Photoaffinity Labeling by 4-Thiodideoxyuridine Triphosphate of the HIV-1 Reverse Transcriptase Active Site during Synthesis

SEQUENCE OF THE UNIQUE LABELED HEXAPEPTIDE*

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The active site of HIV-1 reverse transcriptase (HIV-1 RT) was investigated by photoaffinity labeling based on catalytic competence. A stable ternary elongation complex was assembled containing enzyme, DNA template (RT20), DNA primer molecule (P12), and the necessary dNTPs (one of which was α-32P-labeled) needed for primer elongation. The photoaffinity probe 4-thiodideoxyuridine triphosphate was incorporated uniquely at the 3' terminus of the 32P-labeled DNA product. Upon photolysis, the p66 subunit of a HIV-1 RT heterodimer (p66/p51) was uniquely cross-linked to the DNA product and subsequently digested by either trypsin or endoprotease Lys-C. The labeled HIV-1 RT peptide was separated, purified, and finally subjected to Edman microsequencing. A unique radioactive hexapeptide (V\textsuperscript{276}RQLCK\textsuperscript{281}) was identified and sequenced. Our photoaffinity labeling results were positioned on the HIV-1 RT-DNA-Fab complex x-ray crystallography structure and compared with the suggested aspartic triad active site.

The enzyme reverse transcriptase (RT)\textsuperscript{1} derived from the HIV-1 virus is a heterodimer composed of two subunits p66 and p51, which are derived from the same sequence. A variety of experimental techniques have been directed at the elucidation of the active site and the mechanism of catalysis of the RT DNA polymerase activity, to assist in developing a strategy for treating HIV infection. Kinetic studies have established that an ordered sequential assembly of components forms a ternary complex, which then conducts a processive polymerization during the elongation phase (1–7). Genetic subtitution experiments have shown that several single amino acid interchanges D110Q, D185H, or D186N in the p66 subunit produce an inactive HIV-1 RT enzyme (8, 9). Studies involving specific amino acid derivatizations have suggested that Lys\textsuperscript{263} (10) and Arg\textsuperscript{277} (11) are critically involved at the active site. Photoaffinity labeling studies have yielded additional suggestions for the nucleotide binding site components: Lys\textsuperscript{273} (12), residues 288–307 (13), and residues 288–423 (14).

Several x-ray structures have been solved for the HIV-1 RT unliganded enzyme (Rogers et al. (15) reported a structure at 3.2 Å and Hsiou et al. (16) at 2.7 Å resolution), and for various ligands complexed with the holoenzyme; Kohlstaedt et al. (17) reported a nevirapine-RT enzyme structure at a resolution of 3.5 Å, and Jacobo-Molina et al. (18) reported a RT-dsDNA-Fab x-ray structure at a resolution of 3.0 Å. Model building efforts based primarily on the RT-dsDNA-Fab x-ray structure have yielded a detailed mechanistic proposal for the HIV-1 RT enzyme (19). The model is consistent with the critical involvement of the Asp-triad residues and two Mg\textsuperscript{2+}, as originally proposed by Steitz et al. (20).

The results reported in this paper utilized the photoaffinity probe S4-ddUTP to derivatize the active site of HIV-1 RT during productive synthesis involving a ternary complex. The chain terminating probe is located specifically at the 3'-OH end of the nascent product. The hexapeptide VRQLCK of the p66 subunit was the only target peptide that was detected. A general discussion of the possible mechanistic implications of these results is directed at making all the known topologic information compatible.

EXPERIMENTAL PROCEDURES

Materials—Recombinant HIV-1 reverse transcriptase purified from an Escherichia coli clone was kindly supplied by Dr. Christine Debouck and Dr. Jeffrey Culp from SmithKline Beecham Pharmaceuticals. The template and primer were a gift from Dr. Xiaolin Zhang at the Nucleic Acid Facility of the University of Pennsylvania Cancer Center. The sequence of the DNA templates RT19 and RT20 were 3'-d-[GCGCGGGGGCGCGGTGTA]-5' and 3'-d-[GCGGCGGGGGCGCGGTGTA]-5'. The sequence of the DNA primer P12 was 5'-d-[GCGCGGGGGCGCGGTGTA]-5'.

