Mutational Studies of Human DNA Polymerase α

LYSINE 950 IN THE THIRD MOST CONSERVED REGION OF α-LIKE DNA POLYMERASES IS INVOLVED IN BINDING THE DEOXYNUCLEOSIDE TRIPHOSPHATE*

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The function of a lysine residue, Lys950, of human DNA polymerase α located in the third most conserved region and conserved in all of the α-like polymerases was analyzed by site-directed mutagenesis. Lys950 was mutated to Arg, Ala, or Asn. The mutant enzymes were expressed in insect cells infected with recombinant baculoviruses and purified to near homogeneity. The mutant enzymes had variable activities ranging from 8 to 22% of the wild type. All three Lys950 mutants utilized Mn2⁺ as a metal activator more effectively than the wild type enzyme and showed an increase in Kₘ values for deoxyribonucleoside triphosphate but not kₗ values in reactions with either Mg²⁺ or Mn²⁺ as the metal activator. Although mutation of the Lys950 residue caused an increase in Kₘ values for deoxyribonucleoside triphosphates, mutations of Lys950 to Arg, Ala, or Asn did not alter the mutant enzymes' misinsertion efficiency in reactions with Mg²⁺ as a metal activator as compared with that of the wild type, suggesting that the base of the incoming deoxyribonucleoside triphosphate is not the structural feature interacting with the Lys950 side chain. In reaction with Mn²⁺ as a metal activator, all three Lys950 mutants had an improved fidelity for deoxyribonucleotide misinsertion compared to wild type. Inhibition studies of the three Lys950 mutant derivatives with an inhibitor, structural analogs of deoxyribonucleoside triphosphate, and pyrophosphate suggest that the deoxyribose sugar and β,γ-phosphate groups are not the structural feature recognized by the Lys950 side chain. Comparison of the mutant enzymes to the wild type enzyme for their affinities for dCTPₐS versus deoxyribonucleoside triphosphate suggests that this highly conserved Lys950 is involved in interacting either directly or indirectly with the oxygen moiety of the α-phosphate of the incoming deoxyribonucleoside triphosphate.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Ultrapure deoxyribonucleotides and poly(dA) and dId(pT) were purchased from Pharmacia LKB Biotechnology Inc. dCTP and dCTP-5' in S$_2$, diastereomer form and the (β-35S)dpCTP-s were from Amersham Corp. Aphidicolin and phosphonoacetic acid were obtained from Sigma. Butylyphenyl-dGTP (BuPdGTP), butylyphenyl-dGMPCH$_2$PP (BuPdGMPCHP), carboxyribonucleosides were gifts from G. Wright (University of Massachusetts, Boston). All other chemicals were from commercial sources and were analytical grade. Oligonucleotides for restriction enzyme analysis and DNA sequencing.

**Methods**

Site-directed Mutagenesis—The cloning and in vitro mutagenesis strategy was as described (9). Briefly, a 1.44-kilobase Sall-BamHI fragment of the human polymerase α DNA containing the conserved regions (13) was cloned into the Sall-BamHI site of M13mp19 for site-directed mutagenesis in vitro (9). The mutations were verified by restriction enzyme analysis and DNA sequencing.

Construction of Recombinant Baculovirus—The strategy for construction of transfer vector was performed as described in Ref. 9. Briefly, the Sall-BamHI fragments containing the site-directed mutation was ligated to EcoRI/BamHI sites of pVL1393, to generate the transfer vector pVL1393/SDM containing the site-directed mutation in the polymerase α DNA.

Recombinant baculoviruses were generated by co-transfection of Sf9 cells with the transfer plasmids and linear baculovirus DNAs as described (9). Five micrograms of transfer plasmid DNA were co-transfected with 1 μg of linear AcTGal viral DNA, and the recombinant viruses were selected using standard baculovirus techniques.

