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Pyridoxal 5′-Phosphate Mediated Inactivation of *Escherichia coli* DNA Polymerase I: Identification of Lysine-635 as an Essential Residue for the Processive Mode of DNA Synthesis†

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ABSTRACT: Inactivation of *Escherichia coli* DNA polymerase I by pyridoxal 5′-phosphate treatment results from its reactivity at multiple lysine residues. One of these residues, lysine-758, has been shown to be located at the substrate binding site in DNA polymerase I [Basu, A., & Modak, M. J. (1987) *Biochemistry 26*, 1704-1709]. We now demonstrate that lysine-635 is another important target of pyridoxylation; modification of this site results in decreased rates of DNA synthesis. Addition of template-primer with or without substrate deoxynucleoside triphosphate protects lysine-635 from pyridoxylation. Analysis of the initiation versus elongation phase of DNA synthesis by lysine-635-modified enzyme revealed that elongation of the DNA chain is severely affected by the lysine-635 modification. We therefore conclude that this lysine residue plays an important role in the processive mode of DNA synthesis by *E. coli* DNA polymerase I.

Enzymatic synthesis of DNA is a complex process since it requires an orderly interaction of multiple components. Resolution of the crystal structure of the Klenow fragment of *Escherichia coli* DNA polymerase I (Ollis et al., 1985) has provided the first glimpse of the three-dimensional architecture of this unique protein which carries domains responsible for template-primer and substrate deoxynucleoside triphosphate binding. With the help of site-specific reagents, we have shown that a simple process of substrate binding involves multiple amino acid residues which are situated quite apart in the primary sequence but are actually in close atomic proximity in the three-dimensional structure. For example, with pyridoxal 5′-phosphate (PLP)[1] as a substrate binding site directed labeling reagent (Modak, 1976; Hazra et al., 1984), we demonstrated involvement of lysine-758 (Basu & Modak, 1987) in the process of substrate binding. Yet, with the technique

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

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of UV-mediated cross-linking of substrate dNTP to *E. coli* DNA pol I (Abraham & Modak, 1984), we found histidine-881 to be the singular cross-linking target of dTTP (Pandey et al., 1987). Similarly, using NMR measurements on the pol I–dNTP complex, Ferrin and Mildvan (1985) observed resonance changes characteristic of Ile-Phe, residues which are clearly present in the vicinity of lysine-758. Our recent studies with an ATP analogue, FSBA, which has the overall configuration of ATP but contains reactive sulfonyl fluoride in place of α,β-phosphate groups, have also revealed involvement of arginine-682 in the process of substrate binding (Pandey & Modak, 1988). These observations suggest that dNTP substrate is probably held by multiple residues which presumably react with the purine/pyrimidine ring moiety, sugar moiety, and the metal–triphosphate group of dNTP. During our investigations on the PLP-mediated inactivation of pol I, we identified Lys-758 as one of the reactive sites in pol I which seems to be required for substrate dNTP binding (Basu & Modak, 1987). The location and functional role of other PLP-reactive sites remained to be elucidated. This paper describes the identification of Lys-635 as a second reactive site in pol I, and the functional analysis indicates that this residue plays an important role in binding of the enzyme to the template-primer such that DNA synthesis occurs in a processive mode.

Materials and Methods

E. coli DNA polymerase I large fragment (Klenow fragment) was purified from an overproducing strain (Joyce & Grindley, 1983), generously provided to us by Catherine Joyce of Yale University. Pyridoxal 5′-phosphate, trifluoroacetic acid, and sodium borohydride ($NaBH_4$) were obtained from Sigma. Trypsin (TPCK treated) was from Worthington. Acetonitrile and HPLC-grade water were obtained from Fisher Scientific Co. All other chemicals were of analytical grade.

Template-Primer and Nucleotides. Calf thymus DNA and all nonradioactive deoxynucleoside triphosphates were purchased from P-L Biochemicals. Poly(dT), poly(dA), and poly(rA)·$(dT)_8$ were also obtained from P-L Biochemicals. Tritiated TTP ([^{3}H]TTP) and NaB^3H_4 were from ICN, while [α-^{32}P]TTP (400 Ci/mmol) was from Amersham Inc.

Enzyme Assays: DNA Polymerase Assay. The standard incubation mixture was the same as described earlier (Srivastava & Modak, 1980). Reactions were carried out in a final volume of 100 μL and consisted of 20 mM Hepes, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, 10 μg of bovine serum albumin, 1 mM dithiothreitol, a 20 μM sample of each deoxynucleoside triphosphate, 1 ηCi of [^{3}H]TTP (1000 cpm/pmol), and 1 μg of activated calf thymus DNA. Samples were incubated at 37 °C for 15 min and the reactions terminated by the addition of cold 5% TCA containing 1 mM pyrophosphate. Acid-insoluble material was collected on Whatman glass fiber filters (GF/B). Radioactivity on the filters was measured after they were extensively washed and dried.

