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Site-Specific Modification of *Escherichia coli* DNA Polymerase I Large Fragment with Pyridoxal 5′-Phosphate†

Anup K. Hazra, Sevilla Detera-Wadleigh, and Samuel H. Wilson*

ABSTRACT: Pyridoxal 5′-phosphate (PLP) is an inhibitor of DNA polymerase activity of *Escherichia coli* DNA polymerase I large fragment. Kinetic studies indicated that overall PLP inhibition was noncompetitive with respect to dNTP, and Hill plot analysis revealed that two molecules of PLP were involved in the inhibition. Reduction of the PLP-treated enzyme with sodium [^{3}H]borohydride resulted in covalent incorporation of 3 mol of PLP/mol of enzyme. This incorporation was at lysine residues exclusively, and the PLP-modified enzyme was not capable of DNA polymerase activity. The presence of dNTP during the modification reaction blocked the incorporation of 1 mol of PLP/mol of enzyme. Similar results were obtained in the presence or absence of template-primer. These data indicate that a PLP target lysine is in or around a dNTP binding site that is essential for polymerase activity and that this binding site is functional in the absence of template-primer. The enzyme modified in the presence of dNTP, containing 2 mol of PLP/mol of enzyme, was capable of DNA polymerase activity but was unable to conduct elongation of product molecules beyond a short oligonucleotide length.

Pyridoxal 5′-phosphate (PLP) modification of proteins is an effective approach toward active-site identification and dissection of distinct phases of the enzymatic mechanism and the ligand–receptor interaction. For example, Ohsawa & Gualerzi (1981) used this approach to identify the 30S ribosomal binding site of *Escherichia coli* intiatin factor IF3 and to distinguish this site from an AUG trinucleotide binding site. Benesch et al. (1982) found that PLP specifically modified the polyphosphate binding site of deoxyhemoglobin. Papas et al. (1977) used PLP modification to identify a dNTP binding site in the α-subunit of avian myeloblastosis virus DNA polymerase and to demonstrate that the β-subunit of the enzyme is devoid of a dNTP binding site.

We are using the PLP modification approach to examine the mechanism of *E. coli* DNA polymerase I large fragment (Pol I lf). It had been shown earlier (Modak, 1976) that PLP is an inhibitor of the polymerase activity of Pol I. In this paper, we report pyridoxylation of a lysine residue in or around a dNTP binding site that appears to be essential for polymerase activity. a template-primer was not required for dNTP to protect the enzyme against pyridoxylation at this binding site. In addition, we report that pyridoxylation in the presence of dNTP results in an enzyme containing 2 mol of PLP/mol of enzyme. This modified enzyme has altered polymerase properties such that each cycle of template-primer binding plus synthesis results in addition of only a few dNMP residues to the primer. These results are discussed in the context of current kinetic models for the polymerase activity of Pol I (McClure & Jovin, 1975; McClure & Chow, 1980; Detera & Wilson, 1982).

Materials and Methods

Materials. E. coli DNA polymerase I large fragment was from New England Nuclear Corp. (catalog no. NEE-102, lot no. 1607-023). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoretic analysis and Coomassie blue staining revealed that the only visible protein in the preparation was the M_r 68 200 enzyme polypeptide. The enzyme was stored at −20 °C in 100 mM KP_i buffer, pH 7.0, 1 mM dithiothreitol, and 50% glycerol. Tritium-labeled thymidine 5′-triphosphate and sodium borohydride also were from NEM. The latter was stored in crystalline form at 4 °C and was dissolved in 5 mM NaOH immediately before use. Polynucleotides and unlabeled deoxyribonucleoside 5′-triphosphates were from P-L Biochemicals. PLP and analogues were from Sigma, V8 protease was from Miles, and equipment and chemicals for gel elec-

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trophoresis were from Bio-Rad Laboratories. All other chemicals used were of analytical grade. [5′-^{32}P](pdA)$_9$ and (pdA)$_8$-[^{32}P]dA were prepared as described by Becerra et al. (1983). Silica gel thin-layer chromatography plates (0.25 mm) were from E. Merck.

