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DNA Binding Domain of *Escherichia coli* DNA Polymerase I: Identification of Arginine-841 as an Essential Residue†

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ABSTRACT: To identify the DNA binding site(s) in *Escherichia coli* DNA polymerase I (pol I) (Klenow fragment), we have used an active-site-directed reagent, phenylglyoxal (PG), which specifically reacts with arginine residues. Preincubation of DNA pol I with PG resulted in the loss of polymerase, 3′–5′-exonuclease, and DNA binding functions. Furthermore, the presence of DNA but not deoxynucleoside triphosphates protected the enzyme from inactivation. Labeling studies with [7-^{14}C]PG indicated that two arginine residues were modified per mole of enzyme. In order to locate the site of PG modification, we digested the PG-treated enzyme with trypsin and V-8 protease. The resulting peptides from each digest were then resolved on reverse-phase hydrophobic columns. An appearance of a new peptide peak was observed in both tryptic and V-8 protease digests. Since inclusion of template-primer during PG modification of enzyme blocks the appearance of these peaks, these peptides were concluded to represent the template-primer binding domain of pol I. Indeed, the extent of inactivation of enzyme by PG treatment correlated very well with the quantitative increase in the new tryptic peptide peak. Amino acid composition analysis of both tryptic peptide and V-8 peptide revealed that the two peptides were derived from the same general region; tryptic peptide spanned between residues 837 and 857 while V-8 peptide spanned between residues 841 and 870 in the primary sequence of pol I. Sequence analysis of tryptic peptide further identified arginine-841 as the site of PG modification, which implicates this residue in the DNA binding function of pol I.

Escherichia coli DNA polymerase I is a monomeric protein (M_r 103K) with three distinct enzyme activities, viz., polymerase, 5′–3′-exonuclease, and 3′–5′-exonuclease. Mild proteolysis cleaves the protein into two separate domains: the large C-terminal fragment [67 kilodaltons (kDa)][1] retains the polymerase and 3′–5′-exonuclease activities while the smaller N-terminal fragment harbors the 5′–3′-exonuclease activity (Brutlag et al., 1969; Klenow & Henningson, 1970). The gene for DNA Pol I has been cloned and the primary structure of the protein determined (Joyce et al., 1982; Brown et al., 1982). Recently, the crystal structure of the large fragment (Klenow) has also been elucidated (Ollis et al., 1985). All these studies together with the early kinetic studies (Englund et al., 1969; Huberman & Kornberg, 1970; Que et al., 1978) have provided some insight into the possible arrangement of the different domains which participate in the expression of the various enzyme activities of pol I.

Studies in our laboratory are aimed at defining the structure–function relationships in DNA polymerases by utilizing various active-site-directed reagents (Srivastava & Modak, 1980a,b, 1982, 1983; Srivastava et al., 1983; Abraham & Modak, 1984). We have been able to define some of the structural domains of DNA polymerases which are responsible for the binding of substrate dNTPs and template-primer. Pyridoxal 5′-phosphate, which has been shown to be a substrate binding site directed reagent (Modak, 1976; Modak & Du-

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[1] Abbreviations: pol I, *Escherichia coli* DNA polymerase I; PG, phenylglyoxal; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; dNTP, deoxynucleoside triphosphate; HPLC, high-performance liquid chromatography; TPCK, tosylphenylalanine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PTH, phenylthiohydantoin; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane.

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maswala, 1981), has been found to react at lysine-758 in *E. coli* pol I, implicating this residue to be involved in the binding of the metal chelate form of substrate dNTP (Basu & Modak, 1987). Using the technique of UV-mediated cross-linking of substrate dNTPs to their acceptor site (Modak & Gillerman-Cox, 1982), we have also identified His-881 as another residue involved in the binding of dNTPs (Pandey et al., 1987). Similarly, ferrate ion (a specific reagent for phosphate group binding sites) mediated oxidation of methionine-512 was found to result in the pol I inactivation (Basu et al., 1987). This methionine residue lies at the proposed hinge region (Ollis et al., 1985) and appears to play some important role in binding of enzyme to DNA (Basu et al., 1987). We have earlier shown that phenylglyoxal (PG), which specifically reacts with arginine residues in proteins, strongly inhibits template-dependent DNA polymerases (Srivastava & Modak, 1980c) as well as associated nuclease activities (Srivastava & Modak, 1980a). Furthermore, in avian myeloblastosis virus DNA polymerase, a selective inhibition of the initiation of DNA synthesis was observed as a result of reaction with PG. This suggested that the DNA binding domain was the target of PG reaction. Earlier, arginine residues have been implicated as a target of PG action in *E. coli* DNA polymerase I (Salvo et al., 1976). Our present work is an extension of the above studies with *E. coli* DNA pol I (Klenow fragment). We have identified Arg-841 as the PG reactive residue which is responsible for the template binding function in pol I.

Materials and Methods

All the unlabeled dNTPs, calf thymus DNA, poly(dA-dT)$_{12-18}$, poly(dA), poly(A), poly(dT), and (dT)$_{12-18}$ were obtained from P-L Biochemicals. [^{3}H]dNTPs and [7-^{14}C]-phenylglyoxal were purchased from Amersham. Nonradioactive phenylglyoxal was from ICN Pharmaceuticals. Trypsin (TPCK) was from Worthington. Acetonitrile, HPLC-grade water, and other chemicals were purchased from Fisher Scientific Co. *N*-Ethylmorpholine (sequanal grade) was a product of Pierce Chemical Co. DNA pol I (Klenow fragment) was purified from an *E. coli* clone generously supplied by Dr. Catherine Joyce of Yale University (Joyce & Grindley, 1983).

Enzyme Assays. Polymerase and 3′–5′-exonuclease activities of pol I (Klenow) were measured as described earlier (Basu et al., 1987). For polymerase, poly(dA-dT)$_{12-18}$ was used as the template-primer, and the incorporation of [^{3}H]TTP was monitored. For 3′–5′-exonuclease activity, the loss of acid-precipitable counts from [^{3}H]TTP-labeled poly(dA-dT) was measured.