Modification with Pyridoxal Phosphate. DNA polymerase I was modified by PLP essentially as describeds by Basu and Modak (1987). The Klenow fragment (adjusted to 200 μg/mL) was incubated with PLP in the dark for 8 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 pyridoxal phosphate. The mixture was immediately chilled to 0 °C, and $NaBH_4$ or NaB^3H_4 was added to a final concentration of 10 mM. After standing for 15 min on ice, aliquots were withdrawn and diluted in 0.1% BSA to a final concentration of 1 μg/mL; 5 μL was used to assay for polymerase activity.

Protection experiments were carried out either with template-primer alone [poly(rA)·$(dT)_{12-18}$, poly(dA), or activated DNA], or with substrate alone (TTP or all four dNTPs), or with template-primer and substrate in the incubation mixture. When both the template-primer and substrate were included, the enzyme was first incubated for 8 min with template-primer and PLP followed by 2-min incubation with the added substrate.

Processivity. Processivity was analyzed by performing a primer extension analysis on poly(dA)·$(dT)_8$ as described earlier (Detera & Wilson, 1982; Hazra et al., 1984). The reaction conditions for analysis of product chain lengths were similar to those used for assaying DNA polymerase activity. The incubation mixture contained, in a volume of 10 μL, 50 mM Hepes–KOH, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, 10 μg of BSA, 1 μg of poly(dA)·$(dT)_8$, 10 μCi of [α-^{32}P]TTP, and 5 μM TTP. The components were mixed at 4 °C and incubated for 2 min at 37 °C. The reaction was initiated by the addition of 2 ng of enzyme and terminated at the end of 2.5 min with 60% formamide containing bromophenol blue and xylene cyanol. The samples were heated at 70 °C for 5 min and analyzed by electrophoresis in a 12% polyacrylamide–7 M urea gel and autoradiography using Kodak X-AR5 films.

Cross-Linking Studies. To examine whether modification of the Klenow fragment by PLP led to its inability to bind to DNA, the extent of UV-mediated cross-linking between enzyme and template-primer was determined. The enzyme was modified by PLP in the absence/presence of template-primer, substrate dNTP, or both. After reduction with $NaBH_4$, samples were passed through a 0.5-mL DEAE-cellulose column, equilibrated with buffer containing 50 mM Hepes, pH 7.8, and 20% glycerol. The enzyme samples were eluted with buffer containing 0.3 M NaCl. For cross-linking, 1 μg of enzyme was incubated over ice for 10 min with 0.5 μg of labeled template-primer [poly(dA)·[^{32}P]$(dT)_{12-18}$, 0.5 μCi] in the presence of 1 mM EDtA. The reaction mixture was then exposed to UV irradiation, (A mineral lamp Model R52 manufactured by Ultraviolet products Inc. with the output of 1300 μW/cm^2 at a distance of 15 cm was used for 15 min.) The UV-cross-linked enzyme species were analyzed by SDS–polyacrylamide gel electrophoresis. The extent of cross-linking was quantitated by excising the radioactive bands and measuring the Cerenkov counts associated with them.

Protein Chemistry Studies. The TCA-precipitated proteins were subjected to proteolytic cleavage with trypsin. Precipitates were suspended in 200 μL of 50 mM ammonium bicarbonate, pH 8.0, and digested at 37 °C with a 50:1 (w/w) ratio of the Klenow fragment to trypsin. A second aliquot of trypsin was added after an interval of 2 h and the incubation continued overnight. The digests were either frozen or injected directly onto a Vydac C-18 reverse-phase column that had been equilibrated with 0.1% trifluoroacetic acid (solvent A). Peptides were eluted at a flow rate of 0.7 mL/min, using linear gradients of solvent B (70% acetonitrile containing 0.1% TFA): 0–37.5% solvent B (0–90 min), 37.5–75% solvent B (90–135 min), and 75–100% solvent B (135–150 min). The HPLC analyses were performed by using a Varian Vista 5500 liquid chromatography system equipped with a Polychrom 9060 diode array detector.

Peptides that were pyridoxylated could be identified by three different criteria: (1) detection of tritium label associated with individual peptides; (2) absorption maxima at 325 nm characteristic of pyridoxylation; and (3) analysis of the fractions for emission at 410 nm after excitation at 325 nm. The

Table I: Effects of Template-Primer and Complementary or Noncomplementary dNTPs on Inactivation of Pol I by Pyridoxal 5′-Phosphate

components	pmol of TMP incorporated/ 15 min	% act.
control (no PLP)	53.0	100
PLP	2.0	4
PLP + poly(dA)	4.0	7
PLP + 5 μg of activated DNA	10.5	20
PLP + 5 μg of DNA + 2 mM dNTPs	40.0	75
PLP + 10 μg of poly(rA)·$(dT)_{12-18}$	6.5	12
PLP + 10 μg of poly(rA)·$(dT)_{12-18}$ + 2 mM dTTP	45.0	85
PLP + 10 μg of poly(rA)·$(dT)_{12-18}$ + 2 mM dCTP	21.0	40
PLP + 2 mM dTTP	16.0	30

Table II: Effect of Template-Primer and dNTPs on the Extent of PLP Incorporation in *E. coli* Pol I

components	DNA polymerase act. (%)	mol of PLP/mol of enzyme
control (no PLP)	100	
PLP	5	4.2
PLP + 70 μg of poly(rA)·$(dT)_{12-18}$	12	3.1
PLP + 5 mM TTP	37	3.2
PLP + 70 μg of poly(rA)·$(dT)_{12-18}$ + 5 mM TTP	70	2.5

peptides of interest were dried in vacuo, redissolved in solvent A, and repurified on a C-18 column using a shallow gradient of solvent B: 0–10% solvent B (0–10 min), 10–30% solvent B (10–110 min).