DNA Polymerase Assay. Pol I lf was assayed in a final volume of 20 μL containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (Hepes) or tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8, 10 mM NaCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 400 μg/mL bovine serum albumin, 20% glycerol, 10 or 40 μM [^{3}H]dTTP (specific activity 3000 dpm/pmol), PLP as indicated, the template-primer poly(dA)·(dT)$_{10}$ at 10 and 5 μg/mL, respectively, and 0.7 nM enzyme. Reaction mixtures were incubated at 37 °C for 8 min (in the dark when PLP was used), and reactions were terminated by transferring the tubes to dry ice and then to a 0–1 °C ice–water bath. To each tube was added 0.5 mL of solution T [100% Cl_3CCOOH, 10 mM thymidine, saturated sodium pyrophosphate, and saturated sodium orthophosphate (1:1:4:4)] at 4 °C. The contents of each tube were mixed and kept at 0–1 °C for 15 min. DNA was then collected on a nitrocellulose filter and washed on the filter with approximately 40 mL of 10% Cl_3CCOOH at 4 °C followed by 20 mL of chloroform–methanol (1:1) at 4 °C. Filters then were transferred to scintillation vials and dissolved in 1 mL of methyl cellosolve at 24 °C; 10 mL of Triton X-100–toluene–Permafluor (32:64:4) scintillation mixture was added, and after thorough mixing, radioactivity was determined.

Exonuclease Assay. (dA)$_9$ molecules labeled at either the 3′ or the 5′ end were used as substrate (Becerra et al., 1983). A portion of the stocked enzyme solution was assayed in a final volume of 20 μL containing 50 mM Hepes, pH 8.3, 10 mM NaCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 400 μg/mL bovine serum albumin, 20% glycerol, 2.5 μg/mL [^{32}P](pdA)$_9$ as substrate, 2.5 μg/mL poly(dT), and 0.7 nM enzyme. Reaction mixtures were incubated for 30 min at 37 °C and then frozen. A portion of each reaction mixture was subjected to a denaturing treatment by heating at 90 °C for 3 min in the presence of 60% formamide. The sample was then subjected to analysis by electrophoresis in a 23% polyacrylamide–7 M urea gel as described (Becerra et al., 1983).

Chain-Length Analysis of Products Formed by Pol I lf. The chain-length distribution of polynucleotides formed during DNA polymerase reaction was evaluated by electrophoresis in an 8% polyacrylamide–7 M urea gel. A poly(dA)·(dT)$_{10}$ template-primer system was used at a micromolar concentration of primer and a nanomolar concentration of enzyme (Detera & Wilson, 1982). Sample preparation, electrophoresis, and autoradiography were as described (Detera & Wilson, 1982).

Preparation of Pyridoxylated Enzyme. Pol I lf was incubated in the dark in a final volume of 20 μL containing 50 mM Hepes, pH 8.0, 10 mM NaCl, 5 mM magnesium acetate, 1 mM dithiothreitol (DTT), 20% glycerol, and 1 mM PLP at 37 °C for 8 min. In some cases, as indicated, the template-primer system of 30 μg/mL poly(rA) and 15 μg/mL oligo(dT) was present. The incubation mixture then was placed at 0–1 °C, and a freshly prepared solution of either unlabeled $NaBH_4$ or ^{3}H-labeled $NaBH_4$ in 5 mM NaOH was added in a 10-fold molar excess with respect to PLP. The mixture was held at 0–1 °C for 15 min to ensure complete reduction, as indicated by a change in the reaction mixture from yellow to colorless. To decompose excess $NaBH_4$, 0.1 N acetic acid was added. In some experiments, the entire reaction mixture then was treated with sample application buffer, and the mixture was subjected to SDS–polyacrylamide gel electrophoresis. The Coomassie blue stained M_r 68 200 band was cut from the gel and counted. The specific activity of NaB^3H_4 used was 5 Ci/mmol, and the number of PLP residues incorporated was calculated by assuming one tritium atom per PLP incorporated. In other experiments, reaction mixtures containing pyridoxylated enzyme were treated with cold acetone, and the mixtures were centrifuged at 15000*g* for 20 min. The pellet fractions were suspended in 100 mM KP_i buffer, pH 7.0, 1 mM DTT, and 50% glycerol. Aliquots of the enzyme were used for DNA polymerase assay, exonuclease assay, and product chain-length experiments.

