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The Affinity Labeling of Amino Acids in or about the Active Center of DNA-Dependent DNA Polymerase I†

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ABSTRACT: The use of an affinity label and an inhibitor that shows relative specificity for one amino acid has led to the identification of two amino acid residues in or near the active center of DNA-dependent DNA polymerase I. [^{35}S]-β-D-Ribosyl-6-methylthiopurine periodate oxidation product ([^{35}S]MMPR-OP) and [^{14}C]phenylglyoxal ([^{14}C]PG) were used to elucidate the presence of a single lysine and arginine in or about the active center of the enzyme.

Since the earliest description by Kornberg et al. (1956a,b) and Bessman et al. (1957) of enzyme extracts from *Escherichia coli* that catalyzed the incorporation of deoxyribonucleotides into DNA, much work has been done to elucidate the structure and mechanism of action of the enzyme. These investigators, together with Richardson et al. (1964) and Jovin et al. (1969a), using further sophisticated purification techniques, demonstrated the requirements and structure of the Kornberg DNA polymerase I. In addition to polymerization, this enzyme possesses various other catalytic activities which have been described in excellent reviews by Kornberg (1969) and Goulian (1971). Recently, the possible mechanism of action of DNA polymerase I as a zinc metalloenzyme was reviewed (Mildvan, 1974). DNA polymerase I has recently been shown to function in DNA replication along with DNA polymerase II and III (Tait and Smith, 1974). However, even though the catalytic activities and functions are known, the exact enzymatic mechanisms involved in mediating catalysis are still open to investigation. To that eventual end, this communication describes the identification of two amino acid residues found in the active center of the Kornberg enzyme which demonstrate a role primarily in polymerization.

The role of lysine was determined by using an affinity label, the oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP).[1] This compound was found by Kimball et al. (1968) to be an inhibitor of DNA polymerase of the Ehrlich ascites tumor. This was one of the earlier works utilizing a compound that alters the catalytic mechanism of DNA polymerase by binding to specific sites on the enzyme. Spoor et al. (1970), using cell-free extracts of this tumor line, also found this compound to inhibit the activity of RNA polymerase. In addition, they showed that MMPR-OP would inhibit and affinity label RNase A (Spoor et al., 1973). Nixon et al. (1972) also found that the compound would affinity label the β subunit of RNA polymerase. Specifically, it was shown that there was the formation of a Schiff base between one of the aldehyde moieties of MMPR-OP and the ϵ-amino group of a catalytically active lysine residue in the initiation subsite of the enzyme. Wu and Wu (1974) have modified the MMPR-OP to produce a fluorescent compound that also affinity labels the β subunit of RNA polymerase. Since the above enzymes are enzymes of nucleic acid metabolism, this possible effect of MMPR-OP on DNA polymerase I was used to determine if the same affinity labeling action would apply to this enzyme.

Takahashi (1968) was able to inactivate RNase A some 80–90% with phenylglyoxal (PG) under mild conditions; pH 7–8 and 25 °C. The mechanism of inhibition involved the condensation of two phenylglyoxal molecules with the guanidino group of arginine. The arginine residues of RNase A affected were Arg-39 and Arg-85 which were shown to be those closest to the active center. In addition, experimental conditions were employed so that phenylglyoxal was specific for arginine residues. These properties of the compound led to its use for the investigation of a possible catalytic role for arginine residues in DNA polymerase I.

The evidence obtained from Lineweaver–Burk and Hill plots in conjunction with binding and affinity-label studies showed the presence of single reactive lysine and arginine residues in or about the polymerizing active center of DNA polymerase I.

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[1] Abbreviations used are: MMPR-OP, β-D-ribosyl-6-methylthiopurine oxidation product; PG, phenylglyoxal; NCMIA, *N*-carboxymethylisatoic anhydride.

Materials and Methods

Isolation and Purification of DNA Polymerase. DNA-dependent DNA polymerase was isolated and purified to homogeneity from late-log phase *E. coli B* (Calbiochem; General Biochemicals) by the method of Jovin et al. (1969).