Expression and Purification of Mutants—The expression and harvest of recombinant baculoviruses expressing the mutant polymerase α were performed as described (9). The recombinant DNA polymerase α proteins were purified to near homogeneity from Sf9 insect cell lysates by the one-step immunopurification protocol with monoclonal antibody SJK237–71 cross-linked to Sepharose 4B as described (9).

**RESULTS**

**Sitedirected Mutation of a Highly Conserved Lysine Residue**

A lysine residue located in the third most conserved regions of the α-like DNA polymerases is invariably present in all three major mammalian cellular DNA polymerases α, β, and ε, in yeast POLI, -II, and -III, in several DNA virus polymerases, in E. coli polI, in polymerases of bacteriophage T4, PRD1, and δ29, and in polymerase-like proteins such as PGK1L1 and mitochondria S1 (Fig. 1). In this study, we used human DNA polymerase α as the model for all of the three cellular polymerases to analyze the functional role of this highly conserved lysine residue. By site-directed mutagenesis, we changed Lys$^{962}$ to Arg, thereby replacing the side chain with a larger positively charged side chain, Lys$^{962}$ to Ala, thereby completely abolishing the positively charged side chain, and Lys$^{962}$ to Asn, thereby replacing the ε-NH$_3$ group of the positively charged lysine side chain with a polar amide group. The three mutant polymerases were produced in recombinant baculovirus-infected insect cells and purified with the one-step immunopurification protocol to near homogeneity in high yield (7). Analysis of these three mutant proteins produced from recombinant baculovirus-infected insect cells showed that all three had a predominant protein of 180 kDa like the wild type enzyme with minor species of 165–140 kDa (data not shown). Furthermore, these three mutant proteins like the mutant proteins reported by us before had no detectable global structural alterations (4, 5, 9). The specific activities of each mutant DNA polymerase were measured by using optimally gapped calf thymus DNA as primer-template and with either Mg$^{2+}$ or Mn$^{2+}$ as metal activator. In reactions with Mg$^{2+}$ as the metal activator, mutant K950A had 22% of the specific activity of the wild type enzyme, while mutants K950R and K950N had 7.6% and 8% of the wild type specific activity, respectively. In reactions with Mn$^{2+}$ as metal ion, mutant K950R had 42% of the wild type specific activity, mutant K950A had 70% of the wild type specific activity, while mutant K95ON had specific activity identical with, if not slightly higher than, the wild type enzyme (Table I).

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1 The abbreviations used are: dCTP$\beta$S, deoxyctytidine 5'-O-(α-thiophosphosphate); BuPdGMPCHP, N$\beta$-[(p-butylyphenyl)-2'-deoxyguanosine 5'-[(p$\beta$)-2'-methylene]-triophosphate.)
Kinetic Parameters of the Lys950 Mutants

Kinetic parameters of these three mutants in reactions with either Mg\(^{2+}\) or Mn\(^{2+}\) as the metal activator were measured and compared to the wild type (Table I). We found that mutations of Lys\(^{950}\) to Arg, Ala, or Asn had a profound effect on the Km values of dNTPs in reactions with Mg\(^{2+}\) as the metal activator. Mutants K950R, K950A, and K950N had 8-, 4-, and 6-fold increases in their Km values compared to the wild type are due to the combination of larger size charged side chain or is completely abolished. Inhibition by single-stranded DNA was compared, and no apparent difference was found between wild type and all three mutant enzymes (data not shown). This result indicates that this lysine residue is not involved in template interaction.

Results of these kinetic parameter studies suggest that mutation of this highly conserved Lys\(^{950}\) residue primarily affects the affinity for the incoming dNTP substrate and does not affect catalysis.