Gel Electrophoresis of Substituted Enzyme and Measurement of Radioactivity Incorporated. Discontinuous polyacrylamide slab gels were run by using the Tris–glycine–SDS buffer system described by Laemmli (1970). A typical gel was 0.75 mm thick and had a 1-cm 4% acrylamide–0.11% bis(acrylamide) stacking zone and a 9.5-cm separating zone of 8% acrylamide –0.22% bis(acrylamide). The sample application buffer was 4% SDS, 2% 2-mercaptoethanol, and 36% glycerol. Each 35-μL sample of enzyme was mixed with 15 μL of this sample application buffer and heated at 100 °C for 3 min. The solution was then brought to 25 °C, and a 25-μL portion was applied to a 15 × 4 × 0.75 mm well made in the stacking zone of the gel by using a 20-well comb. Electrophoresis was run at a constant current of 25 mA/gel. The gels were stained in a solution containing 0.1% Coomassie brilliant blue R-250, 50% methanol, and 10% acetic acid, and destaining was in 10% acetic acid and 10% 2-propanol. Molecular weight marker proteins were run in parallel. Stained gels were scanned with an Ortec Model 4310 densitometer fitted with a 570-nm narrow band-pass 3 cavity filter. Radioactivity incorporated from NaB^3H_4 reduction of enzyme samples was detected essentially by the method described by Bonner & Laskey (1974). The gel was allowed to stand in dimethyl sulfoxide (Me_2SO) for 1 h at 25 °C. It then was immersed in 25% 2,5-diphenyloxazole (PPO) in Me_2SO for 3 h at 25 °C. The gel was washed in water for 1 h and dried under vacuum. Fluorography was at −70 °C using Kodak XAR-5 or SB-5 film. Typical results are shown in Figure 1. Prominent bands of ^{3}H-labeled material comigrated with the Coomassie-stained enzyme, and this result was dependent upon PLP, in both the presence and absence of dTTP. After autoradiography, each band was cut from the gel and solubilized in 0.5 mL of 30% hydrogen peroxide at 37 °C for 18 h. Radioactivity was determined by scintillation counting.

Hydrolysis and Two-Dimensional Analysis. N^6-Pyridoxylysine was prepared according to the method described by Ohsawa & Gualerzi (1981). Briefly, poly(lysine hydrobromide) was incubated with 1 mM PLP in 50 mM Hepes buffer, pH 8.0, for 15 min at 37 °C. The reaction mixture was adjusted to 10 mM NaB^3H_4 and held at 0–1 °C until the yellow color disappeared. To remove unreacted $NaBH_4$, 0.1 N acetic acid was added, and the solution was dialyzed against 2% CH_3COOH for 48 h at 25 °C. The dialysate was lyophilized, and the residue was dissolved in a small volume of 0.1 N HCl. The solution was dried under vacuum. The residue was dissolved in 0.5 mL of constant-boiling 6 N HCl and placed in a hydrolysis tube. A crystal of phenol was added, and the tube was flushed with N_2 and sealed. The tube was incubated for 20 h at 110 °C in vacuo. The residue was lyophilized, dissolved in a small volume of H_2O, and relyophilized. This was repeated once. The residue was dissolved in 20 μL of electrophoresis buffer, pH 3.7, prepared from

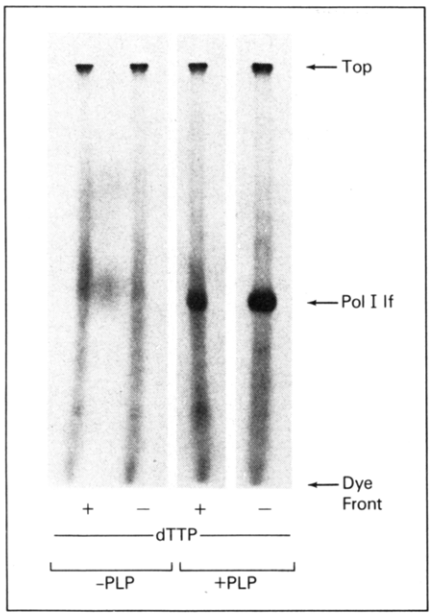

FIGURE 1: Autoradiograms showing the results of 10% SDS–polyacrylamide gel electrophoretic analysis of PLP-substituted ^3H-labeled Pol I lf. The enzyme first was incubated with PLP, and this was followed by NaB^3H_4 reduction as described under Materials and Methods. PLP and dTTP were present at 1 mM each, as indicated. The radioactivity bands overlapped exactly with the Coomassie blue stained enzyme (not shown). Exposure was for 5 days.