Amino Acid Composition and Sequence Analyses. Aliquots of the repurified peptides were hydrolyzed with 6 N HCl containing 0.2% phenol for 16 h at 115 °C. The resulting amino acids were converted to their phenylthiocarbamyl derivatives and analyzed by HPLC according to the method described by Stone and Williams (1986). The amino acid sequence analyses were carried out at the protein chemistry facility of Yale University by Dr. Kenneth Williams as described earlier (Merril et al., 1984).

Results

Inactivation of DNA Polymerase I by Pyridoxal Phosphate and the Effect of Template-Primer and Substrates. Our earlier studies with pyridoxal phosphate (Basu & Modak, 1987) and the results shown in Table I indicate that incubation of the Klenow fragment with PLP, followed by reduction with borohydride, results in an irreversible inactivation of the DNA polymerase activity. The presence of natural (activated DNA) or synthetic DNA [poly(dA)·$(dT)_{12-18}$ or poly(rA)·$(dT)_{12-18}$] in the PLP inactivation mixture provided only limited protection (about 10–15%) from PLP inactivation. Similarly, only partial recovery of DNA polymerase activity (~30%) was observed when substrate dNTPs alone were included in the inactivation mixture [Table I and Basu and Modak (1987)]. However, when the enzyme was modified by PLP in the presence of template-primer followed by addition of substrate dNTP, more than 80% of the activity was recovered (Table I). These results indicate that essential lysine residues both in the substrate and in the template-primer binding sites are modified by PLP. The protection afforded by complementary substrate dNTPs in the presence of template-primer was found to be significantly more than that obtained upon addition of noncomplementary substrate dNTPs (Table I).

Stoichiometry of PLP Binding to Enzyme under Different Conditions. Table II shows the incorporation of tritiated borohydride into PLP-treated enzyme under different conditions of modification. The results show that in the absence of template-primer or substrate dNTPs, about 4 mol of PLP is incorporated per mole of the enzyme. Template-primer, as well as substrate dNTPs individually, reduces the extent of PLP incorporation by approximately 1 mol, while the presence of both template-primer and substrate dNTPs reduces the extent of PLP incorporation by about 2 mol. These results indicate that pyridoxylation of two distinct lysine residues is involved in the process of enzyme inactivation.

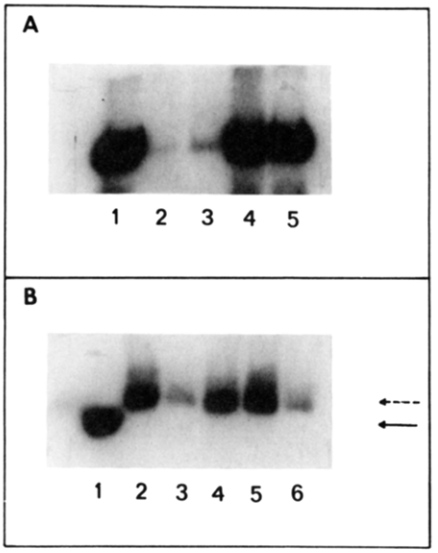

FIGURE 1: UV-mediated cross-linking of pol I (Klenow fragment) to substrate dNTP and template-primer. DNA pol I (Klenow fragment) was modified by 1 mM PLP under various conditions. The modified enzyme was recovered by binding to DEAE-cellulose matrix, and 1 μg of protein was used in the cross-linking assay. The substrate dNTP and the template-primer binding abilities were assayed by the ability of the modified enzyme to cross-link to labeled substrate and template-primer as described under Materials and Methods. (A) Cross-linking to [α-^{32}P]dTTP: lane 1, control enzyme; lane 2, enzyme modified with PLP; lane 3, enzyme modified with PLP in the presence of activated DNA; lane 4, enzyme modified with PLP in the presence of both activated DNA and dNTPs; lane 5, enzyme modified with PLP in the presence of only dNTPs. (B) Cross-linking to poly(dA)·[^{32}P](dT)$_{12-18}$: Description of the enzyme used in lanes 2–6 is the same as in lanes 1–5 in panel A. Lane 1 in this frame shows the position of the control enzyme cross-linked to [α-^{32}P]dTTP. The solid arrow indicates the position of the enzyme cross-linked to TTP, while the broken arrow indicates the position of the enzyme cross-linked to oligo(dT).