DNA Polymerase Assay. The routine assay reaction, in a 0.50-ml volume, consisted of 50 μmol of potassium phosphate buffer (pH 7.6), 100 μg of denatured highly polymerized calf thymus DNA (Sigma), 25 nmol each of dGTP, [5-^{3}H]TTP, dATP, dCTP, 12.5 μmol of $MgCl_2$, and 0.018–0.025 μg of enzyme. The assay mixtures were then incubated for 25 min at 37°C. The reactions were terminated by cooling to 0 °C in an ice–water bath. This was followed by the addition of 1.0 mg of bovine serum albumin (Schwarz/Mann) as carrier protein plus 4.0 ml of trichloroacetic acid. After centrifugation, the precipitates were washed three additional times with Cl_3CCOOH. They were then solubilized by the addition of a toluene scintillator. One unit of enzyme is defined as that amount of enzyme that incorporates 1 mol of [^{3}H]TTP in an acid-insoluble product under these assay conditions. The variations in these assay techniques are described in the legends.

Synthesis of MMPR-OP. The periodate oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP) and its sulfur-35 derivative were synthesized according to the method described by Nixon et al. (1972). The initial specific activity of the radiopure compound was 7.9×10^{12} cpm/mol.

Synthesis of [^{14}C]Phenylglyoxal Hydrate. [^{14}C]Phenylglyoxal ([^{14}C]PG) hydrate was prepared by the oxidation of [^{14}C]acetophenone (ICN) with selenious acid (J. T. Baker) according to the method of Riley and Gray (1943) used originally to prepare the nonradioactive compound. The specific activity of the pure [^{14}C]PG was 2.4×10^{12} cpm/mol.

Binding of [^{35}S]MMPR-OP to DNA Polymerase I. The reaction mixture contained in 0.02 M potassium phosphate buffer (pH 7.6), 10^{-2} μmol of enzyme, and 1 μmol of [^{35}S]MMPR-OP in a volume of 7.0 ml. It was incubated for 45 min at 37 °C and then cooled to 0 °C in an ice–water bath. This was followed by the addition of 20 mg of $NaBH_4$ per ml of reaction mixture and the reduction was allowed to proceed for 21 h at 4 °C. Excess $NaBH_4$ and unbound [^{35}S]MMPR-OP were removed by dialysis for 48 h in 0.02 M potassium phosphate (pH 7.6) with four buffer changes. This was followed by another dialysis in distilled water for 12 h. After dialysis, the sample was concentrated to one-half its volume under a thin stream of nitrogen. An aliquot was removed and its radioactivity measured in order to determine the amount of [^{35}S]MMPR-OP bound to the enzyme by comparison with analogously treated control reactions not containing enzyme.

The amino acid binding site of [^{35}S]MMPR-OP was determined by treating 0.5 mg of the DNA polymerase/[^{35}S]MMPR-OP derivative with 100 μg of Pronase (Calbiochem) in 2.0 ml of distilled water. (The pH of the water was adjusted to 7.6 with 0.1 N NaOH.) The proteolytic products were concentrated further under a stream of nitrogen gas and analyzed on silica gel N-HR (Brinkman) thin-layer chromatography plates in two solvent systems. (See Table II for solvent systems, components, and R_f's.) Free amino acids which were analogously reacted with [^{35}S]MMPR-OP, reduced with $NaBH_4$, but not dialyzed, were chromatographed and served as standards. The chromatograms were cut into 1 × 2 cm strips and radioactivity was determined by scintillation counting after adding 10 ml of a toluene-based fluor.

Binding of [^{14}C]Phenylglyoxal Hydrate to DNA Polymerase. The technique employed was a modification of Takahashi's (1968) method of binding [^{14}C]PG to ribonuclease A. In a volume of 7.0 ml, the reaction mixture contained 0.02 M potassium phosphate buffer (pH 7.6), 10^{-2} μmol of enzyme, and 7 μmol of [^{14}C]PG. The reaction mixture was incubated at 25 °C for 60 min. After incubation, the DNA polymerase/[^{14}C]PG complex formed was stabilized by immediately decreasing the pH of the reaction mixture to pH 2.0 by the addition of 2 N HCl. Unbound [^{14}C]PG was eliminated by a 48-h dialysis against distilled water, pH 2.2 (adjusted with 2 N HCl), with four water changes. After dialysis the sample was concentrated under a thin stream of nitrogen gas to one-third its original volume. An aliquot was removed to determine the amount of [^{14}C]PG bound to the enzyme by comparison with the analogously treated control reactions. The site of [^{14}C]PG binding to the enzyme was determined by moderate hydrolysis. A sample of the complex, still in an acidic medium, was placed in a boiling water bath for 1.5 h. The hydrolytic products were concentrated further to one-sixth the original volume under a thin stream of nitrogen gas. Thin-layer chromatographic techniques similar to those described for the [^{35}S]MMPR-OP binding studies were employed to analyze the hydrolytic products. (See Table II for solvent system, components, and R_f's.)

