dNTP Binding to HIV-1 Reverse Transcriptase and Mammalian DNA Polymerase β as Revealed by Affinity Labeling with a Photoreactive dNTP Analog*

Olga I. Lavrik‡, Rajendra Prasadš, William A. Beardš, Igor V. Safronovš, Mikhail I. Dobrikovš, Deepak K. Srivastavš, Gennadii V. Shishkinš, Thomas G. Woodš, and Samuel H. Wilsonš†

From the §Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1068 and the ¶Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, 630090 Novosibirsk, Russia

The dNTP binding pocket of human immunodeficiency virus type 1 reverse transcriptase (RT) and DNA polymerase β (β-pol) were labeled using a photoreactive analog of dCTP, [N-(β-[p-azidotetrafluorobenzamido]-ethyl)-deoxycytidine-5-triphosphate (FABdCTP). Two approaches of photolabeling were utilized. In one approach, photoreactive FABdCTP and radiolabeled primer-template were UV-irradiated in the presence of each enzyme and resulted in polymerase radiolabeling. In an alternate approach, FABdCTP was first UV-cross-linked to enzyme; subsequently, radiolabeled primer-template was added, and the enzyme-linked dCTP analog was incorporated onto the 3'-end of the radiolabeled primer. The results showed strong labeling of the p66 subunit of RT, with only minor labeling of p51. No difference in the intensity of cross-linking was observed with either approach. FABdCTP cross-linking was increased in the presence of a dideoxynucleotidated primer-template with RT, but not with β-pol, suggesting a significant influence of prior primer-template binding on dNTP binding for RT. Mutagenesis of β-pol residues observed to interact with the incoming dNTP in the crystal structure of the ternary complex resulted in label with kinetic characterization of these mutants indicated specific labeling of the dNTP binding pocket.

DNA polymerases must recognize and incorporate the correct dNTP with high fidelity to maintain genetic stability. The interaction of the polymerase with the DNA primer-template is essentially nonspecific, since the enzyme must bind to an "infinite" number of sequences during replication, while dNTP-template base recognition must be highly specific for DNA replication of high fidelity. Since the role of the polymerase in dNTP recognition and selection is poorly understood, elucidation of polymerase-dNTP interactions under a variety of conditions will aid our understanding of this fundamental process.

During the last several years our knowledge of the structure of DNA polymerases and their interactions with substrates has evolved significantly. The three-dimensional structures of several DNA polymerases have been solved by x-ray crystallography including the Klenow fragment of DNA polymerase I (1), HIV-1 reverse transcriptase (RT) (2-5), Taq polymerase (6), and rat/human DNA polymerase β (β-pol) (7-11). The overall architecture of these enzymes is similar, including a cleft that binds DNA. These polymerases have been described using analogy to the anatomical features of a hand as fingers, palm, and thumb subdomains (2). Catalytically essential carboxylates that bind to the incoming deoxynucleoside 5'-triphosphate (dNTP) via Mg2⁺ are found in the palm subdomain. Whereas the palm subdomains of these polymerases are structurally similar, the fingers and thumb subdomains are structurally distinct. The dNTP binding pocket for DNA polymerase β has been defined by the x-ray structure of the β-pol-ddCTP-primer-template complex (9). The triphosphate moiety of the incoming ddCTP is observed to be interacting with residues primarily in the palm subdomain, whereas the sugar and base moieties primarily interact with residues in α-helices M and N of the thumb subdomain. Although structures of binary-dNTP complexes have been solved for Klenow fragment (12) and HIV-1 RT (13, 14), the specific protein-ligand interactions were suggested to be different in the catalytically active complex (i.e. in the presence of primer-template). Additionally, since the orientation of the DNA in the β-pol structure is different to that observed with HIV-1 RT, the catalytic significance of the β-pol ternary complex structure has been questioned (14, 15).

Mammalian β-pol is the smallest DNA polymerase identified to date and is responsible for filling short DNA gaps during DNA repair (16-18). The dNTP binding pocket has been defined in detail by x-ray crystallography (9). Thus, solution studies to characterize the dNTP binding pocket of β-pol permit comparison with recent crystallographic structures. HIV-1 RT utilizes both RNA and DNA templates during proviral DNA synthesis, and chemical modification and cross-linking approaches have been used to define the substrate binding sites (19-22). These studies can be interpreted in the context of the crystallographic structure of a DNA-RT complex (3).