Effect of Metal Activator

The observed differences in these mutant enzymes' kinetic parameters in reactions with Mg\(^{2+}\) from that of Mn\(^{2+}\) led us to investigate these mutant enzymes' preference of metal activator. The optimal concentrations of each metal activator for the wild type and the three mutant enzymes were compared and are shown in Fig. 2. Like what we observed before (9), the wild type enzyme prefers to utilize Mg\(^{2+}\) as the metal activator with an optimal concentration at 10 mM and utilizes Mn\(^{2+}\) as metal activator poorly with an optimal concentration at approximately 0.5 mM. In contrast, all three Lys\(^{950}\) mutant enzymes were able to utilize Mn\(^{2+}\) as metal activator more effectively than the wild type enzyme (Fig. 2). Mutant enzymes K950R and K950N had similar optimal concentrations for Mg\(^{2+}\) and
Lys\textsuperscript{950} Side Chain Has a Role in the Active Site and Is Involved in Interacting with dNTPs

Our finding of mutations of Lys\textsuperscript{950} to Arg, Ala, or Asn affecting apparent \(K_m\) values for dNTP in reactions with either Mg\textsuperscript{2+} or Mn\textsuperscript{2+}, as the metal activator suggests that the side chain of Lys\textsuperscript{950} may have a role in active site interacting with the incoming dNTP substrate. We, thus, used an inhibitor and several dNTP structural analogs to verify this notion.

We first tested the effect of an inhibitor, aphidicolin, on these three Lys\textsuperscript{950} mutant enzymes. Aphidicolin is a general inhibitor for all three major cellular \(\alpha\)-like DNA polymerases, \(\alpha\), \(\beta\), and \(\epsilon\) (3). Aphidicolin acts as a competitive inhibitor of pyrimidine deoxyribonucleoside triphosphate, but, structurally, aphidicolin is not an analog of dNTPs. We have previously proposed a model of how aphidicolin forms hydrogen bonds with the purine base of the nucleotide in the template in the active site of \(\alpha\)-like DNA polymerases (5). To test if Lys\textsuperscript{950} plays a role in the active site in interacting with metal activator(s) or the dNTP-metal activator complex, we tested the inhibitory effect of aphidicolin on the three mutant derivatives of Lys\textsuperscript{950} and compared their 50% inhibition point to that of the wild type reactions with Mg\textsuperscript{2+} as metal activator. The three Lys\textsuperscript{950} mutant enzymes, K950R, K950A, and K950N, were 10, 14, and 33 times more sensitive to aphidicolin inhibition than the wild type enzyme, respectively (Table III). These results suggest that Lys\textsuperscript{950} functions in the active site and aphidicolin affects the interaction between the Lys\textsuperscript{950} side chain and the dNTP substrate.

We next compared the three Lys\textsuperscript{950} mutant derivatives to wild type enzyme for their 50% inhibition points by an analog of dGTP, BuPdGTP, and its \(\alpha\),\(\beta\)-methylene derivative, BuPdGMPCH\(_2\)PP (Table III). All three Lys\textsuperscript{950} mutant derivatives showed higher sensitivity to both of these compounds than the wild type enzyme. Mutant enzyme K950R had about 3 times higher sensitivity to both BuPdGTP and BuPdGMPCH\(_2\)PP. Mutant enzyme K950A and mutant enzyme K950N both showed much higher sensitivity to the inhibition by BuPdGTP and BuPdGMPCH\(_2\)PP than did the K950R (Table III). These indicate that the Lys\textsuperscript{950} side chain indeed has a role in the active site and is involved in interacting with the incoming dNTP.

Results of the kinetic and inhibitor studies suggest that the positively charged side chain of Lys\textsuperscript{950} has a function in the active site and is involved in interacting with the dNTP-metal activator complex.

Structure Feature of dNTP Recognized by Lys\textsuperscript{950}

We next investigated what structural feature of dNTP interacts with the Lys\textsuperscript{950} side chain. The finding that all three Lys\textsuperscript{950} mutant derivatives have misinsertion fidelity efficiency identical with the wild type enzyme in reactions with Mg\textsuperscript{2+} as the metal activator (Table II) rules out the possible interaction of Lys\textsuperscript{950} with the nucleoside base moiety of the dNTP substrate. The abilities of all three Lys\textsuperscript{950} mutant enzymes to use Mn\textsuperscript{2+} as metal activator effectively suggest that the structural feature of dNTPs interacting with the Lys\textsuperscript{950} side chain are either the deoxyribose sugar or the phosphate groups. We therefore systematically tested the interaction between these structural features of dNTP and the Lys\textsuperscript{950} side chain.