The nonradioactive deoxynucleotides (dATP, dCTP, and dGTP) and Promase E were purchased from Sigma. Radioactive nucleotide triphosphate [α-32P]dCTP (3,000 Ci/mol), reflection autoradiography film, and reflection intensifying screen were purchased from NEN Life Science Products. The nucleoside 4-thiodideoxyuridine was prepared and kindly provided by Dr. Robert Coleman of Ohio State University. The corresponding triphosphate was prepared from the nucleoside according to the method of Ruth and Cheng (21). Exonuclease III was purchased from Amersham Life Science. Sequencing grade endoprotease Lys-C was purchased from Promega. Sequencing grade modified trypsin was purchased from Boehringer Mannheim. HinP1I restriction endonuclease was purchased from New England Biolabs. All reagents for gel electrophoresis were purchased from Bio-Rad. Spectra/Por 6 molecular porous dialysis membrane (M\textsubscript{r} cut-off = 1,000) was purchased from Spectrum. Mini ProBlott\textsuperscript{TM} membranes were purchased from Applied Biosystems. The high intensity black lamp (model B-100A, long wave UV, which peaks at 365 nm) was purchased from Caplan.
Photoaffinity Labeling of HIV-1 Reverse Transcriptase

Eastern Corp. Computer program Insight II was purchased from Bio-sym Technologies.

Photoaffinity Labeling of HIV-1 RT—The standard reaction mixture (100 μl) for photoaffinity labeling was: 20 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 1.0 mM HIV-1 RT heterodimer (M, = 117,000), 10 mM DNA template, 70 mM DNA primer, and 32P radioactivity was excised from a 20% urea-PAGE method. The DNA primer was added and the mixture was incubated at 37 °C for 5 min. Immediately upon adding the substrates, the reaction mixture was placed in a depression well of an aluminum foil-covered, temperature-regulated aluminum block. The aluminum block was covered with an inverted Petri dish to prevent extensive evaporation of the reaction mixtures. The UV lower wavelength cut-off of a Pyrex Petri dish is 290 nm. An additional Petri dish, filled with water, was positioned on top of the first one to provide cooling. The lamp was positioned to shine the light from a distance of about 2 cm onto the top of the water-filled Petri dish. The reaction mixture was irradiated with UV (365 nm) for 60 min at 37 °C. To minimize the damage from the heat generated by the UV lamp, the water in the top Petri dish was replaced with cool water at 20 and 40 min.

A small aliquot of the photoaffinity labeling reaction mixture was removed and analyzed for protein and DNA product. Radioactive labeled and unlabeled HIV-1 RT was analyzed by 20% SDS-PAGE (22). The DNA fragment of the HIV-1 RT was analyzed by 20% urea-PAGE. In both cases, the radioactive bands were detected by autoradiography. For a quantitative analysis, radioactive bands of interest were excised for Cerenkov counting.

DNA Product Analysis—The DNA products were separated on the basis of molecular weight using 20% polyacrylamide (acrylamide:bisacrylamide = 19:1) gel electrophoresis containing 7 mM urea (dimensions: 170 × 140 × 1.7 mm). The electrophoresis buffer was 0.89 mM Tris, 0.89 mM boric acid, 20 mM EDTA, pH 8.3. The gel was preelectrophoresed with bromphenol blue and xylene cyanol dye markers in deionized formamide for 1 h prior to loading the samples. Sample mixtures of 15 μl were applied and electrophoresed for 3.5–4 h at 600 V until the xylene cyanol dye marker was about 5 cm from the bottom of the gel. The DNA products were visualized by autoradiography. The radioactive bands were excised and counted in Eppendorf tubes by the Cerenkov method.

Protein Analysis—For protein labeling analysis, samples from the photoaffinity labeling experiments were transferred with 2 × Laemmli buffer containing the bromphenol blue dye marker (22) at 1.1 (v/v) ratio and boiled at 100 °C for 3 min. The total samples were loaded onto a 10% polyacrylamide (acrylamide:bisacrylamide = 30:0.8) gel containing 0.1% SDS (dimensions: 275 × 140 × 7 mm). The electrophoresis buffer was 25 mM Tris glycine, pH 8.3. The electrophoresis was carried out at 150 V for 1 h and 300 V for another 4.5 h. The radioactive bands were visualized by autoradiography. For quantitative analysis, the radioactive bands were excised and counted in Eppendorf tubes by the Cerenkov method.