Chromatographic analysis of the products formed by the mild hydrolysis above described method showed minimum disassociation. In addition, hydrolysis of the enzyme into individual amino acids although incomplete, was adequate for our analysis. However, acid hydrolysis of the complex, using 6 N HCl at 100–110 °C for 24 h, produced a marked disassociation of the [^{14}C]PG from the enzyme.

Miscellaneous. Protein determinations were done according to the method of Lowry et al. (1951). All slopes and intercepts used in evaluating data were calculated on an Olivetti programmable 101 calculator utilizing a least-squares program.

Results

Effect of MMPR-OP on DNA Polymerase Activity. A Lineweaver–Burk (1934) plot of total DNA synthesis showed that MMPR-OP ($K_i = 1.64 \times 10^{-3}$ M) gave noncompetitive inhibition. The formation of the Schiff base is reversible and only becomes a stable covalent bond upon reduction. This allowed the determination of inhibition kinetics. The noncompetitive inhibition suggests that MMPR-OP binds at a site other than the triphosphate binding site. The active center of this enzyme is considered as having a multisite active center. In this regard, since the effect of MMPR-OP occurs at a site other than the triphosphate binding site, then the possibility exists that it may bind in the 3′-OH terminus subsite and be involved in the formation of the phosphodiester bond. Alternatively, MMPR-OP could have bound nonspecifically to lysine residues on the enzyme causing it to undergo a conformational change and, thereby, exhibiting inhibitory effects. Evidence lending support to the former case was demonstrated by employing Hill plots. Loftfield and Eigner (1969) have derived expressions from the Hill equation where the slope obtained from the logarithmic plots determines the number of bound inhibitor molecules and, consequently, the number of specific reactive amino acid residues in or near the active center. A Hill

Table I: Binding Ratios of [^{35}S]MMPR-OP and [^{14}C]PG to DNA Polymerase I.

Binding Compound	Specific Activity (cpm/nmol)	μg of Enzyme	nmoles of Enzyme	cpm Bound	nmoles of Label Bound	nmoles of Label/ nmoles of Enzyme
[^{35}S]MMPR-OP	7740	24.5	0.224	1750	0.226	1.01
[^{14}C]PG	2380	26.5	0.244	1100	0.461	1

[a] [^{35}S]MMPR-OP/DNA polymerase and [^{14}C]phenylglyoxal/DNA polymerase binding ratios. The binding reactions were performed as described in Materials and Methods. The data are the analysis of 0.1-ml aliquots of the radiolabel/DNA polymerase complex taken from each binding experiment.

Table II: R_f Values of Enzyme–Inhibitor Complexes.

Solvent	Component	R_f Values
Ethanol–ammonium hydroxide (7:3 v/v)	[^{35}S]MMPR-OP	0.510
	Lysine	0.170
	Lysine/[^{35}S]MMPR-OP (Pronase)	
	a. Nonbound	0.170
	b. Bound radiolabel	0.586
	Enzyme/[^{35}S]MMPR-OP (Pronase) 8th ninhydrin spot radiolabel	0.576
	[^{14}C]Phenylglyoxal hydrate ([^{14}C]PG)	0.645
	Arginine	0.131
	Arginine/[^{14}C]PG (hydrolyzed)	
	a. Nonbound	0.143
	b. Bound radiolabel	0.496
	Enzyme/[^{14}C]PG (hydrolyzed) 6th ninhydrin spot radiolabel	0.504
2-Propanol–ammonium hydroxide (7:3 v/v)	[^{35}S]MMPR-OP	0.500
	Lysine	0.150
	Lysine/[^{35}S]MMPR-OP (Pronase)	
	a. Nonbound	0.150
	b. Bound radiolabel	0.406
	Enzyme/[^{35}S]MMPR-OP (Pronase) 7th ninhydrin spot radiolabel	0.403
Butanol–acetic acid–water–hydrochloric acid (160:40:40:1 v/v)	[^{14}C]Phenylglyoxal hydrate	0.647
	Arginine	0.177
	Arginine/[^{14}C]PG (hydrolyzed)	
	a. Nonbound	0.201
	b. Bound radiolabel	0.422
	Enzyme/[^{14}C]PG (hydrolyzed) radiolabel found between the 5th and 6th visible ninhydrin spots	0.422