Does the Lys\textsuperscript{950} Side Chain Recognize the Deoxyribose Sugar of the Incoming dNTP Substrate?—Two dCTP analogs with modifications in the deoxyribose sugar moiety, araCTP and ddCTP, were used to test their 50% inhibitory point (IC\textsubscript{50}) on the three Lys\textsuperscript{950} mutant enzymes and compared to the wild

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Metal titration assays for the wild type and mutant enzymes. Reactions were carried out as standard DNA synthesis reactions with varying concentrations of MgCl\(_2\) (C—C) or MnCl\(_2\) (A—A). Titration curves are plotted as specific activity of enzyme versus metal ion concentration for each enzyme.}
\end{figure}
The primer-template substrate used for this fidelity assay was the following:

- 5′- TGA CCA TGT AAC AGA GAG -3′ (18 mer)
- 3′- ACT GGT ACA TGG TCT CCT ATC TCT CTC TTC TCT TCT-5′ (36 mer)

Where the bold face nucleotide on the template marks the position of insertion of either dTMP or dCMP. Metal concentrations were 10 mM MgCl₂ and 0.75 mM MnCl₂ for wild type enzyme and 20 mM MgCl₂ and 10 mM MnCl₂ for mutant enzymes.

### Table II

| Protein   | Metal | Correct | Incorrect |
|-----------|-------|---------|-----------|
|           | Km (dCTP) | Vmax (dCTP) | Km (dCTP) | Vmax (dCTP) |
| Wild type |       |         |           |
| K950R     |       |         |           |
| K950A     |       |         |           |
| K950N     |       |         |           |
| Wild type |       |         |           |
| K950R     |       |         |           |
| K950A     |       |         |           |
| K950N     |       |         |           |

The reactivities of the three Lys950 mutant enzymes thus, investigated the effect of an altered phosphate group of primer 3′-phosphate moiety of the incoming dNTP for metal activator. The dCTP on the reactivities of the three Lys950 mutant enzymes compared to the wild type enzyme (Table IV). Thus, alteration of the furyl phosphate and was 7-fold more resistant to araCTP than the wild type enzyme did. In contrast, all three Lys⁹⁵⁰ mutant enzymes showed higher sensitivity to ddCTP inhibition than the wild type enzyme. Mutant enzyme K950A with the Lys950 side chain abolished had a 64-fold increase in its sensitivity to ddCTP and was 7-fold more resistant to araCTP than the wild type enzyme (Table IV). Thus, alteration of the furanose ring conformation of a dNTP has an effect on the interaction between a dNTP and the Lys⁹⁵⁰ side chain. Since alterations of the deoxyribose sugar also affects the orientation of the triphosphate group of dNTP, we, therefore, analyzed whether the α-, β-, or γ-phosphate group was the structural feature of a dNTP recognized by the Lys⁹⁵⁰ side chain.

### Table III

Inhibition of the wild type and mutant DNA polymerase α by aphidicolin, BuPdGTP, and its derivative

| Enzyme     | IC₅₀ BuPdGTP | IC₅₀ BuPdGMPCH₃PP | IC₅₀ Aphidicolin |
|------------|--------------|------------------|-----------------|
| Wild type  | 2.50 ± 1.06  | 50.0 ± 10.0      |                 |
| K950R      | 0.88 ± 0.17  | 30.2 ± 2.2       | 4.90 ± 0.65     |
| K950A      | 0.030 ± 0.003| 2.2 ± 0.0        | 3.60 ± 1.30     |
| K950N      | 0.020 ± 0.004| 0.65 ± 0.43      | 0.50 ± 0.43     |