Nitrocellulose Filter Assay for the Binding of Template-Primer to E. coli Pol I. The reaction mixture in a final volume of 200 μL contained 50 mM Hepes–KOH (pH 7.8), 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 20 μg of BSA, 200 ng of pol I, and 200 ng of poly(dA-[^{3}H]dT) equivalent to 50 000 cpm [poly(dA)·(dT)$_{12-18}$ was labeled with [^{3}H]TTP using *E. coli* pol I and purified by phenol/chloroform extraction and ethanol precipitation]. The above reaction mixture was diluted with 3 volumes of filter binding buffer containing 50 mM Hepes–KOH (pH 7.8), 1 mM DTT, 50 mM KCl, and 10 mM $MgCl_2$ and incubated on ice for 15 min. The mixture was then filtered through a nitrocellulose membrane (BA-85, 13 mm, 0.45 μm; Schleicher & Schuell). The filter was washed with 2 mL of filter binding buffer, dried, and counted.

Modification of Pol I with Phenylglyoxal. Seventy micrograms of pol I (1 nmol) was incubated with PG in a final reaction volume of 400 μL containing 50 mM Hepes–KOH (pH 7.8) and 20% glycerol at 37 °C. At desired time intervals, 2-μL aliquots were diluted to 200 μL, and 2 and 20 μL of the diluted enzyme were used for polymerase and nuclease activities, respectively. For template-primer binding, 200 ng of the undiluted enzyme was used directly. For protection experiments with template-primer, pol I (70 μg) was preincubated with an equivalent amount (70 μg) of activated DNA, poly(dA)·(dT)$_{12-18}$, poly(dA), poly(A), poly(dT), or (dT)$_{12-18}$ for 5 min at 37 °C. PG was then added to a final concentration of 2 mM and further incubated at 37 °C for 60 min. For protection with substrate triphosphates, the dNTPs were used at a concentration of 10 mM each along with 10 mM $MgCl_2$. Aliquots were removed for the determination of various activities as described above, and the remaining protein was precipitated with 10% TCA and used for the preparation of peptides.

Stoichiometry of [7-^{14}C]PG Binding to Pol I. Ten nanomoles of pol I was incubated with 2 mM [7-^{14}C]PG (specific activity 5 mCi/mmol) in a 2-mL reaction volume containing 50 mM Hepes–KOH (pH 7.8) and 20% glycerol (v/v) at 37 °C. At regular time intervals, 200-μL aliquots were withdrawn, and the incorporation of [7-^{14}C]PG into protein was determined by precipitation of the protein with 10% TCA. The precipitates were collected on nitrocellulose filters and washed (2–3 times) with 5% TCA and finally with water. The filters were dried and counted for radioactivity. Simultaneously, at each time period, 2-μL aliquots were diluted in 200 μL of 0.1% BSA, and 2 μL of this solution was used to determine polymerase activity.

Protease Digestion and HPLC Mapping of Peptides. In order to locate the site of PG modification, the desired quantity of pol I was modified with PG under different conditions. Modified enzyme protein was precipitated with 10% TCA, and the precipitates were washed 2–3 times with 5% TCA and finally with 95% ethanol. Pellets were dried under vacuum and resuspended in 100 μL of 50 mM *N*-ethylmorpholine acetate buffer, pH 7.8, for tryptic digestion or in 100 μL of 50 mM ammonium acetate buffer, pH 4.0, for V-8 protease digestion. At pH 4.0, V-8 protease preferentially cleaves after glutamic acid residues. Trypsin (TPCK) or V-8 protease was added at a ratio of 1:25 (protease to protein w/w) and incubated at 37 °C. Tryptic digestions were limited to 6 h, while V-8 digestions were carried out for 24 h. The digests were acidified with 0.1% TFA and centrifuged, and the clear supernatants were injected into a Vydac C-18 column (0.45 × 25 cm) that was preequilibrated with 0.1% TFA. The peptides were eluted (0.7 mL/min) from the column attached to a Varian HPLC system by increasing the concentration of solvent B (70% acetonitrile in 0.1% TFA). The gradients used for resolution of peptides are described in the figure legends.

Purification of PG–Peptide. From the comparative analyses of control and PG-treated enzyme digests, emergence of a new peptide peak in PG-treated enzyme digested with trypsin was consistently noted. This peptide elutes at about 85 min (Figure 3). To purify this peptide further, fractions eluting from 82–88 min from control as well as PG-treated enzyme were pooled, concentrated, and repurified on a Vydac C-4 column using a shallow gradient as follows: 0–10 min, 25% solvent B; 10–120 min, 45% solvent B (see Figure 5). By comparing the peptide elution patterns, the new peptide peak which elutes after the major UV-absorbing peak was detected and carefully isolated (see Figure 5). HPLC maps of V-8 protease digested enzyme showed the presence of two radioactive peaks, one major and one minor, eluting at about 24 and 38 min. The major radioactive-containing peak eluting at 24 min was further purified on a Vydac C-4 column (Figure 7) using the