A useful technique for structure-function analysis in solution is affinity labeling based on the "catalytic competence" of a covalently incorporated substrate molecule. This approach has been utilized with other template-dependent systems for the study of the substrate binding sites of RNA polymerase (23), DNA polymerases (24-26), and DNA polymerase α-prime (27). The chemically reactive substrate analog is covalently linked to the enzyme, and the modified enzyme is then purified and used to define the substrate binding sites. This approach has been used to define the substrate binding sites for HIV-1 reverse transcriptase (28).

DNA polymerases recognize and incorporate the correct dNTP with high fidelity to maintain genetic stability. The interaction of the polymerase with the DNA primer-template is essentially nonspecific, since the enzyme must bind to an "infinite" number of sequences during replication, while dNTP-template base recognition must be highly specific for DNA replication of high fidelity. Since the role of the polymerase in dNTP recognition and selection is poorly understood, elucidation of polymerase-dNTP interactions under a variety of conditions will aid our understanding of this fundamental process.

During the last several years our knowledge of the structure of DNA polymerases and their interactions with substrates has evolved significantly. The three-dimensional structures of several DNA polymerases have been solved by x-ray crystallography including the Klenow fragment of DNA polymerase I (1), HIV-1 reverse transcriptase (RT) (2-5), Taq polymerase (6), and rat/human DNA polymerase β (β-pol) (7-11). The overall architecture of these enzymes is similar, including a cleft that binds DNA. These polymerases have been described using analogy to the anatomical features of a hand as fingers, palm, and thumb subdomains (2). Catalytically essential carboxylates that bind to the incoming deoxynucleoside 5'-triphosphate (dNTP) via Mg2⁺ are found in the palm subdomain. Whereas the palm subdomains of these polymerases are structurally similar, the fingers and thumb subdomains are structurally distinct. The dNTP binding pocket for DNA polymerase β has been defined by the x-ray structure of the β-pol-ddCTP-primer-template complex (9). The triphosphate moiety of the incoming ddCTP is observed to be interacting with residues primarily in the palm subdomain, whereas the sugar and base moieties primarily interact with residues in α-helices M and N of the thumb subdomain. Although structures of binary-dNTP complexes have been solved for Klenow fragment (12) and HIV-1 RT (13, 14), the specific protein-ligand interactions were suggested to be different in the catalytically active complex (i.e. in the presence of primer-template). Additionally, since the orientation of the DNA in the β-pol structure is different to that observed with HIV-1 RT, the catalytic significance of the β-pol ternary complex structure has been questioned (14, 15).

Mammalian β-pol is the smallest DNA polymerase identified to date and is responsible for filling short DNA gaps during DNA repair (16-18). The dNTP binding pocket has been defined in detail by x-ray crystallography (9). Thus, solution studies to characterize the dNTP binding pocket of β-pol permit comparison with recent crystallographic structures. HIV-1 RT utilizes both RNA and DNA templates during proviral DNA synthesis, and chemical modification and cross-linking approaches have been used to define the substrate binding sites (19-22). These studies can be interpreted in the context of the crystallographic structure of a DNA-RT complex (3).

A useful technique for structure-function analysis in solution is affinity labeling based on the "catalytic competence" of a covalently incorporated substrate molecule. This approach has been utilized with other template-dependent systems for the study of the substrate binding sites of RNA polymerase (23), DNA polymerases (24-26), and DNA polymerase α-prime (27). The chemically reactive substrate analog is covalently linked to the enzyme, and the modified enzyme is then purified and used to define the substrate binding sites. This approach has been used to define the substrate binding sites for HIV-1 reverse transcriptase (28).
incorporated into the enzyme, and polymerase is subsequently labeled by the addition of radioactively labeled substrate. Only substrate analogs bound at the active site will be labeled in this manner. Substrate analogs not bound at the active site would be catalytically inactive and invisible in this analysis. This procedure for affinity labeling permits discrimination between specific and nonspecific labeling of the enzyme.

In the present study, a photoaffactive analog of dCTP, carrying a photoactive group on the 4-aminogroup of the base (Fig. 1), was used for labeling the dNTP binding sites of HIV-1 RT and DNA polymerase β. Two approaches of photolabeling were utilized. The first approach utilizes the introduction of the substrate analog (i.e. FABdCMP) onto the 3′-end of a primer by the activity of the polymerase. After the analog has been incorporated into the DNA, the polymerase is UV-irradiated to covalently attach the 3′-end of the primer to the polymerase. In an alternate approach, the dNTP analog is first covalently attached to the polymerase by UV-irradiation and subsequently labeled by the addition of a 32P-labeled primer-template allowing incorporation of “catalytically competent” cross-linked analog onto the primer. The influence of a chain-terminating primer-template on the level of catalytically competent complex was also examined.