Cerenkov Counting Calibration—The same size gel bands containing variable amounts of 32P radioactive activity were excised from a 20% urea-PAGE (or 10% SDS-PAGE) and counted in Eppendorf tubes once. Each band was then transferred to a scintillation vial containing scintillation fluid and minced. The scintillation vial was filled with scintillation fluid and counted in the 32P channel for 5 min. The data from the Cerenkov counting was fitted with a linear function. The slope is the calibration factor for Cerenkov counting.

Proteolytic Digestion of the Labeled HIV-1 RT and Amino Acid Sequencing of the Labeled Proteolytic Product—The photoaffinity labeling reaction was followed by subsequent digestion. The reaction mixture was mixed with 10 μl of ExoIII digestion buffer and 5,000 units of ExoIII. The sample was subjected to centrifugation at 4 °C for 5 min. After centrifugation, the pellet was washed once with cold (–20 °C) 100% acetone and centrifuged at 4 °C for another 5 min. The pellet was air-dried and immediately dissolved into a buffer suitable for proteolytic digestion to form peptides (trypsin or Lys-C).

In the case of trypsin digestion, the pellet was recovered by dissolving it in 100 μl of trypsin digestion buffer (800 mM ammonium bicarbonate, pH 7.9, and 8 μl urea). The solution was incubated at 50 °C for 30 min, and then it was diluted 8-fold with H2O. Trypsin was added at a ratio of 1:20 (w/w). The trypsin digestion was incubated at 37 °C overnight.

In the case of Lys-C digestion, the pellet was recovered by dissolving it into 100 μl of denaturation buffer 2 (125 mM Tris-HCl, pH 7.7, 5 mM EDTA, and 5 μM urea). The solution was incubated at 50 °C for 30 min and then was diluted 5-fold with H2O. The Lys-C (1 μg/μl) was added at a ratio of 1:60 (w/w). The Lys-C digestion was incubated at 37 °C overnight.

To remove small radioactive nucleotides, excess salts, and urea, the peptide digestion solution was dialyzed against H2O at 4 °C overnight, then concentrated to 30 μl by SpeedVac. The molecular weight cut-off of the dialysis membrane was 1,000. The concentrated peptide solution was mixed with buffer (98% formamide, 0.1% bromphenol blue) at a ratio of 1:10 (v/v), and then heated to 90 °C for 30 min. The radioactive bands in the gel were visualized by autoradiography. A small amount of labeled peptide sample, which was digested with Pronase, was also loaded on to the same 20% urea-PAGE. The Pronase-sensitive radioactive band was the labeled peptide band of interest. This band was excised, quantified by Cerenkov counting, mined in a 1.5 ml Eppendorf tube, and then soaked in 0 ml of H2O.

The eluate was separated from the gel by centrifugation. The elution was repeated two more times so that the recovery of labeled peptide reached 90%. The eluates were combined, dialyzed against H2O, and concentrated again by SpeedVac.

The recovered peptide (about 20 pmol) was dissolved into 100 μl of H2O. To purify the peptide adduct, “gel band shift” procedure was conducted using the endonuclease HinP11. The DNA template (RT20) was added to the peptide solution at a ratio of 80:1 (template:peptide). The standard reaction buffer was also added to the peptide solution to give a final peptide solution containing 1 × HIV-1 RT reaction buffer. The DNA template and DNA product double helix (total volume was 178 μl) were annealed by boiling the mixture at 100 °C for 3 min and cooling it down gradually (30 min). About 20 μl of 1 × NEBuffer 2 (100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM MgCl2, and 10 mM dithiothreitol) and 2 μl of HinP11 (5 unit/μl) were added to the mixture. The final volume was 200 μl. HinP11 digestion was performed at 37 °C for 30 min. The reaction mixture was then dialyzed against H2O at 4 °C overnight. The molecular weight cut-off of the dialysis membrane was 1,000. The dialyzed solution was then concentrated to 15 μl by SpeedVac. The sample was analyzed on a 20% urea-PAGE, and the shifted labeled peptide was visualized by autoradiography.