1:10:89 (v/v) pyridine:acetic acid:water, and the solution was stored at −20 °C. A 2–4-μL portion was applied to a silica gel thin-layer plate, and peptides were resolved by using electrophoresis in the first dimension and ascending chromatography in the second dimension (Gracy, 1977). Electrophoresis was at 2–4 °C and at 1000 V for 60 min in the electrophoresis buffer described above. The plate was dried at room temperature for 6–8 h. The plate then was suspended in a thin-layer chromatography chamber equipped with a hanging vertical plate holder. About 200 mL of butanol–pyridine–acetic acid–H_2O (50:33:1:40) was added to the bottom of the chamber. After equilibration at 22 °C, the plate was lowered into the liquid and allowed to develop for 5 h or until the solvent front reached the top of the plate. The plate was dried at room temperature. A single fluorescent ninhydrin-positive spot of N^6-pyridoxylysine was detected. Pol I lf (50 μg) containing 4 mol of [^3H]PLP/mol was hydrolyzed and analyzed by using the above procedure.

Peptide Mapping. Following PLP substitution and reduction with NaB^3H_4, the reaction mixture was extensively dialyzed against 2% acetic acid (Ohsawa & Gualerzi, 1981) and then lyophilized. The sample was divided into two portions—one portion was taken for cyanogen bromide (CNBr) treatment. The lyophilized sample was dissolved in a suitable volume of 50 mM ammonium bicarbonate, pH 7.8, containing 2 mM ethylenediaminetetraacetic acid (EDTA) (Drapeau, 1977). The solution was brought to 0.12 M 2-mercaptoethanol and incubated at 37 °C for 18 h. The sample was dried under vacuum, and the residue was dissolved in water. Approximately 100 μg of protein was dissolved in 35 μL of formic acid containing 0.5 mg of cyanogen bromide (Walker et al., 1976). Reaction was allowed to proceed at room temperature for 24 h. The reaction mixture was centrifuged and lyophilized. The residue was dissolved in a small amount of electrophoresis buffer (acetic acid:formic acid:H_2O, 15:5:80) and then centrifuged. The clear supernatant fraction was applied to a 20 × 20 cm silica gel thin-layer plate. Electrophoresis and chromatography were carried out as described by Planck & Wilson (1980). The plates were then dried in air and sprayed lightly with 10% triethylamine, dried for a few seconds, and sprayed and 0.1% fluorescamine and then with triethylamine. Spots were viewed under long-wavelength UV light (336 nm) as described by Gracy (1977). A solution of 10% diphenyloxazole (w/v) in ether was poured over the plate. The plate was dried, and autoradiography was at −70 °C using Kodak XAR-5 or SB-5 film.

The other portion of the PLP-substituted enzyme was oxidized with performic acid and digested with *Staphylococcus aureus* V8 protease. Briefly, 1 mg of 30% H_2O_2 and 9 mL of 88% formic acid were mixed and allowed to stand at 24 °C for 1 h. The solution was cooled and used immediately (Hirs, 1967). For each milligram of protein, 2 mL of this solution was added, and the tube was sealed. Oxidation was allowed to proceed at −1 °C for 4 h. The contents of the tube were dried under pressure at 40 °C. The residue was dissolved in 50 mM NH_4HCO_3, pH 7.8, containing 2 mM EDTA, and for each milligram of starting protein, 33 μg of V8 protease was added, such that the enzyme:substrate ratio was about 1:30. The solution was incubated at 37 °C for 20 h, as described by Drapeau (1977). After digestion, the solution was dried and dissolved in electrophoresis buffer. An aliguot was spotted on a silica gel thin-layer plate, and peptides were separated by electrophoresis followed by chromatography, as described for CNBr digestion.