The Lys⁹⁵⁰ Side Chain Recognize the β- or γ-Phosphate Group of the Incoming dNTP Substrate?—We tested whether the Lys⁹⁵⁰ side chain has a role in properly positioning the triphosphate moiety of the incoming dNTP for metal activator chelation. Interaction between the phosphate groups of the dNTP substrate and the positively charged side chain of Lys⁹⁵⁰ could also facilitate a nucleophilic attack of the incoming primer 3′-hydroxyl group for deoxynucleotidyl transfer. To verify this observation, we tested the effect of pyrophosphate (PPi) and two analogs of PPi, carbonyldiphosphonate and phosphonoacetic acid, for their 50% inhibition point (IC₅₀). The three mutant enzymes were inhibited by pyrophosphate to an extent similar to the wild type enzyme (Table IV). Inhibition patterns of the three mutant enzymes and the wild type enzyme by the two pyrophosphate analogs were variable (Table IV). However, like the inhibition studies with araCTP and ddCTP, K950A, with the Lys⁹⁵⁰ side chain abolished, showed the highest sensitivity to carbonyldiphosphonate and phosphonoacetic acid, as well as to BuPdGTP and BuPdGMPCH₃PP. This suggests that the side chain of Lys⁹⁵⁰ might be involved in interacting with the triphosphate moiety of the incoming dNTP substrate. Since the three mutant enzymes showed similar if not identical sensitivity to pyrophosphate, BuPdGTP, and its β-phosphate analog BuPdGMPCH₃PP as the wild type enzyme, the β- and γ-phosphates of the incoming dNTP are not likely to be the structural feature directly interacting with the Lys⁹⁵⁰ side chain.

### Table IV

| Protein   | Km (dNTP) | Km (ddCTP) | Km (araCTP) |
|-----------|-----------|------------|-------------|
| Wild type | 0.53 ± 0.20| 0.23 ± 0.07| 1.40 ± 0.13 |
| K950R     | 4.80 ± 0.70| 0.82 ± 0.07| 3.90 ± 0.42 |
| K950A     | 4.10 ± 0.97| 0.75 ± 0.10| 1.30 ± 0.45 |
| K950N     | 1.70 ± 0.66| 0.94 ± 0.06| 1.05 ± 0.10 |
| Wild type | 0.005 ± 0.004| 0.03 ± 0.02| 1.73 ± 0.32 |
| K950R     | 0.08 ± 0.00| 0.81 ± 0.16| 1.87 ± 0.07 |
| K950A     | 0.02 ± 0.00| 0.22 ± 0.12| 1.64 ± 0.65 |
| K950N     | 0.01 ± 0.00| 0.99 ± 0.12| 0.82 ± 0.26 |

We tested whether the Lys⁹⁵⁰ mutant enzymes showed a higher resistance to araCTP inhibition than the wild type enzyme did. In contrast, all three Lys⁹⁵⁰ mutant enzymes showed higher sensitivity to ddCTP inhibition than the wild type enzyme. Mutant enzyme K950A with the Lys950 side chain abolished had a 64-fold increase in its sensitivity to ddCTP and was 7-fold more resistant to araCTP than the wild type enzyme (Table IV). Thus, alteration of the furanose ring conformation of a dNTP has an effect on the interaction between a dNTP and the Lys⁹⁵⁰ side chain. Since alterations of the deoxyribose sugar also affects the orientation of the triphosphate group of dNTP, we, therefore, analyzed whether the α-, β-, or γ-phosphate group was the structural feature of a dNTP recognized by the Lys⁹⁵⁰ side chain.