RESULTS

Photoaffinity Labeling of HIV-1 RT with S4-ddUTP—A previous photoaffinity labeling study employing S4-ddUTP as a probe for the active site subunit of HIV-1 RT has been reported by Sheng and Dennis (14). The basic features of that study were repeated using the chain terminator S4-ddUTP, and similar results were obtained in that the labeling of the p66 subunit was light- and photoprobe-dependent and the efficiency of
digestion can be seen by comparing lanes 1 and 4 in panel A, after treatment with either trypsin (panel A) or lys-C (panel B). The autoradiogram of electrophoretically separated components in a 20% urea-PAGE after specific treatments and purification of the photoaffinity labeling reaction mixture. See procedure for details of the ExoIII digestion, trichloroacetic acid precipitation, trypsin or lys-C hydrolysis, and Pronase digestion.

Figure 1. Preparation of labeled HIV-1 RT peptide by trypsin (panel A) or lys-C (panel B). The autoradiogram of electrophoretically separated components in a 20% urea-PAGE after specific treatments and purification of the photoaffinity labeling reaction mixture. See procedure for details of the ExoIII digestion, trichloroacetic acid precipitation, trypsin or lys-C hydrolysis, and Pronase digestion.

The efficiency of labeling was calculated to be 2%, by measuring the total combined radioactivity of the p66 subunit isolated as p1 and p2 bands on a 10% SDS-PAGE (14) and assuming that all of the HIV-1 RT enzyme added to the reaction was catalytically active. The reaction mixture produced a total of 200 pmol of derivatized p66 subunit. The treatment with ExoIII followed by trichloroacetic acid precipitation yielded 150 pmol of p66 subunit. The labeled peptide recovered from the (n-p) gel band after the 20% urea-PAGE procedure was 45 pmol.

Figure 2. Purification of n-p by HinP1I band shift method. The autoradiogram of the 20% urea-PAGE separated components of the HinP1I-digested peptide derived from the p66 subunit treated with trypsin (panel A) or lys-C (panel B). Lane 1 serves as a marker for the reaction components T-n and n. The band denoted n-p is the component containing the peptide fragment covalently linked to the radioactive DNA 20-mer product, and the band denoted n'-p is the digestion fragment produced by treatment of n-p with the HinP1I nuclease (lane 2). Details can be found under “Experimental Procedures.”

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(CGCCACAACAS4ddU-peptide). The cleavage site for HinP1I endonuclease on the target adduct is shown below.

\[
\text{n-p: } 5'-\text{CGCGCCCCG} \downarrow \text{CGCCACAACAS4ddU-peptide} \\
\text{Template RT20: } 3'-\text{GGCGGGGCGC} \downarrow \text{GATTTTTA-5'}
\]

**Sequence 1**

**TABLE I**

| n'-p          | Amino acid detected in successive cycles (pmol) |
|---------------|-------------------------------------------------|
|               | Val | Arg | Gln | Leu | Cys | Lys |
| 1) Trypsin generated | 0.6 | 0.2 |
| 2) Lys-C generated   | 1.4 | 0.9 | 0.4 | 1   |     |     |
| 3) Lys-C generated   | 1.7 | 0.75| 0.4 | 0.76|
| 4) Lys-C generated   | 1.6 | 0.8 | 0.65| 0.9 |
| 5) Lys-C generated*  | 2.3 | 0.9 | 1.6 | 1   |

* n'-p peptide isolated when RT19 template replaced RT20 template in photoaffinity reaction mixture.

Sequence Analysis of the n'-p Fragments—The n'-p fragments resulting from HinP1I treatment of the n-p component shown in Fig. 2 were transferred by electroblotting to a PVDF membrane and autoradiographed to visualize the band of interest, which was excised and a portion subjected to a Edman microsequencing. The total sample was calculated to be 12–20 pmol. The analytical data for several sequence analysis are presented in Table I for either the trypsin- or Lys-C-generated n'-p samples.