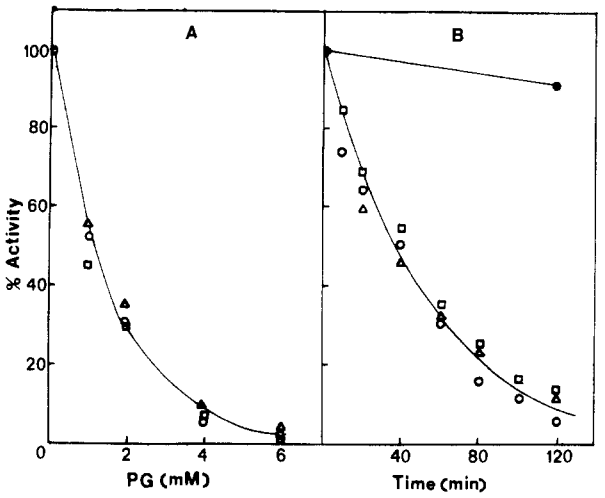

FIGURE 1: Effect of phenylglyoxal on polymerase, 3′–5′-exonuclease, and template-primer binding activities of DNA pol I (Klenow fragment). (A) DNA pol I (1 nmol) was incubated with various concentrations of PG in a 400-μL reaction volume containing 50 mM Hepes–KOH buffer (pH 7.8) and 20% glycerol at 37 °C for 60 min. Suitable aliquots were diluted in BSA (0.1%) for polymerase (○), 3′–5′-exonuclease (□), and template-primer binding (△) activities (see Materials and Methods). For polymerase, 100% activity corresponds to 360 pmol of [^{3}H]TMP incorporated/15 min at 37 °C. For 3′–5′-exonuclease, 100% activity is equal to 20000 cpm of [^{3}H]TMP released (acid-soluble fraction) in 30 min at 37 °C from poly(dA-[^{3}H]dT) (50000 cpm). For template-primer binding control, 100% activity is equivalent to 20000 cpm bound to nitrocellulose filter. Panel B represents a time course of inactivation of DNA pol I activities by PG. DNA pol I (1 nmol) was incubated with 2 mM PG as described above, aliquots were removed at various time intervals, and polymerase (○), 3′–5′-exonuclease (□), and template-primer binding (△) activities were determined (see Materials and Methods). A control without PG was also incubated for 120 min (●).

following gradient: 0–10 min, 20% solvent B; 10–90 min, 40% solvent B. A small aliquot of each fraction was used to monitor the presence of radioactivity. The new peptide peak associated with radioactivity, appearing in the chromatogram of PG-modified enzyme (Figure 7B), was isolated and subjected to amino acid composition analysis.

Amino Acid Composition and Sequence Analyses. Peptides purified on the C-4 reverse-phase column were hydrolyzed in 6 N HCl containing 0.2% phenol for 16 h at 115 °C in a Waters pico-tag work station. Resulting amino acids were converted to their phenylthiocarbamyl derivatives and analyzed by HPLC using Nova-pak columns (two 0.45 × 15 cm columns attached in series) by the method described by Stone and Williams (1986). 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 Pol I (Klenow Fragment) by Phenylglyoxal (PG). Preincubation of DNA pol I with increasing concentrations of PG at 37 °C for 60 min resulted in the loss both of polymerase and 3′–5′-exonuclease activities and of DNA binding ability in a dose-dependent manner (Figure 1A). There is a linear loss of the above three functions of pol I up to 2 mM PG concentration.

A typical time course of inactivation of polymerase, 3′–5′-exonuclease, and DNA binding ability using a fixed concentration of PG (2 mM) is shown in Figure 1B. Once again, all three activities of pol I were found to be inactivated linearly with time. Thus, at 2-h incubation time, 85–95% inactivation was apparent with PG, while under similar conditions of incubation of control pol I (without PG) only a 10% loss of enzyme activity was noted.

Table I: Effect of Templates and Substrate dNTPS on Polymerase and 3′–5′-Exonuclease Activities of DNA Pol I[a]

addition	polymerase: pmol of TMP incorporated/15 min	polymerase: % inactivation	3′–5′-exonuclease[b] (% inactivation)
control (no PG)	332	0	0
none	23	93	85
activated DNA	282	15	22
poly(dA-dT)$_{12-18}$	269	19	26
poly(dA)	199	40	30
poly(dT)	202	39	47
dT$_{12-18}$	49	85	81
dATP	7	98	82
dGTP	17	95	86
dCTP	19	94	79
dTTP	4	99	85

[a] DNA pol I (1 nmol) was preincubated with templates (70 μg) and dNTPs (10 mM) for 5 min at 37 °C, and then PG was added to a final concentration of 2 mM in a 400-μL reaction volume containing 50 mM Hepes–KOH buffer (pH 7.8) and 20% glycerol. The incubation was at 37 °C for 60 min. Suitable aliquots were diluted in BSA (0.1%) for assay of polymerase and 3′–5′-exonuclease activities (see Materials and Methods). [b] Control activity corresponds to 20000 cpm of [^{3}H]TMP released at 37 °C in 15 min.

Protection of Pol I from PG Inactivation by Template-Primer or Substrate dNTPs. In order to determine the target of PG inactivation in pol I, we examined the effects of activated calf thymus DNA and various synthetic template-primers as well as substrate dNTPs on the inactivation of pol I. The results are tabulated in Table I. Activated DNA, poly(dA)·(dT)$_{12-18}$, poly(dA), poly(dT), and poly(rA) protected both polymerase and nuclease activities, ranging from 45% to 85%. However, neither (dT)$_{12-18}$ nor the substrate dNTPs showed significant protective effect (Table I). In order to rule out the possibility that DNA-mediated protection of pol I from PG inactivation was due to reactivity of PG toward DNA, we examined the presence of DNA on the inactivation of a non-DNA polymerase enzyme, namely, lysozyme. The presence of DNA at comparable concentrations had no protective effect on lysozyme inactivation by PG (data not shown). Similarly, when PG inactivation was carried out in the presence of both DNA and 0.4 M NaCl (a condition that prevents the binding of DNA to enzyme), no protection of pol I by DNA could be observed. These results clearly indicate that DNA protects the enzyme by blocking the PG reactive site(s) on the enzyme.

Stoichiometry of [7-^{14}C]PG Binding to DNA Pol I. Phenylglyoxal is known to irreversibly bind arginine residues in proteins (Takahashi, 1968, 1977). Hence, the number of moles of [^{14}C]PG bound per mole of pol I was determined by TCA precipitation. It was found that approximately 4 mol of [^{14}C]PG is bound per mole of pol I, when the enzyme was inactivated to about 95% level (Figure 2). This suggests that two arginine residues are modified, since two molecules of PG react with one arginine residue (Takahashi, 1977).