UV-Mediated Cross-Linking of PLP-Modified Enzyme to Template-Primer. Our earlier experiments on the treatment of pol I with PLP (Basu & Modak, 1987) showed that, in spite of complete loss of polymerase activity, the binding of DNA to PLP-inactivated enzyme was not affected as judged by the nitrocellulose filter binding assay. However, increased protection from PLP inactivation in the presence of DNA and substrate dNTPs as well as the reduction in PLP incorporation suggests that the association of DNA to the enzyme molecule may also be affected by PLP modification. To determine the extent of template-primer binding to the enzyme, we resorted to a more sensitive assay procedure, i.e., UV-mediated cross-linking of ^{32}P-labeled template-primer to the enzyme. The results of the UV cross-linking of template-primer to the enzyme modified by PLP under various conditions are shown in Figure 1. As can be seen from this figure (panel B), the extent of cross-linking of template-primer to the enzyme decreases upon PLP modification (lane 3), and this decrease closely correlates to the decrease in enzyme activity. Pyridoxylation in the presence of template-primer prevents this

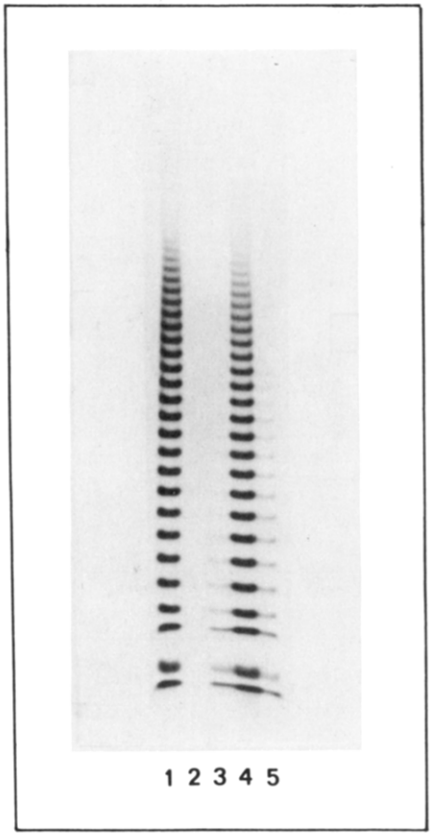

FIGURE 2: Effect of PLP modifications of pol I on the size of DNA product. pol I modified with 1 mM PLP under various conditions was used as a source of enzyme to catalyze the synthesis of poly(dT) using poly(dA)·$(dT)_8$ as a template-primer and [α-^{32}P]dTTP as substrate. An aliquot of the reaction mixture was then applied to a 12% polyacrylamide–7 M urea gel and electrophoresed. Lane 1, control enzyme; lane 2, PLP-modified enzyme; lane 3, enzyme modified with PLP in the presence of activated DNA; lane 4, enzyme modified with PLP in the presence of both activated DNA and four dNTPs; lane 5, enzyme modified with PLP in the presence of only dNTPs.

decrease in cross-linking to a great extent (lanes 4 and 5). The presence of substrate dNTP alone (lane 6) is unable to protect the ability of the enzyme to cross-link to the template-primer. Since UV-mediated cross-linking of the enzyme to template-primer is dependent on the stable binding of DNA to enzyme, we conclude that pyridoxylation of pol I decreases the stable binding of the enzyme to the template-primer.

Effect of PLP-Treated Enzyme on Rates of DNA Synthesis. The rates of DNA synthesis by PLP-modified enzyme, protected either by substrate dNTP alone or by substrate dNTP and template-primer, were linear with time in spite of a nearly 3-fold lower rate of synthesis with the former enzyme (data not shown). Furthermore, the substrate binding ability of both enzyme species was quite similar (Figure 1, lanes 4 and 5) as judged by their ability to cross-link to dTTP. We, therefore, conclude that enzyme treated with PLP in the presence of dNTPs alone is indeed deficient in binding to the template-primer, which may result in abortive synthesis.

Studies on the process of chain elongation by PLP-modified enzyme (Hazra et al., 1984) indicated that the enzyme, pyridoxylated in the presence of dNTP, was capable of initiating the DNA synthesis process but the process of chain elongation was limited to the addition of only a few nucleotides. Thus, it was of interest to know if the lysine residue, which we have identified to be essential for the interaction with DNA, is also involved in the process of chain elongation or the processivity of DNA pol I. On the basis of the chain length distribution of the products formed with a poly(dA)·$(dT)_8$ template-primer