**EXPERIMENTAL PROCEDURES**

**Materials—**[γ-32P]JATP and [α-32P]dTTP were purchased from ICN Radiochemicals or DuPont NEN. High pressure liquid chromatography-purified synthetic oligonucleotides of defined sequence were obtained from Midland Certified Reagent Co., Biosynthesis Inc., or Operon Technologies, Inc. Poly(dA), poly(dT)60, and dNTPs were from Pharmacia Biotech Inc. and Nensorb-20 columns were from DuPont. The base-substituted photoreactive analog of dCTP, FABdCTP, was synthesized as described (28). T4 polynucleotide kinase was from U.S. Biochemical Corp. and T4 DNA ligase was purchased from New England Biolabs. Trypsin and endoproteinase Lys-C were purchased from Sigma.

**Radioactive Labeling of Oligonucleotide Primers—**Dephosphorylated oligonucleotide primers were γ-phosphorylated with T4 polynucleotide kinase as described (29). Unreacted [γ-32P]JATP was separated by passing the mixture over a Nensorb-20 column using the manufacturer’s suggested protocol.

** Primer-Template Annealing—**Liphophilized oligonucleotides were re-suspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and the concentrations were determined from their UV absorbance at 260 nm. Primer-template mixture was annealed by heating a solution of primer with an equivalent concentration of template to 90 °C for 3 min and incubating the solution for an additional 15 min at 50–60 °C, followed by slow cooling to room temperature. The sequences of the primer and template were used as follows: 17-mer primer, 5′-GGATGAGGCCTACTTGAATCCT-3′; 36-mer template, 5′-GGTGGGAGAATTGAGAATGTA-GATCCCCCTAC-3′.

**Photochemical Cross-linking—**HIV-1 RT and β-pol were photoaffinity-labeled using the dCTP analog using the following protocols in a 10-μl reaction mixture containing 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 50 mM KC1, 1 μM polymerase, and 60 μM FABdCTP. (i) In one protocol, 5′-32P-labeled primer-template was included in the reaction mixture, and the mixture was incubated at 25 °C for 45 min to allow polymerization. The mixture was then UV-irradiated (λmax = 312 nm, 3–6 mJ) with a UV-Stratalinker (Stratgene Cloning Systems). (ii) In an alternate labeling protocol, the reaction mixture was first UV-irradiated, and then polymerization was initiated with the addition of 32P-labeled primer-template for 45 min. (iii) In another protocol, dideoxy-primer-template was included in the reaction mixture, and following UV-irradiation, this dideoxy-primer-template was competed out by adding a 7-fold molar excess of 32P-labeled primer-template to initiate polymerization. The same protocols of photochemical cross-linking were followed for FABdCTP. Under these illumination conditions, UV-irradiation alone did not influence enzyme activity. The photochemically cross-linked primer-DNA samples were separated by SDS-PAGE, and dried gels were subjected to autoradiography.

**Proteolytic Digestion—**After UV photochemical cross-linking as described above, the cross-linked DNA/protein mixture (substrate) was digested at room temperature with either endoproteinase Lys-C (for HIV-1 RT) or trypsin (for β-pol) at an enzyme:substrate weight ratio of 1:100. The digested products were then separated by 15% SDS-PAGE and visualized by autoradiography.

**Mutagenesis of the Human β-Pol Gene—**Oligonucleotide site-directed mutagenesis was performed using a procedure described previously (30). M13 phage containing the human β-pol target DNA was propagated using the bacterial host Cj36 (dut ung), and phage DNA was purified for use as template. Synthetic oligonucleotide primers containing the desired codon change were annealed to the template DNA, and the primers were extended with Sequenase version 2.0 (U.S. Biochemical). The following mutations were introduced into the M13 β-pol vector, 5′ to 3′: D276G (GAT to GTG), D276V (GAT to GTG), N279A (AAT to GCC), and N279L (AAT to CTG). To ensure that the resulting β-pol genes contained the desired change, the entire coding sequence of each mutant was confirmed by DNA sequence analysis. The mutated β-pol gene was inserted into the Cial and HindIII sites of the AP9 promoter-based expression system pWL-11 provided by T. A. Patterson (Ares, Inc.) and overexpressed in Escherichia coli TAP56.