Comparative Tryptic Peptide Maps of Native, PG-Inactivated, and DNA-Protected DNA Pol I. PG-reacted arginine residues in proteins are known to be resistant to tryptic cleavage, and, hence, tryptic peptide analysis by reverse-phase HPLC of the native, PG-reacted, and DNA-protected pol I was carried out to identify the PG-modified peptide(s). The pattern of peptides present in the three digests is shown in Figure 3. Comparison of the peptide maps clearly allows identification of a single new peptide eluting at 85 min in the PG-inactivated pol I, which is absent in both native and

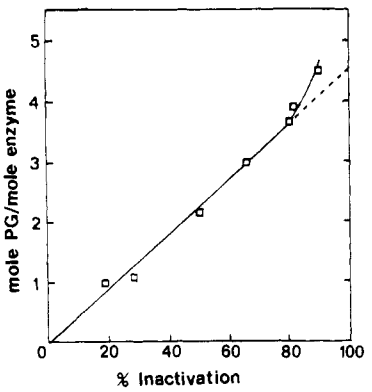

FIGURE 2: Stoichiometry of [7-^{14}C]PG binding to DNA pol I. DNA pol I (10 nmol) was incubated with 2 mM PG (specific activity 5 mCi/mmol) in a 2-mL reaction volume containing 50 mM Hepes-KOH buffer (pH 7.8) and 20% glycerol at 37 °C. At regular time intervals, suitable aliquots were diluted in BSA (0.1%) for polymerase activity, and 200-μL aliquots were removed simultaneously for the determination of [7-^{14}C]PG bound to DNA pol I (see Materials and Methods).

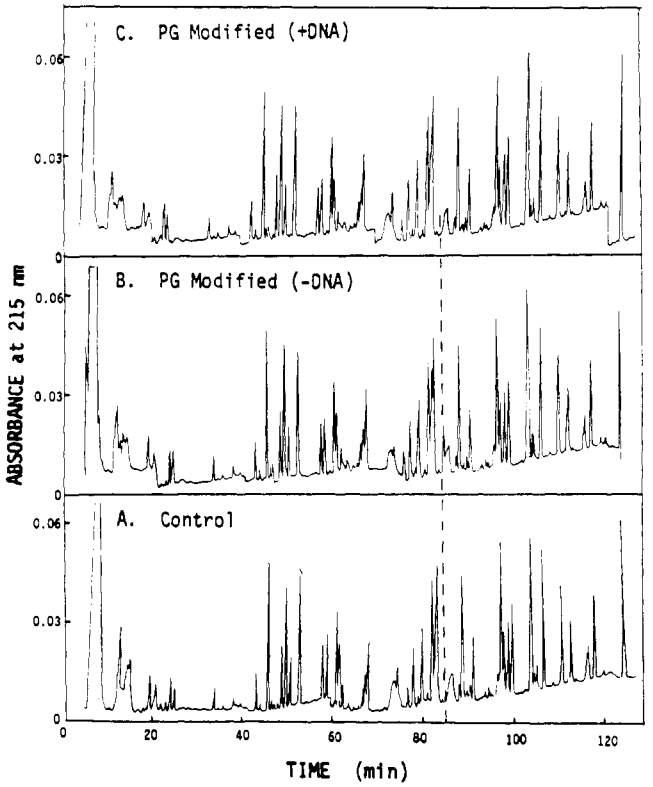

FIGURE 3: Reverse-phase HPLC of tryptic peptides from control, PG-inactivated, and DNA-protected DNA pol I (Klenow fragment). One nanomole of DNA pol I was treated with PG in the presence and absence of DNA, and tryptic digests were prepared as described under Materials and Methods. Peptides were resolved on a Vydac C-18 column preequilibrated with 0.1% trifluoroacetic acid. The peptides were eluted with increasing concentrations of solvent B (70% acetonitrile in 0.1% trifluoroacetic acid) as follows: 0–90 min, 0–40% solvent B; 90–130 min, 40–70% solvent B. They were monitored by their absorbance at 215 nm. The dashed line indicates the new peptide which appears on PG modification. (A) Peptides obtained from control enzyme; (B) peptides from PG-treated enzyme; (C) Peptides from enzyme treated with PG in the presence of 70 μg of DNA.

DNA-protected pol I. It was interesting to note that this peptide, which contains the putative site for PG modification, has no UV absorption at 278 nm. This observation suggests that (a) the PG moiety of the PG–Arg adduct is unstable and (b) this peptide does not contain any aromatic amino acid residues. To confirm that the appearance of this new peptide

Time (min)	% Inactivation: Polymerase activity	% Inactivation: 3'-5' exonuclease activity	PG-Peptide appearance
0	0	0	
20	23	19	
40	63	58	
60	82	83	

FIGURE 4: Correlation between loss of DNA pol I enzyme activities with the appearance of PG–peptide. DNA pol I (5.7 nmol) was incubated with 2 mM PG as described in the legend for Figure 2. Aliquots (1 nmol) were removed at the indicated time, and polymerase and 3'–5'-exonuclease activities were determined after diluting the enzyme suitably in BSA (0.1%). The remainder was used for tryptic peptide analysis. Reverse-phase HPLC on a Vydac C-18 column was performed as described under Materials and Methods. The peptide appearance was monitored at 215 nm. The arrow indicates the position of PG–peptide eluting at about 85 min.