Spectrophotometry and Fluorometric Measurements of PLP-Modified Enzymes. The stoichiometry of incorporation of PLP into the enzyme was determined by spectrophotometric and fluorometric methods in either the absence or the presence of 1 mM dTTP. The PLP and $NaBH_4$ reactions were done as described in a previous section. After reduction with $NaBH_4$, the samples were dialyzed against acetic acid for 48 h at 4 °C. The dialysate was adjusted to 1 mL with water in each tube. Spectrophotometer excitation was at 325 nm, and emission was at 400 nm. Micromoles per liter of PLP was determined by using aldolase as the standard, and micromoles per liter of enzyme was determined from Coomassie blue staining intensity of bands in an SDS–polyacrylamide gel with bovine serum albumin as the standard.

Results

PLP and Enzyme Activity. The initial rate of DNA polymerase activity by Pol I lf was examined by using a poly(dA)-oligo(dT) template-primer system. Activity was inhibited by PLP, whereas the PLP analogue pyridoxal at 4 mM gave only slight inhibition. The shape of the inhibition curve with PLP suggested that the kinetic mechanism was complex. Therefore, we chose to examine the kinetics of PLP inhibition with respect to dNTP by using variable PLP concentrations at two different fixed levels of dTTP. The data were analyzed by using a Hill plot method (Figure 2) as described by Loftfield & Eigner (1969). Enzyme activity as a function of PLP concentration formed linear patterns in both Tris-HCl and Hepes buffer at pH 8, and the slopes were 1.5 and 1.8, respectively. The K_i value was 17-fold lower in the Hepes buffer system than in the Tris-HCl buffer system. These results indicate that the molecular order of inhibition with respect to PLP was 2 and that these molecules did not act in a fully cooperative manner. Also shown in Figure 2 is that the intercept was the same at the two levels of dNTP tested. This indicates that the inhibition was noncompetitive with respect to dTTP, since in noncompetitive inhibition the intercept does not depend upon the dNTP level and for both competitive and uncompetitive inhibition the intercept term contains dNTP levels. In experiments not shown, it was found

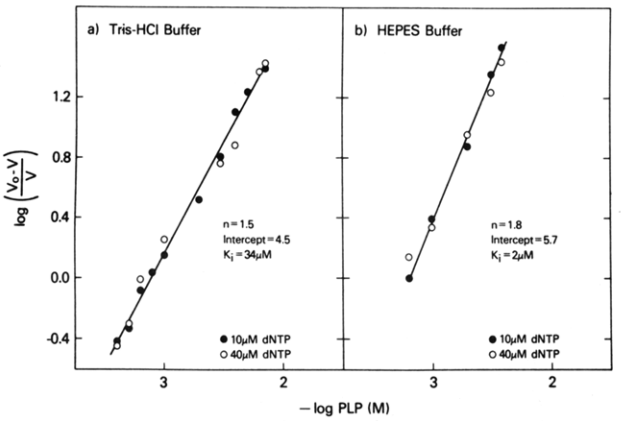

FIGURE 2: Kinetic pattern of PLP inhibition with respect to dNTP. Experiments were conducted as described under Materials and Methods by using variable PLP concentration different fixed levels of dTNP, and 10 and 40 μM dTTP. Hill plot analysis of the data conform to the equation $\log [(V_0 - V)/V] = n \log [PLP] + \log (1/K_i)$.

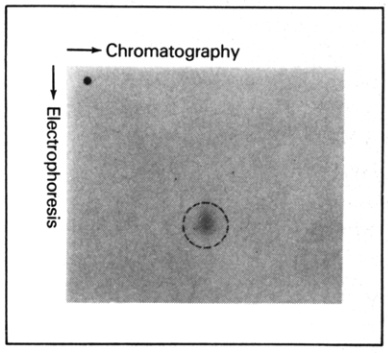

FIGURE 3: Autoradiogram of two-dimensional thin-layer analysis documented incorporation of PLP into pyridoxylysine of Pol I lf. The enzyme (16 μg) first was incubated with PLP, and this was followed by NaB^3H_4 reduction as described under Materials and Methods. The reduced enzyme sample was dialyzed, lyophilized, and acid hydrolyzed. The hydrolysate was lyophilized, and the residue was dissolved in 30 μL of H_2O. A 15-μL portion was applied to a TLC plate along with N^6-pyridoxylysine standard prepared as described under Materials and Methods. Electrophoresis followed by ascending chromatography was conducted. The position of the standard N^6-pyridoxylysine identified by fluorescence overlapped exactly with the radioactivity. Exposure was for 21 days.

that the 3′→5′-exonuclease activity of the Pol I lf also was inhibited by PLP; the kinetic pattern of this inhibition was not determined.