**Protein Purification—**HIV-1 RT was purified as described previously (31). Wild-type human DNA polymerase β, mutant derivatives, and the 14- and 31-kDa domains were purified as described (32, 33).

**β-Pol Polymerization Assays—**Enzyme activities were determined using a standard reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.4 (22 °C), 5 mM MgCl2, and 100 mM KCl. Other reaction conditions are described in the legend of Fig. 6. Reactions were initiated by the addition of enzyme, incubated at 22 °C, and stopped by the addition of 20 μl of 0.5 mM EDTA, pH 8. Quenched reaction mixtures were spotted onto Whatman DE-81 filter disks and dried. Unincorporated [α-32P]dATP was removed, and filters were counted as described (34).

**RESULTS**

**FABdCTP Incorporation and Cross-linking—**The structure of the photoactive base-substituted analog of dCTP (FAB-dCTP) is shown in Fig. 1. This analog is incorporated by HIV-1 RT into DNA. Whereas its Km is approximately 1 order of magnitude greater than for dCTP, Vmax is similar for these alternate substrates (26). This derivative has a photoactive azido group that permits photoactivation at wavelengths that minimize damage to nucleic acids and proteins (i.e. >300 nm). Therefore, we explored the photoaffactive property of the analog for affinity labeling of the dNTP pocket of HIV-1 RT and β-pol by UV irradiation (λmax = 312 nm).

HIV-1 RT and β-pol were UV-cross-linked with the photoaffactive FABdCTP using two approaches (Fig. 2). In the first approach, the dCTP analog was UV-cross-linked in the presence of a 32P-labeled primer-template and polymerase. In the alternate approach, the photoaffactive FABdCTP was first UV-cross-linked to the enzyme, and the labeled primer-template was subsequently added to the reaction to introduce the analog, linked at the dNTP binding pocket, onto the 3′-end of the 32P-labeled primer. The products were separated by SDS-PAGE and visualized by autoradiography. UV irradiation of a primer-template that had been extended previously by FABdCTP resulted in intense labeling of the p66 subunit and only minor labeling of the p51 subunit of HIV-1 RT (Fig. 2A, lane 1).
enhanced in the presence of Mg\textsuperscript{2+}.

The templating nucleotide was not a guanine (data not shown).

In the absence of chain-terminated primer-template, no significant difference in cross-linking was observed. When primer-template was added, a minor cross-linking of a slightly faster migrating contaminant was also observed (Fig. 2B, lane 4). This 31-kDa protein represents the well-known proteolytic degradation product of β-pol and is the carboxyl-terminal catalytic domain (35). A recombinant 31-kDa domain of β-pol could also be labeled in this manner (data not shown), but a recombinant 14-kDa amino-terminal domain that lacks DNA polymerase activity and retains both single- and double-stranded DNA binding activities (32) could not be labeled (Fig. 2B, lane 5).

Specificity of Cross-linking—To investigate whether both approaches of UV cross-linking derivatized the same site, we photolabeled HIV-1 RT and β-pol, utilizing both approaches of cross-linking, and prototyped the resulting complexes. When analog cross-linking occurs prior to primer-template addition, only cross-linked analog, which is catalytically competent, will result in enzyme labeling. When UV cross-linking occurs in the presence of primer-template, then labeling can occur as just described, or the incorporated analog (i.e. a primer with a terminal analog) may also be covalently cross-linked. The cross-linked product of HIV-1 RT, when digested with Lys-C, rapidly generated a radiolabeled fragment of approximately 18 kDa that remained relatively resistant to further proteolysis (Fig. 3, A and B). The proteolytic digestion patterns of the cross-linked RT generated by the two approaches were similar, suggesting that both cross-linking approaches labeled the same dNTP binding site(s) in HIV-1 RT.

In a similar fashion, when the photoaffinity-labeled β-pol was digested with trypsin, two radiolabeled fragments (approximately 18 and 38 kDa) were released within 30 min of digestion, and these remained relatively resistant for 90 min of digestion (Fig. 3, C and D). Again, the proteolytic digestion patterns of these labeled β-pol peptides were found to be very similar, suggesting that both approaches for cross-linking probably labeled the same dNTP binding site(s). From these results it appears that this dCTP analog binds in the dNTP binding pocket and is cross-linked in or near this pocket.