Table II: Amino Acid Sequence Analysis of Tryptic PG–Peptide[a]

cycle	residue[b]	amino acid identified	yield (pmol)
1	837	Ala	63
2	838	Ala	47
3	839	Ala	44
4	840	Glu	22
5	841	Arg	*c*
6	842	Ala	22
7	843	Ala	27
8	844	Ile	16
9	845	Asn	12
10	846	Ala	17
11	847	Pro	11
12	848	Met	11
13	849	Gln	8
14	850	Gly	9
15	851	Thr	1

[a] See Figure 5. [b] The amino acid numbers correspond to the primary sequence of *E. coli* DNA pol I (Joyce et al., 1982). [c] No arginine peak was observed at cycle 5 (see Figure 6) which indicates modification of this residue.

peak at 85 min is indeed related to the loss of polymerase activity by PG treatment, pol I was inactivated to various degrees (25–90%) by varying the incubation time of PG treatment, and the protein was subjected to tryptic peptide analysis. The results (Figure 4) clearly indicate that the quantitative increase in this peptide corresponds very well with the loss in the polymerase activity of pol I. The amino acid sequence of this new peptide was determined. The presence of two overlapping peptides in this peak fraction (obtained from the C-18 column) was deduced from sequence analysis which showed that the two peptides corresponded to residues 795–804 and 837–857 in the primary amino acid sequence of pol I (data not shown). Further resolution of the two peptides was achieved by rechromatography of these peptides on a C-4 matrix (Figure 5). We have identified the PG-reacted tryptic peptide as 837–857, which contains an arginine-841 which was not cleaved by trypsin. This arginine residue, which is the site of PG reaction, was conspicuously missing from the fifth cycle of the sequence analysis (Table II and Figure 6).

Our [^{14}C]PG binding studies with pol I indicated that two arginine residues are modified, but we have been able to locate and identify only one arginine residue by the tryptic digestion. At present, the identity of the second PG-modified peptide is not known.

V-8 Peptide Maps of Native and PG-Modified DNA Pol I. In order to confirm the identity of residues 837–857 as a

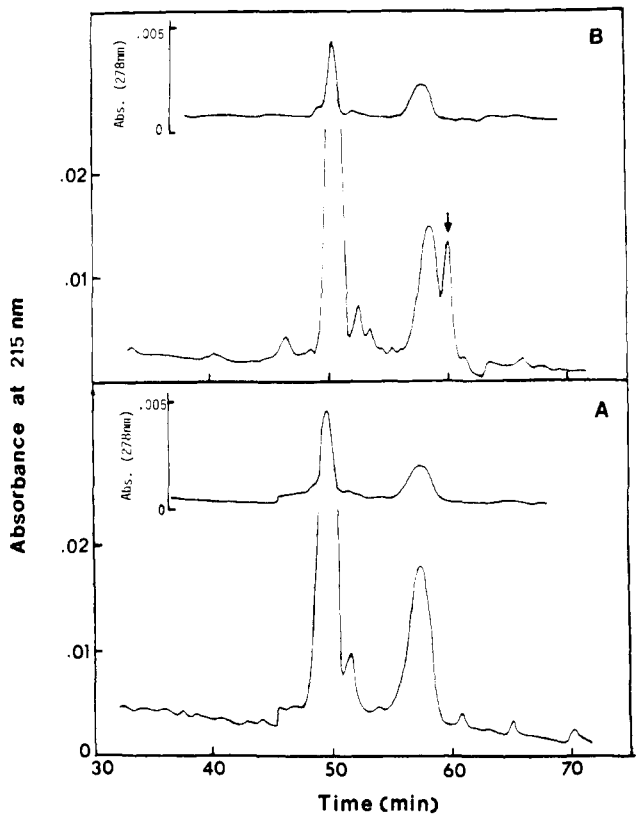

FIGURE 5: Purification of PG–peptide by reverse-phase HPLC. Tryptic peptides were prepared from control and PG-inactivated (80% inactivated) DNA pol I (5.7 nmol) and separated on a C-18 matrix column as described in Figure 3. Peptide fractions eluting between 82 and 88 min were collected, concentrated, and further resolved on a Vydac C-4 column, preequilibrated with 0.1% trifluoroacetic acid. The peptides were eluted at a flow rate of 0.7 mL/min with solvent B (70% acetonitrile in 0.1% trifluoroacetic acid) as follows: 0–10 min, 0–25% solvent B; 10–120 min, 25–45% solvent B. The peptides were detected by their absorbance both at 215 nm and at 278 nm. (A) Control digest; (B) PG-treated enzyme digest. The arrow indicates the position of the new peptide which appears on PG modification of DNA pol I.