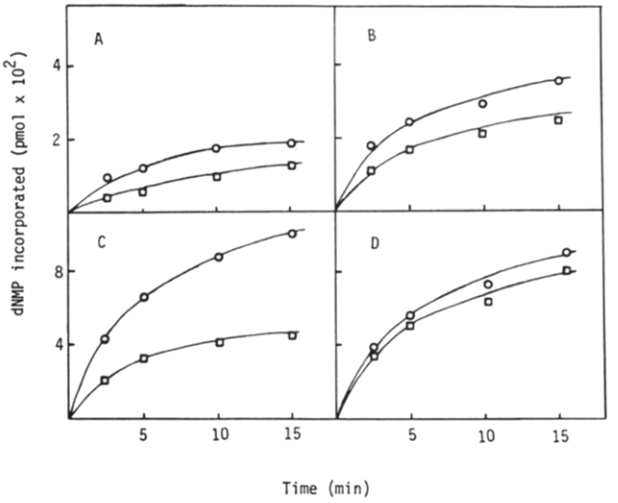

FIGURE 3: Rates of addition of the first and fourth nucleotides by PLP-modified DNA pol I (Klenow fragment). Klenow fragment was modified by PLP in the presence and absence of substrate dNTPs and/or template-primer as described under Materials and Methods. One nanogram of the modified enzyme was then used to determine rates of polymerization of the first and the fourth nucleotides in a reaction mixture (100 μL) containing 50 mM Hepes–KOH, pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 μg of *Hin*dIII fragments of phage DNA as template-primer, and 20 μM each of all four dNTPs with 10 μCi of either [α-^{32}P]dTTP or [α-^{32}P]dATP. The two radioactive substrates repesent the first (○) and the fourth (□) substrate in the polymerization reaction. Reactions were carried out at 25 °C, and at regular time intervals, 20-μL aliquots were withdrawn, and the incorporation of nucleotides was measured by acid precipitation. (A) Enzyme modified with PLP. (B) Enzyme modified with PLP in the presence of activated DNA. (C) Enzyme modified with PLP in the presence of dNTPs. (D) Enzyme modified with PLP in the presence of activated DNA along with all four dNTPs.

(Figure 2), it is clear that the process of chain elongation was restricted to addition of 10–12 dTMPs when the enzyme was modified by PLP in the presence of substrate dNTP. However, the addition of dTMP by enzyme modified by PLP in the presence of both template-primer and dNTP was similar to that by the unmodified enzyme. Thus, it appears that stable binding of both template-primer and substrate dNTPs is required for the efficient elongation of the nascent DNA chains. In the absence of stable binding of template-primer, the enzyme may be expected to fall off the template and must rebind in order to initiate DNA synthesis (addition of dNMP residue to primer terminus). In order to obtain further support for this notion, we designed an experiment where staggered ends of *Hin*dIII-digested λ-phage DNA were used as the source of template-primer. Since *Hin*dIII cleaves double-stranded DNA leaving a 5′ overhang of the four-nucleotide sequence TCGA, with a free 3′-OH on the complementary strand, such a template-primer is efficiently filled by the Klenow fragment of *E. coli* DNA polymerase I. Incorporation of dATP with this template-primer provides the rate of initiation, whereas the rate of incorporation of dTTP, which is the fourth nucleotide from the 3′-OH end, gives a general idea of the processive nature of synthesis. When the rates of the first or the fourth nucleotide addition by PLP-treated enzyme, with modification restricted at Lys-635 (see below), were compared to those of the control enzyme (Figure 3), it became quite apparent that the rate of incorporation of the fourth nucleotide, representing the elongation reaction, was severely curtailed. In contrast, no effect on the initiation reaction, as seen by incorporation of the first nucleotide, was observed as a consequence of pyridoxylation of Lys-635.

Comparative Tryptic Maps of Native and PLP-Treated Enzyme in the Presence or Absence of Template-Primer.

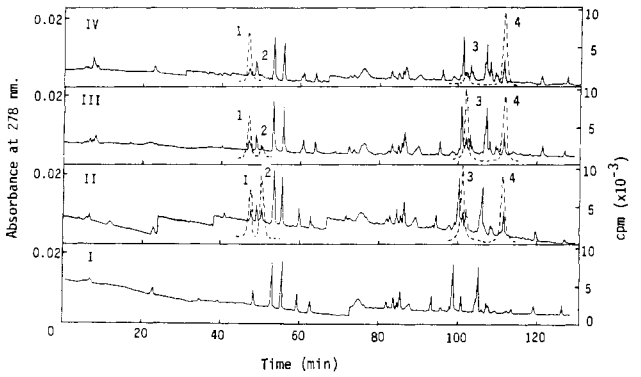

FIGURE 4: Separation of tryptic peptides of *E. coli* pol I (Klenow fragment) by reverse-phase HPLC. The tryptic peptides derived from 1 nmol of Klenow fragment modified by PLP under various conditions were resolved on a Vydac C-18 column equilibrated with 0.1% trifluoroacetic acid. The peptides were eluted at a flow rate of 0.7 mL/min with increasing concentrations of buffer B (70% acetonitrile containing 0.1% trifluoroacetic acid): 0–90 min (0–37.5% B), 90–135 min (37.5–75% B), and 135–150 min (75–100% B). Peptides were monitored by absorption both at 215 nm and at 278 nm simultaneously. The scans monitored at 278 nm (—) are shown here for better clarity. A small aliquot from each fraction was counted for monitoring the distribution of radioactivity (- - -). The plots shown here represent tryptic peptides obtained from control enzyme (I), enzyme treated with PLP (II), enzyme treated with PLP in the presence of template-primer [poly(rA)·(dT)$_{12-18}$ or activated DNA] (III), and enzyme treated with PLP in the presence of substrate dNTPs and template-primer [poly(rA)·(dT)$_{12-18}$ or activated DNA] (IV).