Influence of Primer-Template on dNTP Pocket Labeling—The mechanism by which nucleic acid polymerases bind the correct nucleotide for polymerization with high fidelity is not well understood. HIV-1 RT (36) and β-pol (37) utilize an ordered mechanism for DNA synthesis where primer-template binds first, followed by dNTPs. However, dNTP binding can occur in the absence of primer-template as demonstrated by UV cross-linking for HIV-1 RT (38) and β-pol (39). The influence of the DNA or RNA primer-template on the topology of the dNTP binding pocket is an important aspect of fidelity. Our data confirm that both HIV-1 RT and β-pol can bind to FAB-dCTP in the absence of nucleic acid and that this covalently bound analog can be incorporated into a primer-template (Fig. 2). To analyze the influence of primer-template on dNTP cross-linking, both RT and β-pol were cross-linked with FAB-dCTP in the presence or absence of a chain-terminated primer-template with a dGMP serving as the templating base. This is in contrast to the protocol described above where cross-linking occurred in the presence of primer-template, which allowed extension of the primer. Specific labeling was then achieved by adding a 7-fold molar excess of a 32P-labeled “active” primer-template (i.e. primer-template not terminated) to effectively compete the terminated primer-template for polymerase binding and incorporation. The results using this protocol demonstrate a significant increase in labeling of HIV-1 RT in the presence of dideoxyprimer-template (Fig. 4A). In contrast, when β-pol was cross-linked in the presence or absence of chain-terminated primer-template, no significant difference in cross-linking was observed (Fig. 4B). These results suggested that the binding of HIV-1 RT, but not β-pol, to FAB-dCTP was greater in the presence of primer-template.

DNA Polymerase β Extension of Cross-linked Primer-Template—To determine the degree of primer utilization with a 3'-terminally cross-linked FAB-dCMP, β-pol and FAB-dCTP were UV-irradiated in the presence (Fig. 5, lane 1) or absence (lane 2) of 5'-32P-primer-template; in the latter case, enzyme was subsequently mixed with 5'-32P-primer-template. Equiva-

---

**Fig. 2.** Photoaffinity labeling of HIV-1 RT and β-pol with FAB-dCTP. Photoaffinity labeling of HIV-1 RT (panel A) and β-pol (panel B) using FAB-dCTP was performed as described under "Experimental Procedures." The reaction mixture was UV-irradiated (\(\lambda_{max} = 312 \text{ nm}\)) after polymerization (panel A, lanes 1–4; panel B, lanes 1 and 2) or prior to the addition of radiolabeled primer-template (panel A, lane 5; panel B, lanes 3–5). The UV-cross-linked enzyme-DNA complexes were separated by SDS-PAGE and visualized by autoradiography. In lanes 3 and 4 (panel A), 50 mM EDTA was added prior to UV illumination. In lane 5 (panel B), the purified recombinant 14-kDa N-terminal domain of β-pol was UV-cross-linked under similar conditions as lane 4. This domain has DNA binding activity similar to that of wild-type enzyme (32) but lacks polymerase activity. The dCTP analog was not added to the reaction mixture in lanes 2 and 4 (panel A) and lanes 1 and 3 (panel B). The positions of the complexes formed between the primer-template and the DNA polymerases are indicated as p66 and p51 (p66 and p51 subunits of HIV-1 RT) or β-pol. The positions of the free probe and the protein markers are also indicated.

There is an approximately 6- and 18-kDa retardation in the usual migration of the RT subunits consistent with the cross-linking of one molecule of primer and one molecule of primer-template, respectively. Therefore, two radiolabeled bands, 72 and 84 kDa, were observed as products of covalently cross-linked primer and primer-template to the 66-kDa subunit of RT, respectively. The intense labeling at approximately 84 kDa may also represent the covalent cross-linking of several primers to p66. The presence of EDTA to stop DNA synthesis prior to UV irradiation decreased primer-template RT cross-linking (Fig. 2A, lane 3). The preferential decrease in the higher molecular weight bands suggest that primer-template labeling is enhanced in the presence of Mg\textsuperscript{2+}.

In the alternate approach, photoreactive FAB-dCTP was first UV-cross-linked to the enzyme, and the labeled primer-template was added subsequently to introduce the cross-linked analog onto the 3'-end of the 32P-labeled primer. In this case, only the p66 subunit of RT was labeled (Fig. 2A, lane 5). In control experiments where FAB-dCTP was not added to the reaction mixture, the 32P-labeled primer did not cross-link to RT (Fig. 2A, lanes 2 and 4). In addition, there was no cross-linking in the absence of template or primer-template where the templating nucleotide was not a guanine (data not shown).