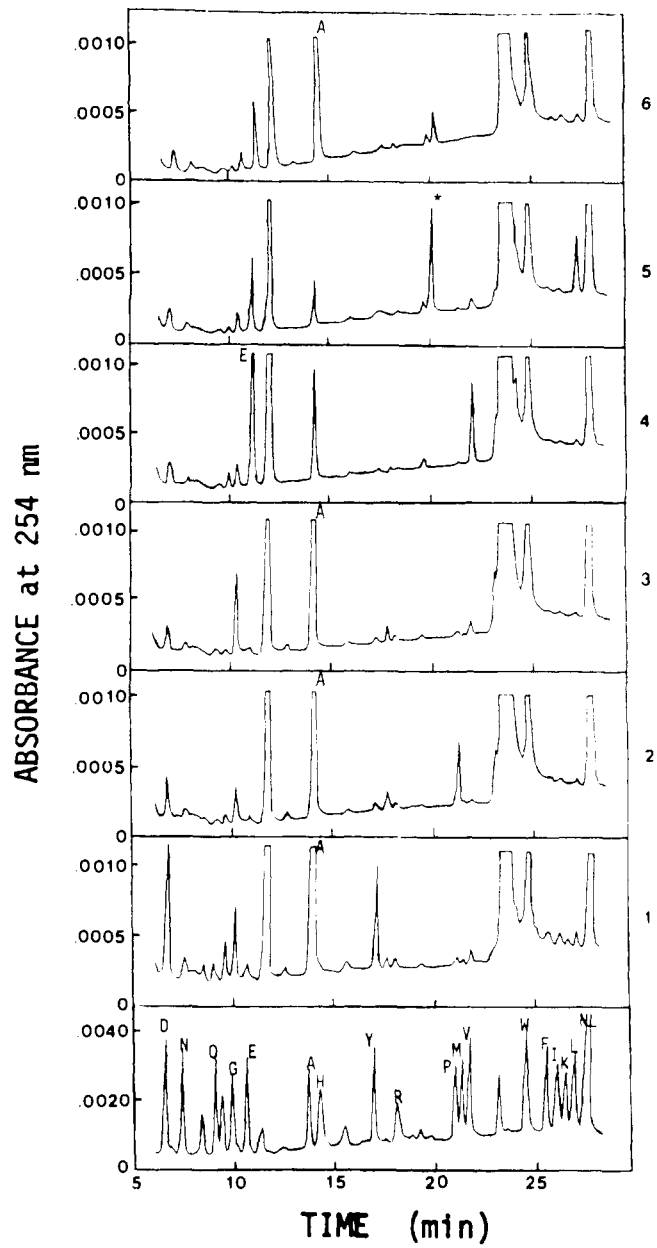

FIGURE 6: HPLC traces of phenylthiohydantoin derivatives from cycles 1–6 of the gas-phase sequencing of PG–peptide. An internal standard of PTH–norleuine (100 pmol) was added to each sequenator cycle. Samples were run on a Waters HPLC system as described by Merril et al. (1984). The tracing at the bottom represents 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. In cycle 5, the peak labeled with an asterisk, eluting at 20 min, does not correspond to any PTH–amino acid. This peak is possibly the PTH derivative of modified arginine. The peaks eluting at 12, 23, and 24.5 min in each cycle are apparently reagent peaks arising from incomplete drying.

region that represents the DNA binding domain (with Arg-841 as the site of PG modification) and to detect the second PG-reactive arginine residue, we resorted to V-8 protease digestion of pol I at pH 4.0. In this set of experiments, enzyme modification was carried out with [^{14}C]PG since the PG–enzyme adduct was expected to be more stable during the protease digestion under acidic medium. The comparative HPLC maps of native and PG-modified enzyme clearly showed the presence of a major radioactive peak eluting at about 25 min (Figure 7A). This peak was also found to be protected from PG modification in the presence of a template-primer [activated DNA or poly(dA·dT)$_{12}$] (results not shown). This peptide was further purified by using a Vydac C-4 column (Figure 7B) and characterized by amino acid composition analysis. The composition (Table III) showed that this peptide corresponds to amino acid residues 841–870, which should contain two arginines. However, the composition analysis showed the presence of only one arginine, indicating that this peptide contains only one PG-reactive arginine. Since both V-8 peptide and tryptic peptide are derived from the same domain (residues 837–870), the identity of PG-reactive arginine at position 841, as noted in the sequence analysis of tryptic peptide (Figure 6 and Table II), seems viable.

DISCUSSION

The presence of arginine in the active center of DNA polymerases has been indicated by the sensitivity of these enzymes to phenylglyoxal treatment (Borders et al., 1975; Salvo et al., 1976; Srivastava & Modak, 1980c). Since PG has been known to selectively react with arginine residues, identification of putative arginine residues in pol I which are reactive toward PG seemed feasible. We had earlier reported a detailed analysis of PG-mediated inactivation of avian myeloblastosis virus and Rauscher leukemia virus reverse transcriptases and had found that the template-primer binding domain was the target of PG action (Srivastava & Modak, 1980c). The investigations of PG-mediated inhibition of pol I revealed that the general properties and characteristics of inhibition of this enzyme are very similar to those observed with reverse tran-