Comparative tryptic mapping by HPLC of native and modified enzyme has proven to be a useful approach to identify the modified peptides. Using this technique, we were successful in identifying the substrate binding domain (Basu & Modak, 1987) and template-primer binding domains (Basu et al., 1987; Mohan et al., 1988) in *E. coli* DNA polymerase I. Thus, in order to identify the domain in the enzyme molecule containing the PLP-reactive lysine residue which interacts with the template-primer, we resorted to comparative tryptic peptide mapping. Figure 4 shows the reverse-phase tryptic peptide maps, on a Vydac C-18 HPLC column, of native enzyme, PLP-modified enzyme and PLP-modified enzyme in the presence of template-primer and/or substrate dNTP. The pyridoxylated peptides were monitored by their absorbance at 215 and 278 nm, by the distribution of radioactivity, and by their characteristic absorption spectra (Basu & Modak, 1987). The tryptic map of pyridoxylated enzyme showed the presence of four distinct peptides associated with pyidoxal phosphate (Figure 4, panel II) eluting at 47, 51, 102, and 112 min. Of these, peak 2, eluting at 51 min, was found to be specifically protected from PLP modification when template-primer was included in the modification mixture (Figure 4 panel III). The level of protection was greater than 90% as judged by the UV absorption as well as by the decrease in associated radioactivity of this peptide. Peptide 3, eluting at about 102 min, was protected only when dNTP was included in the reaction mixture (Figure 4, panel IV). The protection of this peptide by dNTPs was independent of the presence of template-primer and has been characterized by us to represent residues 756–775, with lysine-758 as the site of PLP modification (Basu & Modak, 1987). In our previous report (Basu & Modak, 1987), the dNTP-protected PLP peptide was labeled as peptide 2 since in our earlier chromatograms we were able to locate only three PLP-modified peptides. However, with the use of a new Vydac C-18 column (5-μm particle size and 300-Å pore size), which has better resolution, and using a slightly different gradient system, we were able to resolve four PLP-modified peptides as described above. Thus, the peptide labeled 3 in this paper represents peptide 2 in the earlier report (Basu & Modak, 1987).

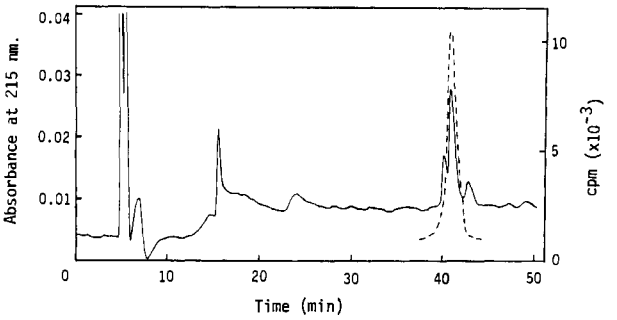

FIGURE 5: Purification of peptide 2 on a C-18 reverse-phase column. The peak fraction eluting at 51 min (Figure 4, panel II) from a preparative run (5 nmol of Klenow fragment) was lyophilized, redissolved in 0.1% trifluoroacetic acid, and loaded on a Vydac C-18 column equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted at flow rate of 0.7 mL/min with increasing concentrations of buffer B (0.1% trifluoroacetic acid in 70% acetonitrile solution): 0–10 min (0–10% B) and 10–100 min (10–30% B). PLP peptide was monitored by absorption at 215 nm (—) and by radioactivity (- - -).

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

amino acid residue	residues/mol of peptide: found	residues/mol of peptide: expected from sequence 632–637[b]
Cys		
Asp		
Thr		
Ser	0.2	
Glu	0.3	
Pro	0.4	
Gly	1.2	1
Ala	0.8	1
Val		
Met		
Ile	1.1	
Leu	1.7	2
Tyr		
Phe		
His		
Lys	1.1[c]	2
Arg		
Trp		

[a] See Figures 4 and 5. [b] Represents amino acid residues 632–637 in the primary sequence of *E. coli* DNA polymerase I (Joyce et al., 1982). [c] Absence of one Lys residue in this peptide is attributed to the modification by PLP (Benesch et al., 1982; Basu & Modak, 1987).

Table IV: Sequence Analysis of Peptide 2[a]

cycle	amino acid residue identified[b]	yield (pmol)
1	Gly (632)	122
2	Leu (633)	109
3	Ala (634)	130
4	Lys (635)	*c*
5	Leu (636)	77
6	Lys (637)	48

[a] See Figures 4 and 5. [b] The amino acid numbers corresponding to the primary sequence of residues in *E. coli* DNA Pol I are shown in parentheses. [c] No Lys peak was observed at cycle 4, indicating modification of this residue by PLP (Basu & Modak, 1987).