Using the same reaction conditions for cross-linking as described above for HIV-1 RT, the dNTP binding site of mammalian DNA polymerase β was also labeled (Fig. 2B). The results indicate an intensive cross-linking of the 39-kDa full-length enzyme. Therefore, as reported earlier for HIV-1 RT (26), β-pol can also utilize this analog as a dNTP substrate. When β-pol was first UV-cross-linked to FAB-dCTP and then a 32P-labeled primer-template was added, a minor cross-linking of a slightly faster migrating contaminant was also observed (Fig. 2B, lane 4). This 31-kDa protein represents the well-known proteolytic degradation product of β-pol and is the carboxyl-terminal catalytic domain (35). A recombinant 31-kDa domain of β-pol could also be labeled in this manner (data not shown), but a recombinant 14-kDa amino-terminal domain that lacks DNA polymerase activity and retains both single- and double-stranded DNA binding activities (32) could not be labeled (Fig. 2B, lane 5).
lent labeling was observed in both cases (Fig. 5, lanes 1 and 2). Next, the ability of the incorporated and cross-linked FAbdCMP 3'-OH to participate in further extension was examined by determining whether the next incoming complementary dNTP (dTTP) was incorporated (Fig. 5, lanes 3 and 4). The FAbdCTP analog was incorporated into an unlabeled primer-template by UV-cross-linking either in the presence (lane 3) or absence (lane 4) of the nucleic acid, as in lanes 1 and 2 (Fig. 5). The catalytic competence of the enzyme-FAbdCMP adduct was ascertained by its ability to incorporate the next complementary nucleotide, $[^{32}\text{P}]d\text{TMP}$. Less $[^{32}\text{P}]d\text{TMP}$ incorporation was observed in the reaction mixture resolved in lane 3 than in lane 4. Since $[^{32}\text{P}]d\text{TMP}$ will be incorporated only onto primers that are catalytically competent, the diminished labeling observed in lane 3, where cross-linking occurred in the presence of primer-template, indicates that a significant level of the labeled complex observed in lane 1 is no longer catalytically competent after FAbdCMP incorporation. This could result from FAbdCMP incorporation onto the primer-template and cross-linking after the nucleic acid dissociated and bound “nonproductively.” In any case, the 3'-OH of the incorporated FAbdCMP can act as a substrate in the next incorporation event during primer elongation, although it is covalently attached to the enzyme.

Site-directed Mutagenesis of the $\beta$-Pol dNTP Binding Pocket—Based on the crystal structure of the DNA-ddCTP-$\beta$-pol ternary complex (9), the side chain of Asn$^{279}$ is within hydrogen bonding distance to the O-2 of the incoming ddCTP (Fig. 6A). To ascertain whether FAbdCTP was binding specifically to this dNTP binding pocket, Asp$^{276}$ and Asn$^{279}$ were altered by site-directed mutagenesis. Expression constructs of human $\beta$-pol were prepared; Asp$^{276}$ was replaced with either glycine or valine, and Asn$^{279}$ was replaced with either alanine or leucine. Each altered human $\beta$-pol gene was expressed in $E$. coli, and the recombinant enzymes were soluble in the crude cell extracts. Following purification, SDS-PAGE analysis indicated that the mutant $\beta$-pol-DNA complexes are indicated as p66 or $\beta$-pol. The positions of the protein markers are indicated on the left of each panel.

![Image](http://www.jbc.org/)