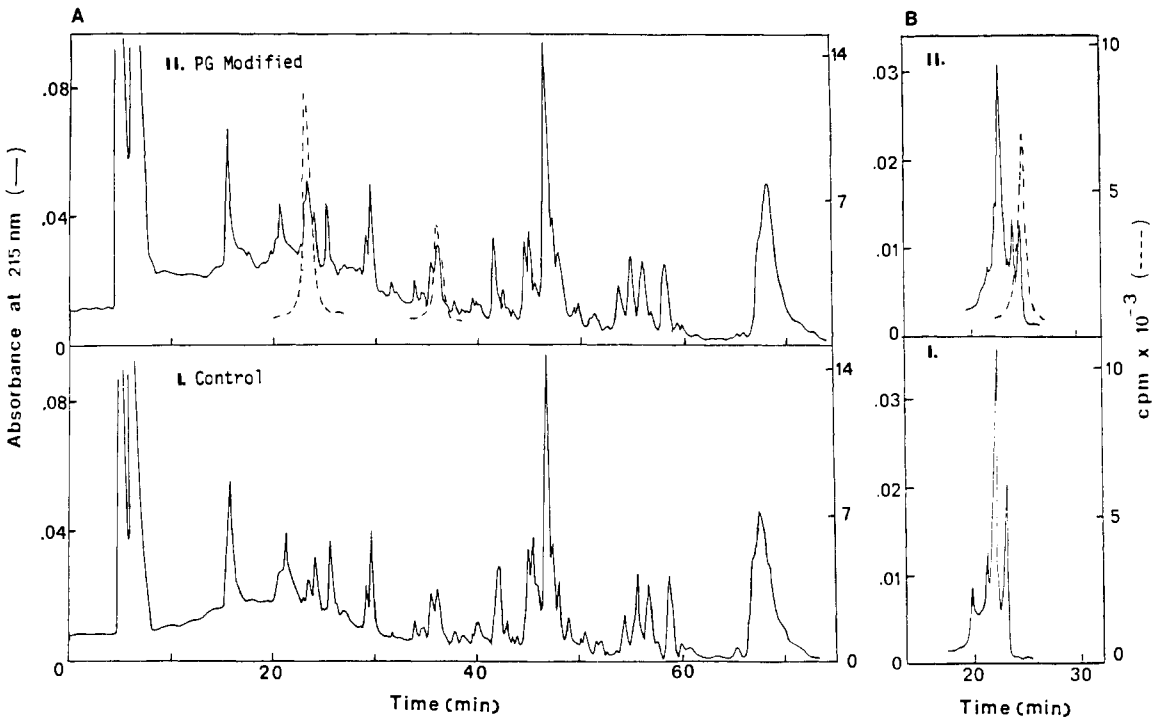

FIGURE 7: Reverse-phase HPLC of V-8 protease peptides from control and PG-inactivated DNA pol I (Klenow fragment). One nanomole of DNA pol I was treated with [^{14}C]PG, and the V-8 peptides were generated as described under Materials and Methods. (A) Comparative peptide maps of control and PG-modified enzyme. The peptides were eluted from a Vydac C-18 column by increasing the concentration of solvent B (70% acetontrile in 0.1% TFA) as follows: 0–40 min, 0–40% solvent B; 40–90 min, 40–60% solvent B. The peptides were monitored at 215 nm (—). A small aliquot from each fraction was used for monitoring the presence of radioactivity (---). (B) Purification of PG–peptide on a C-4 column. The fractions eluting between 22 and 25 min from control and PG-modified enzyme [see (A)] were pooled, lyophilized, and resolved on a Vydac C-4 column using the following gradient: 0–10 min, 0–10% solvent B; 10–90 min, 10–40% solvent B. Only the region containing peptides is shown.

Table III: Amino Acid Composition Analysis of V-8 Protease PG–Peptide[a]

amino acid residue	found	residues/mol of peptide expected from sequence 841–870[b]
Cys		0
Asp	2.7	3
Thr	1.8	1
Ser	1.4	0
Glu	1.7	3
Pro	0.9	1
Gly	1.2	1
Ala	8.8	9
Val	0.6	1
Met	1.6	2
Ile	3.7	4
Leu	1.2	1
Tyr		0
Phe		0
His	1.1	0
Lys	1.1	1
Arg	1.2[c]	2
Trp	ND[d]	1

[a] PG-labeled peptide obtained by V-8 protease digestion and further purification on a C-4 column (see Figure 7). [b] Represents amino acid residues in the primary sequence of *E. coli* DNA polymerase I (Joyce et al., 1982). [c] Absence of one arginine residue is attributed to the modification of that residue with PG (see Figure 6, panel 5). [d] Not determined.

scriptase. Thus, the specificity of PG toward the DNA binding domain in pol I was shown to be due to (a) loss of polymerase as well as nuclease function and (b) protection from PG inactivation by inclusion of template-primer but not by oligomeric DNA or substrate dNTPs. Furthermore, the degree of inactivation either in a dose–response study or as a function of incubation time with PG indicated a remarkable coincidence between the loss of polymerase and nuclease activities and the template binding activity (Figure 1). The stoichiometry of PG binding to Klenow enzyme also indicated a high degree of specificity since only 4 mol of PG was found to react per mole of enzyme, when complete and irreversible inactivation of the enzyme was apparent (Figure 2). All attempts to reactivate the PG-modified enzyme via dialysis against (i) Tris buffers of pH between 7 and 9, (ii) Tris buffer, pH 8, supplemented with either 20 mM dithiothreitol or 20 mM arginine, and (iii) 0.1 M ammonium bicarbonate were not successful. Paradoxically, the majority of [^{14}C]PG that was originally bound to the enzyme and stabilized by acidification was quickly released into the dialysis media when [^{14}C]PG-inactivated enzyme was subjected to any of the above conditions. Therefore, tryptic peptide analysis based on radiolabeling with [^{14}C]PG could not be performed. The use of pepsin in place of trypsin, as a choice of peptidase and to avoid the exposure of PG-treated enzyme protein to nonacidic conditions, resulted in multiple radioactivity-containing peaks. The purification of individual peptide clearly posed a serious problem. However, we had moderate success with the V-8 protease digestion approach, since nearly 50% of the [^{14}C]PG incorporated into the enzyme protein could be recovered in the peptide fractions. Examination of both V-8 and tryptic peptide patterns indicated the appearance of a new peak upon PG modification and its disappearance upon inclusion of template-primer during modification. We, therefore, purified both the tryptic and V-8 peptide peaks containing the PG modification site. The unusual property of the tryptic peptide was the total absence of absorption at 278 nm, indicating the loss of PG moieties from the arginine residue. Nevertheless, this arginine residue had undergone some structural modification since it is not cleaved by trypsin. The appearance of the new peptide seems to be quantitatively related to the degree of enzyme inactivation, and most importantly, the presence

of template-primer in the PG modification mixture completely eliminated the appearance of this peak. We therefore conclude that this peptide must contain the DNA binding domain of pol I and that a residue in this domain is the target of PG reaction. As detailed under Results, this peptide spans from amino acid residues 837–857 with arginine-841 as a reactive site for PG action. The amino acid composition analysis of V-8 peptide, which contained a major portion of the tryptic peptide, confirmed this region as a template-primer binding domain. The presence of radioactive PG within this peptide also confirmed the presence of modified arginine in this peptide. Since sequence analysis of the tryptic peptide has identified Arg-841 as a site of PG modification, we conclude that Arg-841 is also the target of PG modification in the V-8-derived PG peptide. Arg-858 which does not react with PG is clearly scored in composition analysis of this peptide (Table III). We have not been able to clarify the nature of arginine modification nor have we succeeded in identification of the second PG-reactive arginine residue which is a target of PG reactivity.