Covalent Incorporation of PLP after Sodium Borohydride Reduction. Sodium borohydride reduction of the enzyme in the presence of 1 mM PLP resulted in covalent incorporation of 3 mol of PLP/mol of enzyme. The stoichiometry of PLP incorporation was in good agreement by three methods of measurement: UV absorbance at 325 nm, fluorescence emission at 400 nm, and tritium uptake after sodium borohydride reduction. As shown in Figure 3, essentially all of the tritium incorporation after sodium [^{3}H]borohydride reduction was into pyridoxyllysine. No incorporation was observed with pyridoxal or pyridoxamine.

Stoichiometry of PLP Incorporation. The amount of PLP incorporated into Pol I lf was reduced when dTTP was present in the incubation mixture (Table I). This was evaluated in both the presence and absence of 15 mM KP_i, which was found to increase PLP uptake. The protection against PLP incorporation afforded by dTTP was equal to ~1 mol of PLP/mol of enzyme in both the presence and absence of KP_i. The presence of template-primer was not required for protection by dTTP; although protection was somewhat higher in the presence of template-primer in the experiment shown, this generally was not observed in other experiments. Template-primer alone had no effect on PLP uptake. In experiments not shown, we found that the protective effect of dNTP did not require base-pair complementarity with the template.

Table I: Effect of Template-Primer and dNTP on Incorporation of PLP into *E. coli* DNA Pol I Large Fragment

expt	template-primer	dTTP	amount of PLP[a] incorp (pmol)	mol of PLP/mol of enzyme
I	+	+	37	2
	+	−	54	3
	−	+	45	2.5
	−	−	55	3.0
II + 15 mM KP_i[b]	+	+	52	2.9
	+	−	71	3.9
	−	+	60	3.3
	−	−	67	3.7

[a] Incubations for covalent PLP incorporation were used with 1.2 μM enzyme, 1 mM dTTP, and the template-primer 30 μg/mL poly(rA) and 15 μg/mL olido(dT), as indicated. Experiments were as described under Materials and Methods. [b] Experiments were conducted for incubations containing sodium phosphate buffer in addition the components of the usual incubation.

Table II: PLP Incorporation and Polymerase Activity of *E. coli* DNA Pol I Large Fragment

modification[a]	mol of PLP/mol of enzyme	initial rate[b] of dNMP incorpn pmol/4 min	%
none	3	<5	<2
−PLP	0	420	115
−PLP + pyridoxamine	0	380	104
+dTTP	2.5	50	14
+dTTP and template-primer	2	50	14
−$NaBH_4$ reduction	0	380	104
control[c]		365	

[a] Modification of the usual incubation for covalent PLP incorporation; 1 mM dTTP and the template-primer 30 μg/mL poly(rA) and 15 μg/mL oligo(dT) were present as indicated. [b] After the PLP incorporation reaction, the enzyme was isolated. DNA polymerase assays and other experiments were as described under Materials and Methods. [c] An aliquot of the enzyme was acetone precipitated prior to incubation, and the pellet fraction was assayed.

Enzyme Activity and PLP Modification. The enzyme containing 3 mol of PLP/mol was devoid of both DNA polymerase and 3′→5′-exonuclease activity. As observed for the covalent incorporation of PLP, the block in DNA polymerase activity did not occur when either pyridoxal, pyridoxamine, or pyridoxamine phosphate was substituted for PLP or when either PLP or sodium borohydride was omitted from the incubation (Table II). The modified enzyme containing 2 mol of PLP/mol of enzyme had an initial rate of polymerase activity equal to about 20% of that of the unmodified enzyme; this enzyme also was capable of 3′→5′-exonuclease activity.

The ability of the modified enzyme containing 2 mol of PLP/mol to elongate nascent DNA chains (Detera & Wilson, 1982) was evaluated in the experiment shown in Figure 4. The chain-length distribution of products formed by this modified enzyme was markedly different from that of the unmodified enzyme. Most of the products formed by the modified enzyme had a chain length of one to eight newly incorporated dNMP residues. This indicates that the modified enzyme was unable to conduct extensive product elongation under the conditions used in this experiment.