**Photoaffinity Labeling of dNTP Pocket.**
understanding of polymerase structure and function (3, 9, 10). The results described were used to probe the nucleic acid interactions in the dNTP pocket of these DNA polymerases. Our results demonstrate a higher level of UV labeling of HIV-1 RT or b-pol (Fig. 5B). This indicates that these enzymes can bind the dCTP analog and subsequently incorporate the covalently cross-linked analog onto a primer. Thus, these DNA polymerases can productively bind dNTPs in the absence of primer-template, and the dNTP bound to the active site can subsequently be incorporated when a primer-template binds to the enzyme. Although steady-state kinetic analysis indicates that an ordered addition of substrates is preferred with primer-template binding first, it is clearly not obligatory based upon the evidence from the current study. To further investigate this point, we determined whether a primer-template will influence the labeling of HIV-1 RT or b-pol. We compared the dCTP analog cross-linked in the presence and absence of a dideoxynucleoside triphosphate (ddCTP). Asn279 can hydrogen bond indiscriminately to the O-2 of pyrimidines or the N-3 of purines in the DNA minor groove. The C-b of Asn279 makes van der Waals contact with C-4 and C-5 of ddCTP. The van der Waals surface of these atoms is indicated with dots. B, steady-state kinetic parameters for wild-type and mutant b-pol. Assays were performed as described under “Experimental Procedures.” The dNTP concentration was varied from at least 0.3 to 3 × K_m under saturating concentrations of primer-template (i.e., >4 × K_m). Initial velocities were fitted to the Michaelis equation by nonlinear least squares methods. The results represent the mean and S.E. of at least two independent determinations. The kinetic parameters for the Asn279 mutants were taken from Beard et al. (40). The corresponding values for k_cat and K_m of dCTP with wild-type enzyme are 0.8 ± 0.01 s^{-1} and 8.6 ± 1.3 μM, respectively.

Fig. 6. DNA polymerase b-dNTP binding pocket probed by site-directed mutagenesis. A, stereo diagram of Asp276 and Asn279 side chain interactions with the base of the incoming dCTP observed in the rat b-pol-DNA-ddCTP ternary complex (Protein Data Bank file 2pct). The Asn279 side-chain is within hydrogen bonding distance (dashed lines) to O-2 of the incoming dideoxynucleoside triphosphate (ddCTP). Asn279 can hydrogen bond indiscriminately to the O-2 of pyrimidines or the N-3 of purines in the DNA minor groove. The C-b of Asp276 makes van der Waals contact with C-4 and C-5 of ddCTP. The van der Waals surface of these atoms is indicated with dots. B, steady-state kinetic parameters for wild-type and mutant b-pol. Assays were performed as described under “Experimental Procedures.” The dNTP concentration was varied from at least 0.3 to 3 × K_m under saturating concentrations of primer-template (i.e., >4 × K_m). Initial velocities were fitted to the Michaelis equation by nonlinear least squares methods. The results represent the mean and S.E. of at least two independent determinations. The kinetic parameters for the Asn279 mutants were taken from Beard et al. (40). The corresponding values for k_cat and K_m of dCTP with wild-type enzyme are 0.8 ± 0.01 s^{-1} and 8.6 ± 1.3 μM, respectively.

 DISCUSSION

The crystallographic analysis of DNA polymerases complexed with nucleic acids substrates has rapidly advanced our understanding of polymerase structure and function (3, 9, 10, 41). The dNTP binding site of DNA polymerase b has been identified from the crystal structure of the ternary substrate complex (9). It is important to complement these crystallographic studies with solution techniques to probe both structure and function. A promising solution technique is the specific labeling of an enzyme based on the “catalytic competence” of a covalently bound substrate. We used a new photoactive analog of dCTP carrying a photoactive azido group attached to a spacer arm (Fig. 1) to specifically label the dNTP binding pocket of HIV-1 RT and mammalian b-pol. The azido moiety is approximately 10 Å from the site of attachment at N-4 of the nucleotide base.

Using this photoactive dCTP substrate, we have demonstrated catalytically competent labeling of RT and b-pol (Fig. 2B). This indicates that these enzymes can bind the dCTP analog and subsequently incorporate the covalently cross-linked analog onto a primer. Thus, these DNA polymerases can productively bind dNTPs in the absence of primer-template, and the dNTP bound to the active site can subsequently be incorporated when a primer-template binds to the enzyme. Although steady-state kinetic analysis indicates that an ordered addition of substrates is preferred with primer-template binding first, it is clearly not obligatory based upon the evidence from the current study. To further investigate this point, we determined whether a primer-template will influence the labeling of HIV-1 RT or b-pol. We compared the dCTP analog cross-linked in the presence and absence of a dideoxynucleoside triphosphate (ddCTP). The dCTP analog cannot be incorporated onto a terminated primer, but the primer-template could influence the nucleic acid interactions in the dNTP pocket of these DNA polymerases. Our results demonstrate a higher level of UV cross-linking with HIV-1 RT (Fig. 4A), suggesting more favorable interactions within the dNTP binding pocket and concomitant greater labeling in the presence of dideoxynucleoside triphosphate (ddCTP).
The crystal structure of the ternary complex were altered by chains observed to interact directly with the incoming dNTP in the Asn279 generated very low labeling, whereas mutants of Asp276 resulted in a strong increase in FABdCTP labeling. The effect was most pronounced when the acidic side chain was replaced with a hydrophobic side chain (i.e., Val), which could preserve van der Waals contact with the base. These results illustrate that labeling of the β-pol dNTP binding site is specific and that the ternary complex crystal structure can guide further study of dNTP binding and fidelity.