Does the Lys⁹⁵⁰ Side Chain Interact with the α-Phosphate Group of the dNTP?—The finding that these three Lys⁹⁵⁰ mutant enzymes could utilize Mn²⁺ more effectively than the wild type enzyme, had altered sensitivity to araCTP and ddCTP, and were insensitive to pyrophosphate inhibition led us to investigate whether the α-phosphate group of the incoming dNTP is the structural feature recognized by the Lys⁹⁵⁰ side chain. We compared the affinity difference of the three Lys⁹⁵⁰ mutant enzymes for an α-phosphate analog, dCTP₅S, and for dNTP (Table V) in reactions with either Mg²⁺ or Mn²⁺ as the metal activator. The dCTP₅S used in this study is the S₅ diastereomer which has been documented to be an active substrate for E. coli polymerase I (19).

In reactions with Mg²⁺, wild type enzyme had 22-fold higher affinity for dCTP than dCTP₅S (Table V). Mutant enzyme K950R had 3-fold higher affinity for dCTP₅S than dNTP (Kₐ₉ = 86 ± 46 mM and Kₐ₅₀ = 250 ± 43 mM), but comparable affinity for dCTP₅S as the wild type enzyme (Kₐ₉ = 86 ± 46 mM for K950R and Kₐ₅₀ = 74 ± 12 mM for the wild type enzyme). Mutant enzyme K950A had 5- to 6-fold lower affinity to dCTP₅S than the normal dNTP; mutant enzyme K950N had 13-fold lower affinity for dCTP₅S than dNTP (Table V). Mutant enzymes K950A and K950N had 6 and 24 times lower affinity (higher Kₐ₅₀ values) for dCTP₅S than the wild type enzyme. These results indicate that substitution of the oxygen moiety of the α-phosphate by sulfur profoundly affects the affinity of the Lys⁹⁵⁰ mutant enzymes for the α-phosphate analog. This suggests that the Lys⁹⁵⁰ side chain interacts with the α-phosphate group of the incoming dNTP substrate.
The Lys950 Side Chain Interacts with the Oxygen Moiety of the α-Phosphate of dNTP Substrate

We then compared the three mutants with the wild type enzyme for their affinity ratio for dCTPαS to dNTP (Km/dNTP). Mutant enzyme K950N with a polar amide side chain replacing the ε-amino side chain had less than 2-fold lower affinity ratio for dCTPαS to dTTP as compared to that of the wild type enzyme (Km/dNTP = 13 for K950N, and Km/dTTP = 22 for the wild type enzyme) (Table V). In contrast, by replacing the ε-amino side chain of Lys950 with a larger positively charged side chain, the mutant enzyme K950R showed a 73-fold lower affinity ratio of dCTPαS to dTTP than the wild type enzyme (Km/dTTP = 0.3 for K950R compared to the wild type enzyme of Km/dTTP = 22). Mutant enzyme K950A with the ε-amino side chain abolished had an approximately 4.5-fold lower affinity ratio for dCTPαS to dTTP than the wild type enzyme (Km/dTTP = 5.0 for K950A compared to Km/dTTP = 22 for the wild type enzyme).

In contrast to the reactions with Mg2+, as the metal activator, in reactions with Mn2+, as metal activator, all three mutant enzymes, as well as the wild type enzyme had lower affinity (higher Km values) for dCTPαS than the wild type enzyme. Furthermore, the three mutant enzymes and the wild type enzyme had comparable or equal affinity ratio of dCTPαS to dTTP (Km/dTTP) (Table V).

We then compared the three mutant enzymes to the wild type enzyme for their catalysis rate (kcat) in utilizing dCTPαS versus dNTP as substrate. In reactions with Mg2+, the wild type enzyme had 2.6-fold higher kcat in utilizing normal dNTP versus dCTPαS as substrate with a ratio of kcat (dNTP)/kcat (dCTPαS) of 0.38. Mutant enzyme K950R did not show a significant difference in its kcat value when either normal dNTP or dCTPαS was used as substrate. Mutant enzyme K950A like the wild type enzyme had an approximately 2-fold higher kcat when dNTP was used as substrate than when dCTPαS was used as substrate (kcat (dNTP)/kcat (dCTPαS) = 0.68 and kcat (dNTP)/kcat (dCTPαS) = 0.27). Mutant enzyme K950N, interestingly, had higher kcat when dCTPαS was used as substrate versus dNTP (Table V).