[a] R_f values of [^{35}S]MMPR-OP and [^{14}C]phenylglyoxal binding products. The enzyme–[^{35}S]MMPR-OP complex was treated with Pronase for 72 h at 35 °C and spotted on 0.2-mm Silica Gel N-HR thin-layer chromatographic sheets (Brinkman). The controls consisted of free [^{35}S]MMPR-OP, free lysine, and Pronase-treated lysine/[^{35}S]MMPR-OP analogously prepared as the enzyme reaction. The chromatograms were developed with 0.5% ninhydrin and 1 × 2 cm strips were cut and counted as described in Materials and Methods. The enzyme–[^{14}C]PG was hydrolyzed and described in Materials and Methods and spotted on 0.2-mm Silica Gel N-HR thin-layer chromatographic sheets (Brinkman). The controls consisted of free [^{14}C]PG, free arginine, and hydrolyzed arginine/[^{14}C]PG analogously prepared as the enzyme reaction. The chromatograms were developed with 0.5% ninhydrin and cut into 1 × 2 cm strips, and the radioactivity was counted.

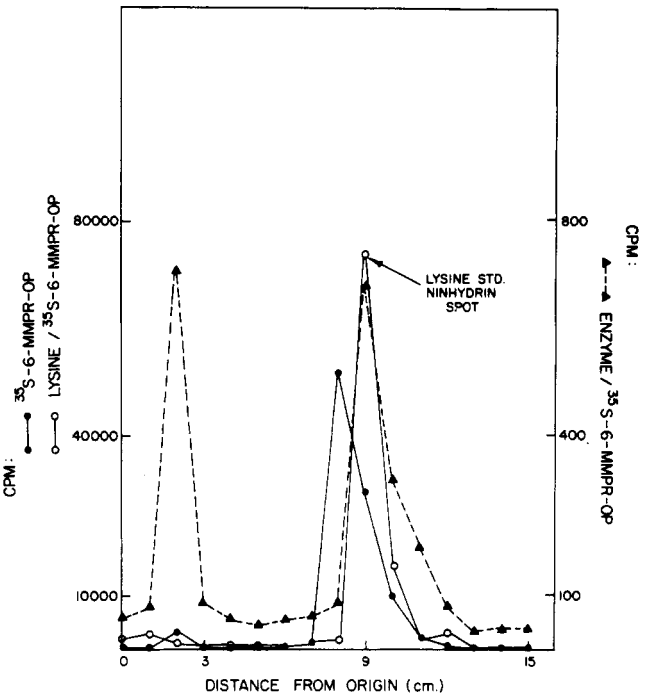

FIGURE 1: The radiochromatographic profile of the Pronase-treated DNA polymerase/[^{35}S]MMPR-OP complex in an ethanol–ammonium hydroxide (7:3 v/v) solvent system. The treatment of the enzyme and the controls are described in the legend to Table II. (●) Free [^{35}S]MMPR-OP, R_f value of 0.510; (○) Pronase-treated lysine/[^{35}S]MMPR-OP, R_f value of 0.586; (▲) Pronase-treated DNA polymerase/[^{35}S]MMPR-OP R_f value of 0.576.

plot of MMPR-OP-treated enzyme gave a slope of 1.16. Since this value approximated unity, this shows that the vicinity of the active center is affected and that there is only one molecule of MMPR-OP binding in the active center at a specific site. Since only one molecule of MMPR-OP combines with one lysine molecule, then the Hill plot data give presumptive evidence for only one lysine residue in or about the active center of DNA polymerase.

[^{35}S]MMPR-OP Binding Studies. Supportive evidence for the conclusion derived from the Hill plot data was obtained by using affinity labeling techniques. When [^{35}S]MMPR-OP was used as the affinity label, it was observed that this compound could be covalently bound to DNA polymerase. Binding experiments (Table I) showed that 1.01 mol of [^{35}S]MMPR-OP was bound per mol of enzyme. This value is in close agreement with the slope of 1.16 obtained from the Hill plot data. Once it was established that MMPR-OP could be covalently bound to the enzyme on an equimolar basis, it was still uncertain as to whether it was bound to lysine or to some other amino acid residue. To resolve this question, Pronase digests of the enzyme/[^{35}S]MMPR-OP complex were chromatographed. The R_f values of the results obtained in different solvent systems are summarized in Table II. The radioactivity of the enzyme/[^{35}S]MMPR-OP Pronase degradation coincided with the Pronase-treated lysine/[^{35}S]MMPR-OP reaction. In the ethanol–ammonium hydroxide system (Figure 1), the R_f value of 0.586 for lysine/[^{35}S]MMPR-OP coincided with the R_f value of 0.576 of the seventh ninhydrin visible spot. It was this ninhydrin spot that had the peak of radioactivity. Analogously, in the 2-propanol–ammonium hydroxide system, the respective R_f values were 0.406 and