It has been demonstrated that the γ-phosphate of the dNTP plays an important role in dNTP binding and selection (44, 45). However, even for noncomplementary dNTPs, the contribution of the triphosphate moiety and particularly the γ-phosphate is important (45). This is consistent with the similar γ-phosphate binding observed in the binary β-pol-dATP and ternary primer-template-ddCTP complexes (8). Therefore, it is likely that interactions of the dNTP base and triphosphate moieties provide essential and nonspecific preliminary arrangement of the incoming dNTP in the dNTP binding pocket of DNA polymerases. DNA polymerases for which the structure has been determined have palm subdomains that are structurally very similar. However, if the active site carboxylic acids are used to align the palm subdomains of HIV-1 RT and β-pol, the primer is observed to be entering the active site from opposite sides (9, 42, 43). This has led some groups to question the significance of the β-pol ternary complex to whether it is catalytically correct (14, 15) and others to suggest that the structural similarity observed upon alignment of the palm subdomains of HIV-1 RT and β-pol should be ignored in favor of alignment of the bound DNA substrate (42). To determine whether dNTP contacts in the β-pol ternary complex structure are specific, two side chains observed to interact directly with the incoming dNTP in the crystal structure of the ternary complex were altered by site-directed mutagenesis; the side-chain of Asn279 is within hydrogen bonding distance to the O-2 of the incoming dCDTP, and the C-β of Asp276 is near C-4 and C-5 of the dCDTP base (Fig. 6A). The catalytic efficiency (kcat/Km) of the Asp276 mutants was similar to that of wild-type enzyme, due to a parallel modest decrease in both kcat and Km (Fig. 6B). In contrast, kcat for the Asn279 mutants was similar to wild-type enzyme, but the apparent binding affinity was decreased, resulting in a lower catalytic efficiency as compared with wild-type enzyme (40). Catalytically competent cross-linking of the mutants of Asn279 generated very low labeling, whereas mutants of Asp276 displayed a significantly increased labeling relative to wild-type enzyme (Fig. 7). Labeling was, therefore, dependent on the apparent dNTP binding affinity (Km) and not catalytic efficiency or kcat. Interestingly, removing the charged side chain of Asp276 resulted in a strong increase in FABdCTP labeling. The effect was most pronounced when the acidic side chain was replaced with a hydrophobic side chain (i.e., Val), which could preserve van der Waals contact with the base. These results illustrate that labeling of the β-pol dNTP binding site is specific and that the ternary complex crystal structure can guide further study of dNTP binding and fidelity.

Acknowledgments—We thank Dr. Robert W. Sobol for helpful discussions, Dr. Thomas A. Kunke1 for critical reading of the manuscript and suggestions, and Kay Miller for typing the manuscript.

REFERENCES

1. Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., and Steitz, T. A. (1985) Nature 313, 762–765

2. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) Science 256, 1783–1790

3. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6320–6324

4. S. N. Khodyreva, A. S. Levina, M. I. Dobrikov, A. A. Koshkin, O. I. Lavrik, M. Buckle, M. Richetti, P. Roux, and M. Buc, submitted for publication.
dNTP Binding to HIV-1 Reverse Transcriptase and Mammalian DNA Polymerase \( \beta \) as Revealed by Affinity Labeling with a Photoreactive dNTP Analog

Olga I. Lavrik, Rajendra Prasad, William A. Beard, Igor V. Safronov, Mikhail I. Dobrikov, Deepak K. Srivastava, Gennadii V. Shishkin, Thomas G. Wood and Samuel H. Wilson

*J. Biol. Chem.* 1996, 271:21891-21897.
doi: 10.1074/jbc.271.36.21891

Access the most updated version of this article at [http://www.jbc.org/content/271/36/21891](http://www.jbc.org/content/271/36/21891)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/271/36/21891.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 42 references, 13 of which can be accessed free at [http://www.jbc.org/content/271/36/21891.full.html#ref-list-1](http://www.jbc.org/content/271/36/21891.full.html#ref-list-1)