When Mn2+ was used as the metal activator, the wild type enzyme had identical kcat values in utilizing either dCTP or dCTPαS as a substrate. Mutant enzymes K950R and K950N both had comparable kcat values in using dCTPαS or normal dNTP as a substrate. Mutant enzyme K950A showed a 2-fold lower kcat in using dCTPαS as the substrate than with dCTP as the substrate, like that observed in the Mg2+-catalyzed reaction.

By comparing the affinity (Km) and catalysis (kcat) of these mutant enzymes to the wild type enzyme for utilizing dNTP and dCTPαS as substrate, it is apparent that substitution of the P-O by P-S in the α-phosphate group of dCTP profoundly affects the affinity of the enzyme's binding to the incoming dNTP substrate, but does not significantly affect the rate of catalysis.

These results together with the findings that the three Lys950 mutant enzymes are able to utilize Mn2+ as metal activator strongly suggest that the positively charged side chain of Lys950 either directly or indirectly participates in interactions with the oxygen moiety of the α-phosphate group of the incoming dNTPs, either to position the dNTP to interact with the metal activator or to facilitate the nucleophilic attack by the 3′OH group of the incoming primer.

DISCUSSION

We have used the recombinant human DNA polymerase α as the prototype model for the three principal cellular DNA polymerases α, δ, and ε to elucidate the functional roles of several highly invariant amino acid residues in the active site. We altered several invariant residues by site-directed mutagenesis based on the rationale that we described (20). We generated a panel of mutants that did not have any detectable gross alteration of the protein structure (4, 5, 9, 20). Thus, it is reasonable to assume that the structural alterations resulting from each mutation are confined to the position of the mutated side chains. By steady-state kinetic analysis of the mutant enzymes, we have defined the functions of several residues in the two most conserved regions (regions I and II) of the active site (4, 5, 8, 9). In this report, we have extended our studies of the active site by investigating the function of a highly invariant lysine residue in the third most conserved region (Fig. 1).

Previous study has documented that the interaction of DNA polymerase α with its substrates obeys a rigidly ordered sequential terreactant mechanism, with template as the first substrate, followed by primer as the second substrate and...
dNTP as the third. Specification of which of the four dNTPs has kinetically significant binding is determined by the base sequence of the template (21). A similar ordered sequential ter-
reactant mechanism was also proposed by Dahlberg and Benkovic (22) for the Klenow fragment of E. coli polymerase I. Given the universal ordered sequential mechanism for both eu-
karyotic DNA polymerase α and prokaryotic E. coli polym-
erase I, and depending on the base sequence of the template, DNA polymerases have different modes of interaction with the incoming dNTP. Studies of Klenow fragment have shown a rate difference in using dTTP versus dGTP (23). In this study, we did not compare the difference of either the wild type or the mutant enzymes for their affinity (K_m(dNTP)) in binding each different incoming dNTP or the difference in binding purine versus pyrimidine deoxyribose trisphosphates. It is possible that the side chain of this highly invariant Lys of α-like DNA poly-
merases like that of E. coli polymerase I has a different mode of interaction with each different incoming dNTP (23). Here, we assume in the enzyme where the protein contacts the dNTP, the Lys950 side chain has the same interaction with all four of the dNTPs in all circumstance. We also only evaluated the side chain function of Lys950 by kinetic analysis in the state of dNTP in reactions with Mg2+ versus Mn2+ or resistance to the dNTP analogs regardless of whether the mutant enzymes have shown that the metal activator utilization and affinity for dNTP, the side chain of Lys950 therefore might interact either directly or indirectly with the second metal ion chelated α-phosphate of the incoming dNTP.