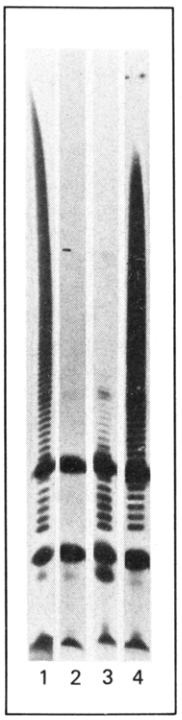

FIGURE 4: Autoradiogram showing results of 8% polyacrylamide–7 M urea gel electrophoresis of $[^{32}P](dT)_n$ molecules formed by Pol I lf and pyridoxylated Pol I lf. DNA polymerase incubations were as described under Materials and Methods and contained 1 nM enzyme and the template-primer system 40 μg/mL poly(dA) and 20 μg/mL $(dT)_{10}$ (cf. Fig. 5; Detera & Wilson, 1982). Lane 1, unmodified Pol I lf; lane 2, no enzyme; lane 3, Pol I lf with 2 mol of PLP/mol of enzyme. The modification reaction contained dTTP and template-primer. Lane 4, enzyme incubated in modification reactions with pyridoxamine instead of PLP. The stepladder pattern formed by product molecules in lanes 1, 3, and 4 begins with $(dT)_{11}$ and includes molecules several hundred nucleotides long. The large diffuse bands in the $(dT)_{18}$ region and below $(dT)_{11}$ are due to material in the commercial preparation of $[\alpha\text{-}^{32}P]$dTTP. Conditions of the electrophoresis were as described under Materials and Methods.

Enzyme Primary Structure and Pyridoxyllysine Modification. Two-dimensional peptide maps after cleavage with staphylococcal V8 protease or cyanogen bromide were obtained. V8 protease cleaves at glutamate and is expected to yield 54 peptides from Pol I lf of which 21 peptides contain lysine (Joyce et al., 1982; Brown et al., 1982). The peptide map after V8 protease cleavage of the enzyme containing 4 mol of PLP/mol of enzyme (Figure 5, top panels) contained four major and several minor radiolabeled peptides. A portion of the labeled material remained unresolved; however, ninhydrin staining revealed that peptide spots were distributed throughout the chromatogram (data not shown). Incorporation into one of the major labeled peptides was blocked by dTTP.

Cyanogen bromide cleaves at methionine and is expected to yield 15 peptides, of which 10 contain lysine. The enzyme with 4 mol of PLP/mol of enzyme contained three or four major spots, all of which failed to migrate substantially in the electrophoresis dimension (Figure 5, bottom panels). Incorporation into one of these peptides was blocked by dTTP. Although a number of minor spots were detected with each method of enzyme cleavage, these results indicate that PLP incorporation into lysine residues was selective and that dTTP blocked incorporation into a specific peptide.

Discussion

Our results from substrate kinetic experiments indicate that PLP binds to the Pol I lf and inhibits the initial rate of DNA

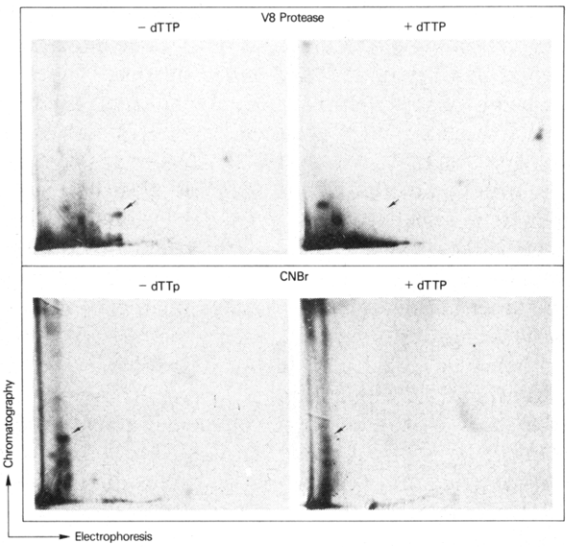

FIGURE 5: Autoradiogram showing the results of two-dimensional peptide mapping of pyridoxylated Pol I lf. Cleavage was with V8 protease or CNBr as indicated. Experiments were as described under Materials and Methods. The arrow indicates the main peptide protected from ^3H labeling by dNTP.

