ferrin and Mildvan (1986), using NMR studies, have suggested that Ile-Tyr (residues 765–766) present in the O segment is involved in the recognition of the purine/pyrimidine ring of the dNTP. Preliminary studies presented by Joyce et al. (1985) indicate that Tyr-766 is the amino acid residue that

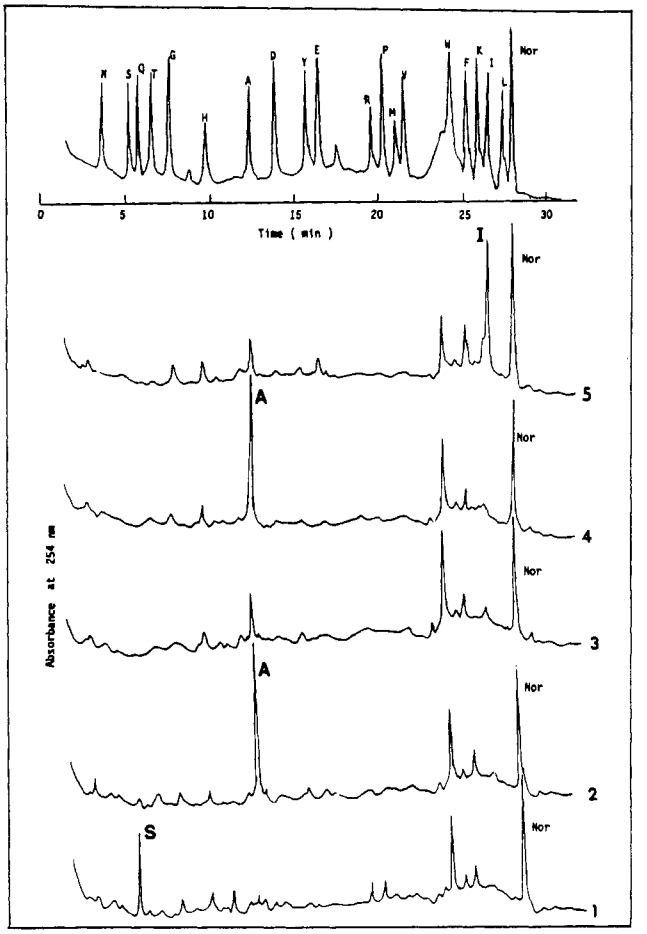

FIGURE 4: HPLC traces of phenylthiohydantoin derivatives from cycles 1–5 of the gas-phase sequencing of peptide 2 containing pyridoxalyllysine. An internal standard of PTH–norleucine (100 pmol) was added to each sequenator cycle. Samples were run on a Waters HPLC system as described under Materials and Methods. The tracing at the top represent 100 pmol of a standard mixture of PTH–amino acids. The sequence is indicated by the one-letter amino acid symbol adjacent to the corresponding peak. The peak eluting at 24.5 min in each cycle is apparently a reagent peak arising from incomplete drying.

cross-links to azido-dATP. Our studies on dTTP cross-linking to Pol I (Abraham & Modak, 1984; A. Basu and M. J. Modak, unpublished results) have also indicated that amino acid residues within this domain exhibit photo-cross-linking to substrate dNTP. It is quite interesting that binding of dNTP in Pol I utilizes Lys-758 and Tyr-766 which are separated by a span of seven amino acid residues in the primary structure. However, considering the α-helical structure of this region, both Lys-758 and Tyr-766 may actually be much closer, and thus, interaction of Tyr-766 with the functional groups of the purine/pyrimidine rings and Lys-758 with the metal phosphate group of the dNTP is quite feasible. It is thus apparent that the recognition or interaction of the sugar moiety may also lie in this vicinity.

Identification of the region and specific residue(s) in the recognition of the template-primer would shed further light on the molecular mechanism of the transfer of the bound nucleotide to the 3′-OH terminus of the primer. Another complexity that needs to be resolved is the influence of template in the selection and/or binding of the complementry dNTP. In the present study, we have identified the site which binds to all four dNTPs (Ferrin & Mildvan, 1985), and modification of this site definitely results in the inability of the enzyme to accept dNTPs for the polymerization reaction. Thus, it appears that the binding of a particular dNTP is quite

independent of template binding (Hazra et al., 1984; Ferrin & Mildvan, 1985, 1986). Yet in the polymerization reaction, noncomplementary substrates do not seem to interfere in the addition of complementary substrate. Therefore, a distinct mechanism for the preferential binding of substrates exhibiting complementarity to template nucleotide must exist.

Registry No. Pol I, 9012-90-2; PLP, 54-47-7; L-lysine, 56-87-1; thymidine 5′-triphosphate, 365-08-2.

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Persistent and Heritable Structural Damage Induced in Heterochromatic DNA from Rat Liver by *N*-Nitrosodimethylamine†

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ABSTRACT: Analysis, by benzoylated DEAE-cellulose chromatography, has been made of structural change in eu- and heterochromatic DNA from rat liver following administration of the carcinogen *N*-nitrosodimethylamine (10 mg/kg body weight). Either hepatic DNA was prelabeled with [^{3}H]thymidine administered 2–3 weeks before injection of the carcinogen or the labeled precursor was given during regenerative hyperplasia in rats treated earlier with *N*-nitrosodimethylamine. Following phenol extraction of either whole liver homogenate or nuclease-fractionated eu- and heterochromatin, carcinogen-modified DNA was examined by stepwise or caffeine gradient elution from benzoylated DEAE-cellulose. In whole DNA, nitrosamine-induced single-stranded character was maximal 4–24 h after treatment, declining rapidly thereafter; gradient elution of these DNA preparations also provided short-term evidence of structural change. Following incubation of purified nuclei with micrococcal nuclease, 10–12% of labeled DNA was solubilized (euchromatin) by 1.0 unit of micrococcal nuclease (5 mg of DNA)$^{-1}$ mL^{-1} after 9 min. In prelabeled animals, administration of *N*-nitrosodimethylamine caused a marked fall in the specific radioactivity of solubilized DNA, while that of sedimenting DNA was not affected. Caffeine gradient chromatography suggested short-term nitrosamine-induced structural change in euchromatic DNA, while increased binding of heterochromatic DNA was evident for up to 3 months after carcinogen treatment. Preparations of newly synthesized heterochromatic DNA from animals subjected to hepatectomy up to 2 months after carcinogen treatment provided evidence of heritable structural damage. Carcinogen-induced binding of heterochromatic DNA to benzoylated DEAE-cellulose was indicative of specific structural lesions whose affinity equalled that of single-stranded DNA up to 1.0 kilobase in length. The data suggest that structural lesions in heterochromatin, which may be a consequence of incomplete repair, are preferentially degraded by endogenous nuclease(s).

In mammalian cells, various stages in the excision repair of DNA have been identified (Collins & Johnson, 1984). Best characterized have been those reactions involved in the initial stages (Teebor & Frenkel, 1983). Most work on nitrosamine-induced DNA repair has concerned monitoring the concentration of alkylated bases in DNA (Preussmann & Stewart, 1984), a parameter of the first stage of DNA repair. The distribution of carcinogen adducts within subfractions of DNA generated by digestion of chromatin has provided evi-

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