The pyridoxylated peptide 2, eluting at 51 min, was further purified after rechromatography on a C-18 column using a shallow gradient (Figure 5) and was used for amino acid composition and sequence analysis. The amino acid composition of this peptide, as shown in Table III, revealed it to span residues 632–637 in the primary amino acid sequence of *E. coli* DNA pol I (Joyce et al., 1982). Further proof for the identify of peptide 2 was obtained after performing sequence

analysis. Table IV indicates the residues appearing at each of six cycles, and the sequence once again corresponds to residues 632–637. As seen from the amino acid composition and sequence analysis, the lysine residue at position 635 is absent in these analyses, indicating that this residue is the site of pyridoxylation.

Discussion

Enzyme kinetic studies of PLP-mediated inactivation of pol I and other DNA polymerases have clearly shown that PLP is a substrate binding site directed reagent (Modak, 1976; Basu & Modak, 1987). However, the fact that substrate dNTPs in the absence of template-primer provided only partal protection against PLP inactivation suggested the existence of other targets for PLP which play a role in the polymerase reaction. Indeed, the stoichiometry of PLP binding to the Klenow fragment of pol I had revealed the presence of four PLP reactive sites, and only one of these sites was protected by dNTPs. As detailed under Results, we found that binding of template-primer was also affected by PLP, which suggested that other PLP-sensitive lysines are involved. The observation that template-primer addition to the enzyme protein reduced the incorporation of PLP by one residue strongly suggested that a selective modification of the involved site could be achieved. Using comparative tryptic peptide mapping of pol I modified with PLP in the presence of appropriate components, we show that the involved residue is lysine-635. The protection of lysine-635 by template-primer and lysine-758 by substrate dNTP together provide nearly complete protection of polymerase activity against PLP inhibition, implying important catalytic roles for these two residues. Lysine-635, however, does not appear to be the primary binding site for the template-primer, since the enzyme modified at this lysine still expresses polymerase activity, although the rate of polymerization is significantly lower. This is in contrast to modification of residues involved in the primary binding of template-primer, that results in complete enzyme inactivation. For example, modification of Arg-841 by phenylglyoxal (Mohan et al., 1988) and of methionine by ferrate (Basu et al., 1987) completely inactivates polymerase and template-primer binding activity of pol I. Some insight into the role of the PLP-sensitive lysine, other than the one located at the substrate binding site, was provided by Wilson and his colleagues (Hazra et al., 1984), who found that enzyme modified with PLP in the presence of dNTPs alone was able to add only a limited number of residues to the nascent chains. We, therefore, investigated the properties of the Lys-635-modified enzyme and confirmed Wilson's observations.

Further analysis using kinetics of the first and fourth nucleotide incorporation clearly showed that the elongation process was affected. In the DNA polymerase reaction, distinction between initiation and elongation reactions is somewhat fuzzy since DNA polymerases require a preformed primer. Nevertheless, judging from the products of the polymerase reaction, it has been possible to deduce whether the enzyme is in a processive or a distributive mode of synthesis. In the latter mode, the enzyme presumably dissociates from the template-primer after addition of a single substrate in contrast to the continuous addition of a few nucleotides in the processive mode. Thus, lower rates of synthesis may be expected for an enzyme that switches from the processive to the distributive mode. The distributive mode may also be considered to represent a continuous initiation reaction since binding of the enzyme to the template-primer, prior to the addition of dNTP, is required, whereas a processive mode may be more analogous to an elongation-type reaction since a bound enzyme will add a few nucleotides before dissociation. Earlier, using this definition, we had demonstrated that a tripartite complex, consisting of P_i–Mn–dNTP (Modak & Marcus, 1977; Modak et al., 1982), inhibits the polymerization of dNTPs by Rauscher leukemia virus DNA polymerase via a more pronounced effect on the elongation reaction. The lysine-635-modified pol I, by product analysis studies (Figure 2) and by kinetics of incorporation of the first and fourth nucleotides (Figure 3), shows that incorporation of the fourth nucleotide is significantly affected by modification of lysine-635. We, therefore, conclude that modification of lysine-635 may result in the inability of the enzyme (a) to bind to the template-primer in a stable manner, leading to dissociation after the addition of a single dNTP (analogous to the distributive mode), and (b) to catalyze DNA synthesis in a processive manner. At the present time, we have not attempted identification and/or functional characterization of two other PLP-reactive lysine residues. Judging from the roles of Lys-635 and Lys-758, it seems unlikely that the other residues play a major role in the catalysis of the polymerase reaction.

In the crystal structure of pol I, Lys-635 is situated in helix I, which forms the roof of the active-site cleft, and is diagonally across lysine-758, which is the primary binding site of substrate dNTPs. Moreover, it is very close to Arg-682, which we find to be an important residue possibly involved in the binding of phosphates of the triphosphate substrate (Pandey & Modak, 1988). The close molecular proximity of Lys-635 and Arg-682 seems to be well-suited for efficient polymerase reaction where Lys-635 may be postulated to bind to the growing chain while Arg-682 may facilitate the cleavage of the PP_i moiety of the incoming dNTP.

Registry No. Pol, 9012-90-2; PLP, 54-47-7; L-Lys, 56-87-1; poly(rA)·$(dT)_{12-18}$, 27156-07-6; poly(dA), 25191-20-2; dTTP, 365-08-2; dCTP, 65-47-4.