synthesis. Hill plot analysis indicates that the inhibition involved two molecules of PLP that were not fully cooperative. The PLP inhibition is overcome to an extent by dNTP, and the kinetic pattern of inhibition with respect to dNTP was noncompetitive, indicating that all molecules of PLP and dNTP do not combine with the same form of the enzyme. These results were consistent with our subsequent findings on properties of the enzyme containing covalently incorporated PLP. Thus, the fully pyridoxylated enzyme, which contained several moles of PLP per mole of enzyme, was not capable of DNA synthesis, whereas the enzyme pyridoxylated in the presence of dNTP and containing 1 less mole of PLP per mole of enyzme was capable of synthesis, albeit at a slower initial rate than the unsubstituted enzyme. These results taken together suggest that two PLP target sites are responsible for the inhibition and that one of these sites is protected by dNTP.

The Pol I lf contains 38 lysine residues and an N-terminal valine (Brown et al., 1982). All of these residues have a free primary amino group and are theoretically potential target sites for pyridoxylation. We have shown in the present study, however, that three or four of the lysine residues in the native protein are preferentially pyridoxylated at pH 8.0. One of these target lysines appears to reside in or around a dNTP binding site, since this residue was protected from pyridoxylation by dNTP and the property of PLP binding to nucleotide binding sites is well recognized. This protected lysine residue is probably identical with the single lysine residue modified by the oxidation product of β-D-ribosyl-6-(methylthio)purine as reported by Salvo et al. (1976) and the active-center lysine residue identified with *N*-(carboxymethyl)isatoic anhydride in the study by Jovin et al. (1969). The basis for the preferential pyridoxylation of the other two lysine groups is unknown.

The observation that the enzyme pyridoxylated in the presence of dNTP was capable of DNA synthesis offered the possibility of studying elongation by a modified form of Pol I lf. This was of interest since earlier work (Detera & Wilson, 1982) had suggested that the enzyme undergoes a structural transition as the synthesis of an individual nascent chain proceeds from an initiation phase to an elongation phase. On the basis of the chain-length distribution of products formed in a poly(dA)·oligo(dT) template-primer system (Figure 5),

we concluded that the enzyme containing 2 mol of PLP/mol of enzyme could initiate synthesis but was able to add only a limited number of residues to the nascent chain. The chain-length distribution of products indicated a constant chance for termination of a nascent chain with each addition of dNMP (Detera & Wilson, 1982). This was in contrast to results with unmodified enzyme which exhibited decreasing termination probability as the nascent chain gets longer. Changes in the enzyme required for this change in termination probability appear to be blocked in the PLP-modified enzyme.

The order of addition of substrates to an enzyme can be deduced from substrate initial velocity patterns obtained in the presence of reaction products. This approach has been particularly useful with enzyme reactions that are freely reversible, as is the case with the polymerase activity of Pol I lf. On the basis of PP_i inhibition data, McClure & Jovin (1975) concluded that template-primer combines first and that dNTP combines with the template-primer–enzyme complex. A disadvantage of this approach, however, is that product inhibition with PP_i is complicated in the study of a processive enzyme such as Pol I; in addition, inhibition can occur by mechanism other than driving the reverse reaction, such as through formation of a dead-end complex as proposed for PP_i inhibition of mouse β-polymerase (Tanabe et al., 1979). Therefore, it was of interest to determine whether Pol I lf is capable of binding dNTP in the absence of template-primer. Our results suggest that the free enzyme has a functional dNTP binding site and that binding to this site in the presence of template-primer does not require base-pair complementarity with the template. Hence, the addition of dNTP and template-primer to the free enzyme need not be ordered and could be random. Further investigation is needed to establish preferred kinetic pathways for the initiation phase of synthesis.

Finally, we observed that PLP modification failed to distinguish the polymerase activity and the 3′→5′-exonuclease activity of Pol I lf. The observation that dNTP protected the enzyme against inhibition of both activities and that this protection corresponded to one PLP residue per mole of enzyme is consistent with the idea that the single dNTP binding site identified here is involved with both activities.

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Registry No. PLP, 54-47-7; DNA polymerase, 9012-90-2; L-lysine, 56-87-1; dTTP, 365-08-2.

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