The sequence analysis for the n'-p peptide derived from a trypsin digestion sample gave evidence of a tetrapeptide that did not allow the identification of the amino acids located in either position 1 or 3. This result did not allow distinction between two possible tetrapeptide sequences ELNK (positions 79–82) or QLCK (positions 278–281). The sequence analysis for the n'-p derived from a Lys-C digestion sample was therefore conducted to make the selection between these choices since the nonomer peptide LVDFRELNK would be produced in place of the ELNK trypsin-generated tetrapeptide and the hex-

![A](image1.png)

**Panel A**, a stereo view of HIV-1 RT looking down the axis of the bound duplex DNA from the top of a closed “right hand” (right-hand analogy of Kohlstaedt et al. (17)). The colored spheres denote certain α carbons of RT: Asp triad (yellow), Lys73 (green), Lys263 (white), Arg277 (purple), Cys280 (red), and Thr290 (orange). The phosphorus atoms of the primer product strand are shown in blue except for the 3'-terminal phosphate, which is striped. The α carbon backbone of RT is traced in pink. **Panel B** is the same view as panel A without the α carbon backbone of RT. **Panel C** is the same view as panel B but rotated 90°.

![B](image2.png)

**FIG. 3. Various derivatized α carbon atoms positioned in the 3 Å x-ray structure of the RT-dsDNA Fab complex (18).**
FIG. 4. Topographic relationship of various derivatized α carbons suggested to be at the active site of HIV-1 RT. Panel A shows the radial location of various α carbons of the RT molecule about the axis of the duplex DNA as viewed in Fig. 3A. The bold arrow indicates the direction of rotation of the duplex DNA during successive translocations that accompany polymerization. Panel B gives the distances (in Å) between certain α carbons of RT.

Photoaffinity labeling of HIV-1 reverse transcriptase

In our present study using S4-ddUTP as the photoprobe, we isolated a 16-kDa labeled fragment (288–423) from the ternary complex and was incorporated as a chain terminator. Exposure of the photoprobe to ultraviolet light resulted in derivatization of the amino acid Cys 280 as an accessible target, presumably in close proximity to the active site. The isolation of a target hexapeptide that is somewhat different from our initial study (14) could reflect the possible derivatization with the photoprobe. Since we were unable to successfully achieve the alkylation, we suggest that the Cys 280 is the most likely choice of the amino acid that is covalently linked with the photoprobe.

DISCUSSION AND CONCLUSION

In our previous study of the photoaffinity labeling of the HIV-1 reverse transcriptase using S4-dUTP as a photoprobe (14), we isolated a 16-kDa labeled fragment (288–423) from the p66 subunit and sequenced the N-terminal portion (288–313). In our present study using S4-ddUTP as the photoprobe, we have isolated a unique labeled hexapeptide (276–281). The photoprobe in our present studies was positioned at (and only at) the 3’ end of the primer terminus of an actively synthesizing ternary complex and was incorporated as a chain terminator. Exposure of the photoprobe to ultraviolet light λ360 nm resulted in derivatization of the amino acid Cys 280 as an accessible target, presumably in close proximity to the active site. The isolation of a target hexapeptide that is somewhat different from our initial study (14) could reflect the possible different binding options available for the different photoprobes. For example, if translocation occurs prior to photolysis, the S4-dUTP photoprobe could engage in a binding interaction at the 3’-hydroxy binding site for the next phosphodiester bond forming event. The S4-ddUTP would have no such new binding option since it does not have a 3’-hydroxyl group.

Extensive studies have been conducted to elucidate the location of the active site and the mechanism of the DNA polymerase activity of HIV-1 RT (24). Attention has been focused on the role of the Asp-triad in catalysis, since initial genetic substitution experiments by Larder et al. (8, 9) and Boyer et al. (25) showed that HIV-1 RT containing either of the mutants D110Q, D185E, D186E, D110E, or D186N (and several others) were essentially inactive. This aspartate triad is a strongly conserved feature of many nucleic acid polymerases (26). The availability of several x-ray structures of HIV-1 RT has greatly stimulated a detailed consideration of the active site of the polymerase activity (17, 18). The structure of RT−dsDNA−Fab at a resolution of 3.0 Å (18) showed that the α phosphate of a modeled incoming nucleotide triphosphate could be positioned in close proximity to the Asp triad, which is located near the 3’-OH terminus of the primer strand of the complexed dsDNA. This observation has promoted several detailed mechanistic proposals and suggestions for a two/three divalent metal Asp triad mechanism (17, 19, 20, 27) for DNA polymerases (HIV-1 RT, Klenow, T7 DNA polymerase, as well as DNA polymerase β).