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Kinetic Mechanism Whereby DNA Polymerase I (Klenow) Replicates DNA with High Fidelity†

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ABSTRACT: A complete kinetic scheme describing the polymerization of correct and incorrect dNTPs by the Klenow fragment (KF) of DNA polymerase I has been developed by using short DNA oligomers of defined sequence. The high fidelity arises from a three-stage mechanism. The first stage of discrimination [$(1.1 \times 10^4 \rightarrow 1.2 \times 10^6)$-fold] comes primarily from a dramatically reduced rate of phosphodiester bond formation for incorrect nucleotides, but it also gains a smaller contribution from selective dNTP binding. After phosphodiester bond formation, a conformational change slows dissociation of the incorrect DNA products from KF and, in conjunction with editing by the 3′→5′-exonuclease, increases fidelity 4–>61-fold. Finally, KF polymerizes the next correct dNTP onto a mismatch very slowly, providing a further 6–>340-fold increase in fidelity. Surprisingly, the 3′→5′-exonuclease did not in its hydrolysis reaction differentiate between correctly and incorrectly base-paired nucleotides; rather, an increased lifetime of the enzyme–DNA complex containing the misincorporated base is responsible for discrimination.

DNA polymerase I (pol I)[1] from *Escherichia coli* is a multifunctional enzyme that catalyzes DNA-directed DNA synthesis and is involved in both replication and repair of DNA in vivo (Kornberg, 1980). In addition to catalyzing DNA polymerization, the enzyme also contains a 3′→5′-exonuclease and a 5′→3′-exonuclease. Recently, the complete kinetic scheme of the polymerization reaction was determined (Kuchta et al., 1987).

A critical feature of pol I is the extremely high fidelity with which it synthesizes DNA. The error frequency may be as low as 10^{-8}–10^{-12} errors per nucleotide polymerized (Englisch et al., 1985). This is much less than the error frequency of 0.2–0.0006 errors per nucleotide polymerized predicted on the basis of energy differences between correct and incorrect base pairs (Loeb & Kunkel, 1982).

The kinetic mechanism by which pol I achieves high fidelity remains obscure, although many models have been proposed (Bernardi et al., 1979; Brutlag & Kornberg, 1972; Clayton et al., 1979; Hopfield, 1974, 1980; Ninio, 1975). In each of these models pol I selects for the correct dNTP during two distinct and separate processes, thereby obtaining increased fidelity. These processes have typically been ascribed to the selection of the correct dNTP (El-Deiry et al., 1984; Fersht et al., 1982) and the removal of misincorporated nucleotides by the 3′→5′-exonuclease (Brutlag & Kornberg, 1972; Kunkel et al., 1981a). More recently, a rate-determining conformational change prior to phosphodiester bond formation has been implicated (Ferrin & Mildvan, 1986; Mizrahi et al., 1985).

In the present study, the kinetic parameters governing misincorporation, polymerization onto a mismatched 3′ terminus, and removal of mismatches by the 3′→5′-exonuclease were measured by using short DNA oligomers of defined sequence. We then constructed a scheme that reveals how fidelity results from amplification of the discrimination occurring in three distinct kinetic steps.

Chart I: DNA Duplexes

9/20-mer[a]	TCGCAGCCG(3′) AGCGTCGGCAGGTTCCCAAA	9A/20-mer[b]	TCGCAGCCGA(3′) AGCGTCGGCAGGTTCCCAAA
13/20-mer	TCGCAGCCGTCCA(3′) AGCGTCGGCAGGTTCCCAAA	9C/20-mer	TCGCAGCCGC(3′) AGCGTCGGCAGGTTCCCAAA
16/19-mer	TGCGTCCGGCGTAGAG(3′) CGCAGGCCGCATCTCCTAG	13T/20-mer	TCGCAGCCGTCCAT(3′) AGCGTCGGCAGGTTCCCAAA
9/36-mer	GCCTCGCAG(3′) CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT		
12/36-mer	GCCTCGCAGCCG(3′) CGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTTT		

[a] The labels denote the length of the primer/template. Those duplexes consisting of only two numbers (e.g., 9/20-mer) are correctly base-paired. The template base(s) to be copied along with the 3′-terminal base(s) of the primer/template is (are) included in the text after each DNA [e.g., 9/20-mer ($^{CG}_{GCAGG}$)]. [b] The labels containing a letter have a mismatched primer 3′-terminus. For example, the 9A/20-mer is the 9/20-mer with an A appended to the primer 3′-terminus and which forms an A–A base pair.

Experimental Procedures

Materials. New England Nuclear supplied (S_P)-[^{35}S]-dATPαS, [^{32}P]PP_i and [^{32}P]dNTPs. Pharmacia provided

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate, sodium salt; KF, Klenow fragment of DNA polymerase I; pol I, DNA polymerase I; PP_i, sodium pyrophosphate; Tris·HCl, tris(hydroxymethyl)aminomethane, hydrochloride salt.

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