Lys950 Side Chain Interacts with Oxygen Moiety of the α-Phosphate of the dNTP Substrate—The abilities of the three Lys950 mutant enzymes to utilize Mn2+ as metal activator more efficiently than the wild type enzyme suggest that the positively charged lysine side chain may have an influence on the configuration of the negatively charged phosphate groups of the incoming dNTP. Comparison of the inhibitory effect of pyrophosphate and the inhibitory effect of BuPdGMPCH2PP on the wild type enzyme and the three mu-
tant enzymes have shown that the β- and γ-phosphates are not likely to be in direct contact with the lysine side chain (Tables III and IV). Analogs of dNTP containing alterations in the ribose moiety had notable effects on the reactivity of the three Lys950 mutant enzymes as compared to the wild type enzyme (Table IV). Mutant enzyme K950A which has the positive charge of lysine side chain abolished always displays higher sensitivity or resistance to the dNTP analogs regardless of whether the analog has deletion of the 3′-OH group as in ddCTP or has a twisted deoxyribose ring as in araCTP (26).

Since alteration of the deoxyribose in either araCTP or ddCTP could also affect the orientation of the oxygen group of α-phosphate, we also tested the interactions between the Lys950 side chain and the oxygen group of α-phosphate with an analog, dCTPαS, in reactions utilizing either Mg2+ or Mn2+ as metal activator. We compared each enzyme’s affinities (K_m) and catalysis (k_cat) for utilizing dCTPαS versus dNTP as sub-
strate in reactions with either Mg2+ or Mn2+ (Table V). We also compared each enzyme’s affinity ratio for dCTPαS versus dNTP (K_m(dCTPαS)/K_m(dNTP)) with the wild type enzyme. In reactions with Mg2+, mutation of the Lys950 side chain by either replacing it with a larger charged side chain (K950R) or abolishing the charged side chain (K950A) had a significant effect on the mutant enzyme’s affinity to dCTPαS versus dNTP substrate. In con-
trast, replacing the e-amino side chain of Lys950 to Asn (K950N) appears not to affect the mutant’s affinity ratio for dCTPαS versus dNTP. In Mn2+−catalyzed reactions, all three mutants showed comparable affinity ratio, K_m(dCTPαS)/K_m(dNTP), as the wild type enzyme. We reason that this difference in metal effect might be due to the polymerase α-Mn2+ complex having less selective preference for C2′-endo deoxyribose. The presence of the C3′
-end form of dNTP could affect the affinity between the dNTP and the side chain of Lys950 resulting in an observed mild effect on the affinity of the mutant enzymes for dCTPαS.

In sum, results presented in this study strongly suggest that
The active site of the α-like polymerases. The results have supported a model of the active site of α-like polymerases. In human polymerase α, Asp1002 and Asp1004, and Thr1003 located in an anti-parallel β-sheet (region I) chelate with the metal activator, Mg²⁺, which in turn chelates to the oxygen moiety of β- and γ-phosphate of the incoming dNTP (8, 9). The phosphodiester side chain of Tyr²⁶⁰ (in region II) interacts with the nucleotide base of the incoming dNTP to properly position the incoming dNTP for Watson-Crick base pairing (5). The oxygen moiety of the Ser²⁶⁰ hydroxyl side chain in region II forms a hydrogen bond either directly or indirectly with the 3'-OH terminus of the primer. The hydrogen bond formation might enhance the oxygen moiety at the 3'-OH primer terminus for nucleophilic attack at the α-phosphate of the incoming dNTP (4). The positively charged side chain of Lys⁹⁵⁰ located in an α-helix in region III of human polymerase α interacts either directly or indirectly with the oxygen group of the α-phosphate of the incoming dNTP. This interaction of a positively charged side chain will neutralize the negative charge on the α-phosphate to facilitate nucleophilic attack of the incoming primer 3'-hydroxy group. This proposed model of the α-like polymerase active site (Fig. 3) is based entirely on biochemical data and can only be verified in the future by crystallographic data of a ternary complex of polymerase α with primer-template and dNTP substrates.

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