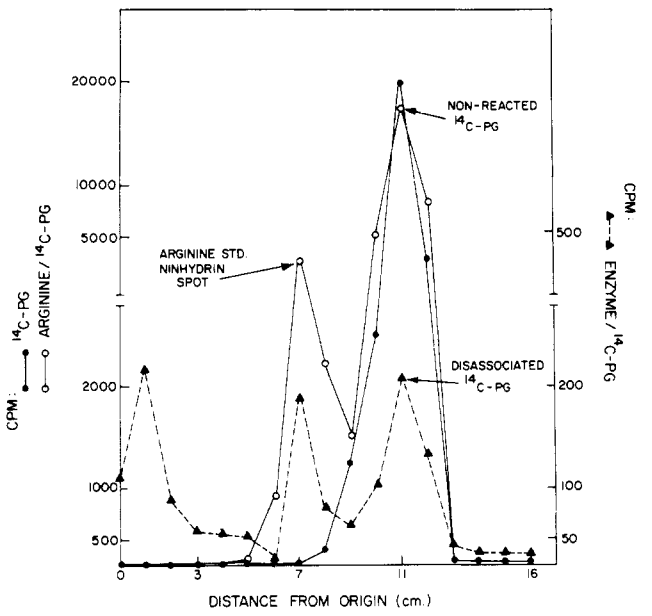

FIGURE 2: The radiochromatographic profile of the hydrolyzed DNA polymerase/[^{14}C]phenylglyoxal complex in an 1-butanol–acetic acid–water–hydrochloric acid (160:40:40:40:1 v/v) solvent system. The treatment of the enzyme/[^{14}C]PG complex and the controls are described in the legend to Table II. (●) Free [^{14}C]PG, R_f value of 0.647; (○) arginine/[^{14}C]PG, R_f value of 0.422; (▲) DNA polymerase/[^{14}C]PG complex, R_f value of 0.422.

0.403. This corresponded to the ninhydrin visible spot which also contained the peak of radioactivity. The large peak evident near the origin in the enzyme/[^{35}S]MMPR-OP profile represents a portion of undigested enzyme. Furthermore, when these proteolytic products were analyzed by two-dimensional chromatographic techniques, the radioactive peak in the enzyme/[^{35}S]MMPR-OP reaction was in a ninhydrin spot that coincided with that produced by the lysine/[^{35}S]MMPR-OP control reaction.

The results of these binding studies, together with the data previously presented, indicate that MMPR-OP produces inhibition of DNA polymerase activity by binding to a single lysine residue in or about the active center of the enzyme.

Effect of Phenylglyoxal Monohydrate on DNA Polymerase Activity. A Lineweaver–Burk plot utilizing PG (K_i = 1.51 × 10^{-3} M) showed mixed kinetics. This suggests that PG binds close to but not directly in the triphosphate binding subsite.

Again, in a manner analogous to that described above for MMPR-OP, inhibition kinetics were possible since it requires an acidic pH to stabilize the binding of PG.

The number of arginine residues that are in or about the active center of the enzyme was determined by a Hill plot which gave a slope of 1.33 and which may be interpreted as one inhibitor molecule reacting with one arginine residue. Since two molecules of PG are known to react with one arginine residue (Takahashi, 1968), then the possibility exists that the product of PG condensation is being recognized as one inhibitor molecule. This would explain the Hill plot data. This premise was shown to be valid by reacting radiolabeled PG with DNA polymerase I. It was found that two [^{14}C]PG moieties were bound to the enzyme. The results of this binding study are found in the following section.

[^{14}C]Phenylglyoxal Hydrate Binding Studies. The binding of [^{14}C]phenylglyoxal hydrate to DNA polymerase showed that 1.89 (Table I), or approximately 2 mol of this compound was bound per mol of enzyme. The Hill plot data showing approximately a 1:1 molar binding ratio may now be explained by the condensation of two PG molecules to one arginine residue which acts as a single inhibitor molecule. However, the fact that PG was bound to the enzyme did not necessarily mean that it was bound to arginine residues in our case. The use of both one- and two-dimensional chromatographic analyses of hydrolytic products of enzyme/[^{14}C]PG complexes resulted in the isolation of arginine/[^{14}C]PG derivatives.