Arginine-841 that we have identified as an important residue in the template-primer binding function of pol I lies in the large C-terminal cleft and is a part of the Q helix, which is in the vicinity of tyrosine-766 and lysine-758, both of which are present on the O helix as well as His-881 present on B sheets 13 (Ollis et al., 1985). All of these residues have been implicated in the binding of deoxynucleoside triphosphate (Joyce et al., 1985; Basu & Modak, 1987; Pandey et al., 1987). Thus, the molecular proximity of substrate and template-primer binding sites seems well justified. Another interesting feature of Arg-841 is the presence of Glu as a preceding residue. In the elegant studies on the crystal structure of the *Eco*RI–DNA complex by Rosenberg and colleagues (McClarin et al., 1986), a similar feature at the DNA binding site was noted. Indeed, overall similarity in the local environment of the *Eco*RI–DNA complex and the pol I–DNA complex seems quite comparable. In the case of *Eco*RI, the amino-terminal ends in α-helices are proposed to face the same general direction of bound DNA, thus generating a favorable interaction between the helix dipoles and the electrostatic field generated by the phosphate backbone of DNA. In the crystal structure of pol I (Ollis et al., 1985), the amino-terminal ends of many α-helical structures, e.g., helices J, K, and L and helix Q containing arginine-841, appear to constitute a major portion of the DNA binding cleft. Thus, Arg-Glu combination at the end of the α-helix may be a general feature that DNA polymerase shares with DNA binding proteins, such as *Eco*RI. It is quite intriguing that both polymerase and nuclease activities of pol I are affected to a similar extent by PG treatment (Figure 1). In DNA pol I, two distinct catalytic domains have been implicated for the polymerase and the 3′–5′-nuclease activities (Que et al., 1978; Joyce et al., 1986). In the Klenow fragment of DNA pol I, polymerase activity has been assigned to the large C-terminal cleft of which arginine-841 is a part. The 3′–5′-nuclease activity is proposed to reside in the N-terminal domain where the deoxynucleoside monophosphate binding site exists (Tyr-497). However, it is not clear if the template-primer binding domain is shared in both polymerase and nuclease catalyses. Such a sharing of domains has been suggested in the case of reverse transcriptase and its associated RNase H activity (Modak & Marcus, 1977; Marcus et al., 1978; Modak & Srivastava, 1979). Thus, modification of Arg-841 may be responsible for the inhibition of DNA binding which is required for both polymerase and nuclease catalyses. Alternatively, a distinct PG-modified arginine that we have not identified may be present in the active-site region, which is required for the nuclease function of pol I. In conclusion, we have identified arginine-841 as an important residue required for the template-primer binding in *E. coli* DNA polymerase I.

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Purification and Characterization of Human Salivary Peroxidase†

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ABSTRACT: Human salivary peroxidase (SPO) has been purified to homogeneity by subjecting human parotid saliva to immunoaffinity, cation exchange, and affinity chromatography. These procedures resulted in a 992-fold purification of the enzyme. When purified SPO was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), three Coomassie stainable bands were apparent, all of which stained positive for enzyme activity. The apparent molecular weights of the three bands were 78 000, 80 000, and 280 000 as analyzed by SDS–PAGE. Reduction with 2-mercaptoethanol resulted in a decreased mobility of these bands, and enzyme activity could no longer be detected on the gels. The SPO preparation had the characteristic peroxidase heme spectrum in the range 405–420 nm. The ratio between the absorbance of the Soret band (412 nm) and the absorbance at 280 nm was 0.81. The enzyme activity was inhibited by the classical peroxidase inhibitors cyanide and azide. Salivary peroxidase is similar to bovine lactoperoxidase (LPO) in amino acid composition, in ultraviolet and visible spectrum, in reaction with cyanide, in susceptibility to 2-mercaptoethanol inactivation, and in thermal stability. The two enzymes differ in carbohydrate composition and content. SPO contains 4.6% and LPO 7% total neutral sugars. The ratio of glucosamine to galactosamine is 2:1 in SPO and 3:1 in LPO. SPO contains mannose, fucose, and galactose in a molar ratio of 1.5:1.5:1.0, while the ratio was 14.9:0.5:1.0 in LPO. Glucose was present in both preparations in minor amounts. The concentration of azide required for 50% inhibition of enzyme activity was 20-fold greater for LPO than for SPO. The specific activity of SPO is approximately 5 times higher than that of LPO when 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) is used as a substrate.

The peroxidase in human saliva is one of the nonimmunoglobulin defense factors found in mucosal secretions [for review, see Tenovuo and Pruitt (1984) and Mandel and Ellison (1985)]. The enzyme catalyzes the oxidation of the thiocyanate ion by hydrogen peroxide to generate oxidized forms of the thiocyanate ion (Thomas, 1985). Several studies have suggested that the peroxidation reaction serves at least two important functions in the human mouth: (i) The products of the reaction inhibit bacterial growth and metabolism [for review, see Pruitt and Reiter (1985)], and (ii) the reaction prevents the accumulation of hydrogen peroxide excreted by many strains of oral streptococci (Thomas et al., 1983; Carlsson et al., 1983) and by host cells (Pruitt et al., 1983). Hydrogen peroxide is highly toxic for mammalian cells, but it is consumed rapidly by the peroxidation reaction, the products of which are nontoxic (Hanstrom et al., 1983; White et al., 1983; Tenovuo & Larjava, 1984).

Both salivary peroxidase (SPO) and lactoperoxidase (LPO) have catalytic properties that are qualitatively similar to those of other mammalian peroxidases, e.g., human myeloperoxidase (Clark et al., 1975), cervical mucus peroxidase (Shindler et al., 1976), and eosinophil peroxidase (Wever et al., 1980). All of these enzymes catalyze the oxidation of halides and thiocyanate in the presence of hydrogen peroxide. However, myeloperoxidase and eosinophil peroxidase catalyze the oxidation of chloride, but LPO and SPO do not.

Although attempts have been made to isolate the peroxidase from human saliva, only partially purified preparations have been obtained, and only limited biochemical characterization has been reported (Morrison & Allen, 1963; Iwamoto & Matsumura, 1966; Slowey et al., 1968). The difficulty of purification is partially due to the fact that the enzyme, at a concentration of 1–10 μg/mL (Tenovuo, 1985), is a relatively minor component of saliva [total protein concentration = 2.8–3.2 mg/mL (Nikiforuk, 1985)]. Furthermore, there is probably low motivation for undertaking the isolation of SPO because of similarities in catalytic properties of SPO and the more readily available bovine LPO (Morrison & Steele, 1968).

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