Kinetic descriptions of the catalytic events have assisted in the formation of mechanistic and structural proposals in that a binary complex involving the complexation of the template−primer with the enzyme appears to be required prior to the binding of the dNTP substrate. A conformational change occurs in the HIV-1 RT−DNA complex coincident with the binding of the substrate (28). The formation of this binary complex is greatly enhanced when the single stranded template extends to 7 or more nucleotides upstream from the 3’ primer terminus (29).

Amino acid derivitizations have been conducted to implicate specific amino acids in the RT polymerization event. Pyridoxal phosphate was complexed with Lys 263 and reduced to form a stable covalent derivative, which produced an inactive RT enzyme (10). Phenyl glyoxal was used to form a unique derivative with Arg 277, which also inactivated the polymerization activity of the RT enzyme (11).

Photoaffinity labeling studies of HIV-1 RT in various stages of assembly or catalysis have been conducted to implicate various amino acids. The photolysis of the holoenzyme HIV-1 RT in the presence of dTTP produced a derivatized Lys 73, which was suggested to be at or near the dNTP substrate binding site (12). The photolysis of RT in the presence of short oligonucleotide primers bound to template yielded derivatized enzyme, which was linked to Leu 296-Thr 299 or Leu 296-Thr 296 (13, 30).

The catalytic competent ternary complex has been derivatized using the photoprobe S4-dUTP (14) or the photoprobe (FABdCTP) (31). A large peptide containing the derivative was reported by Sheng and Dennis (14). Our present study using S4-ddUTP as a photoprobe with a catalytically competent strategy, photoaffinity labeled the p66 subunit of HIV-1 RT and allowed the isolation of the hexapeptide Val 276–Lys 281 with...
Cys$_{280}$ as the derivatized amino acid.

We have collected the data from many diverse experimental approaches to the elucidation of the active site of HIV-1 RT and attempted to integrate the information. In Fig. 3 (A–C), we have examined the topographic locations of the Cα carbons of the various targeted amino acids as they would be positioned in the reported 3.0-Å x-ray structure of the RT-dsDNA-Fab complex (18).

A cluster of α carbons of certain targeted amino acids suggested to be involved at the active site of the polymerase are positioned at about a 90° clockwise rotation from the terminal phosphate. This rotation would correspond to about 2–3 translocations during synthesis (assuming ~36°/dNTP added) if the aspartic 185 served as a fixed marker for the catalytic site of the polymerase event. The location of Lys$_{73}^a$ is about 90° in the opposite direction (counter clockwise) and might correspond to a location of the single-stranded template upstream from the active site (Fig. 4A).

The large distances between these targeted α carbons and the aspartic 185 suggested to be at the catalytic site are problematic (Fig. 4B). The static “snapshot” of the enzyme complexed with the duplex template-primer moiety might misrepresent the location of the 3’ end of the product as being positioned in the double helix when, in fact, the dynamic synthesizing ternary complex might contain a segment (3 or more nucleotides long) that is quite flexible and not fixed in the final duplex helix.

The active site of a polymerase also presents problems with respect to topographic assignments of function to structure since derivatization (e.g. by a photoprobe substrate) could occur either before or after translocation. A substrate probe containing a photoactive group in the binding recognition loci (4-thio moiety) might effectively target the substrate binding site prior to translocation but would be located at a very different site after translocation. In contrast, a substrate probe containing a photoactive group in the 3’ moiety might be positioned at the catalytic site only after bond formation (involving the 5’-phosphate), followed by translocation to position the 3’-hydroxyl for formation of the next phosphodiester bond.

Kinetic studies have indicated certain conformational changes in HIV-1 RT and one could consider that the metal/Asp triad loci not only functionally masks the negative charge of the incoming nucleotide triphosphate but actually escorts the pyrophosphate product away from the newly formed phosphodiester bond at the catalytic site. We are currently investigating a chain terminating photoprobe, which appears to derivatize at the catalytic site of the polymerase, since the probe is located at the 3’-position of the substrate analogue.

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