An analogous treatment of lysine was examined chromatographically since Takahashi (1968) reported that the ϵ-amino group of lysine could combine with a large excess of PG when reacted for a long period of time. This was of special interest to us since any PG reaction with this amino acid could yield misleading data. Chromatographic analysis of this reaction showed no lysine–[^{14}C]PG interactions. The radiochromatographic profiles of the hydrolytic products using different solvent systems are summarized in Table II. The data show that in both chromatograms, the radioactivity in the hydrolyzed DNA polymerase/[^{14}C]PG samples coincide with the analogously treated arginine/[^{14}C]PG standard. In the 1-butanol–acetic acid–water–hydrochloric acid (160:40:40:1 v/v) chromatogram (Figure 2), the R_f value for both reactions was 0.422. In the ethanol–ammonium hydroxide (140:60 v/v) chromatogram the R_f values were 0.496 and 0.504, respectively.

There was a radioactive peak observed at the origin in the enzyme/[^{14}C]PG reaction in both chromatograms which represents unhydrolyzed DNA polymerase that did not migrate in the solvent systems used. In addition, there is a peak of radioactivity, which may represent a partial disassociation of [^{14}C]PG from the enzyme, that probably occurred during hydrolysis. There were no ninhydrin spots associated with these radioactive peaks.

When the hydrolytic products were analyzed by two-dimensional chromatographic techniques, the [^{14}C]PG radiolabel of the enzyme/[^{14}C]PG reaction coincided with that found in the arginine/[^{14}C]PG control reaction.

The results of these binding studies support the premise that PG produces inhibition of DNA polymerase activity by binding to a single arginine residue in or about the active center of the enzyme.

Discussion

The use of inhibitors that show specificity for only one amino acid has led to the identification of amino acid residues in or about the active center of DNA-dependent DNA polymerase. It was found that the enzyme contains a single lysine and arginine residue in or near the active center of the enzyme. The conclusion that these residues lie in this area was made through the use of Hill plots and binding studies. Since active center amino acid residues tend to be more reactive than other identical amino acids (Vallee and Riordan, 1969) on the enzyme, it may be assumed that a lysine and an arginine constitute part of the active center, or else that they are positioned close to the active center.

The possible catalytic role of lysine residues in DNA polymerase has also been shown by Jovin et al. (1969b). By using *N*-carboxymethylisatoic anhydride (NCMIA), these workers concluded that the compound reacted with lysine residues resulting in an alteration of the triphosphate site. When the role of lysine was investigated here with MMPR-OP, the results showed that this compound inhibit-

ed in a noncompetitive manner. NCMIA reacted with 11 different sites on the enzyme, while the reaction with MMPR-OP was specific for one sensitive lysine. It is likely that NCMIA produced inhibition by nonspecific reactions. In the presence of Mg^{2+}, DNA, and deoxynucleoside 5′-triphosphates, DNA polymerase incorporates nucleotides into a DNA polymer. According to the Kornberg (1969) model for the active center of DNA polymerase, this catalytic process involves the interaction of various sites within the active center of the enzyme. There is a template site which binds to the DNA and involves base pairing. The DNA strand that acts as a primer for the extension of the DNA chain binds at a primer site. The enzyme is able to recognize 3′-OH terminal groups of the primer at the primer terminus site, for the formation of the phosphodiester bond. Lastly there is a triphosphate binding site which recognizes (or rejects) the incoming triphosphate as the correct complementary base. We (Spoor et al., 1970; Nixon et al., 1972) and Krakow and Fronk (1969) have proposed a similar model for DNA-dependent RNA polymerase. Based on our affinity labeling study of RNA polymerase (Nixon et al., 1972), we placed a lysine in the initiation subsite on the β subunit of the enzyme—a result later confirmed by Wu and Wu (1974). The research presented here indicates that a similar situation may possibly obtain for DNA polymerase I except that the initiation subsite on RNA polymerase might be translated here to read the 3′-OH primer terminus subsite of DNA polymerase I. The role of the arginine in or near the active center of DNA polymerase I is obscure at the moment since our study shows it to be neither directly in the triphosphate or 3′-OH terminus subsites. Three possibilities present themselves; namely, (1) that the arginine is involved in base recognition; (2) that it is concerned directly in some fashion in bond making or breaking; or (3) that it is involved in template binding.

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