Active Site Labeling of HIV-1 Reverse Transcriptase

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ABSTRACT: The human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT) heterodimer (M_r = 66 000 and M_r = 51 000) has been photoaffinity labeled using 4-thiodeoxyuridine triphosphate (S^4-dUTP) as a probe. A nascent polymerization complex was assembled from a single-stranded DNA template, a 12-mer DNA primer, and the necessary dNTPs (one of which was α-^{32}P-labeled) to extend the primer to produce the $n-1$ product. The photoaffinity probe was then uniquely added at the 3′-terminal position of the extended primer bound at the catalytic site and photolyzed. The larger subunit (p66) was exclusively derivatized. The unique radioactive peptide resulting from proteolysis was isolated and identified by amino acid sequencing.

One of the reactions catalyzed by the reverse transcriptase enzyme (RT)[1] from the HIV-1 is the synthesis of the plus (+) strand DNA using the minus (–) strand DNA as a template. The functional enzyme is a heterodimer composed of a p66 and a p51 subunit.

A number of mechanistic studies of this enzyme have been conducted. 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 (Majumber et al., 1988, 1989; Kedar et al., 1990; Reardon & Miller 1990; Huber et al., 1989; Huber & Richardson 1990).

Several studies have been conducted to identify protein domains involved in specific functions of the reverse transcriptase. Treatment of the RT heterodimer with pyridoxal phosphate (PLP), followed by reduction with sodium borohydride, abolished the polymerase activity (Basu et al., 1989). The addition of the template and substrate dNTP prior to PLP prevented the inactivation of the enzyme. The Lys_{263} residue was shown to be derivatized and was suggested to be at or near the substrate binding domain. The kinetic inhibition by PLP was complex, and both of the subunits, p90 and p100 (these are the fusion protein analogs of p66 and p51), were equally labeled by the derivatization procedure.

Photolytic cross-linking (λ 254 irradiation) of the p66 subunit of the homodimer (p66/p66) or the heterodimer (p66/p51) to either the substrate [α-^{32}P]dTTP or the 5′-^{32}P-labeled template-primer rA_{12-18}·dT_{10} has been reported (Cheng et al., 1991). The photoaffinity labeling is prevented by competing substrates (e.g., dCTP) or an analogue inhibitor (e.g., AZTTP) and therefore is suggested to occur at the primer-template and or substrate binding site.

Recently, complexes between the p66 homodimer form of HIV-RT and suitable primer analogs [$d(T)_8$ and $d(T)_{16}$] have been cross-linked by photolysis (Sobol et al., 1991). The derivatized peptide was identified as the residue 195–300 amino acid segment and was suggested to be the primer binding site of the enzyme.

Photoaffinity labeling of various RNA polymerase enzymes has been achieved in our laboratory [yeast Pol I (Kelly et al., 1990) and yeast Pol II and wheat germ Pol II (Sheng, et al., 1992)] using a single-stranded synthetic DNA template, a trimer primer, and [^{32}P]-NTP's. The components were assembled to produce a bound transcript which then adds the photoprobe (S^4-UTP) uniquely at the 3′ end of the bound product. This procedure was used to identify which subunit was involved in the catalytic event.

This experimental approach has now been successfully applied to the HIV-RT enzyme operating in the elongation mode. The photoprobe 4-thiodeoxy-UTP (S^4-dUTP) derivatizes only the p66 subunit.

EXPERIMENTAL PROCEDURES

Materials. Recombinant HIV-1 reverse transcriptase was purified from an *Escherichia coli* clone IIIb(BH10) and kindly supplied by Dr. Christine Debouck and Dr. Jeffrey Culp (Smith Kine & Beecham Pharamceuticals). Single-stranded synthetic DNA templates and primer were the kind gift of Dr. Lucinda Ivanoff (Smith Kline & Beecham Pharmaceuticals). Nonradioactive dATP, dCTP, and TTP were purchased from Sigma. [α-^{32}P]dCTP and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from New England Nuclear. Photoaffinity probes AzTTP and T505 [a succinylfluorecein derivative of TTP described by Proper et al. (1987)] were kindly supplied by Dr. George Trainer (Du Pont Merck Pharmaceutical Co.), and N_3-dUTP was provided by Dr. Boyd E. Haley (University of Kentucky, Lexington, KY). T4 polynucleotide kinase and protein molecular weight markers were purchased from Promega. DNase I was purchased from ICN. All reagents for gel electrophoresis were of electrophoresis grade and were obtained from Bio-Rad. 4-Thio-2′-deoxyuridine and all other chemicals were from Sigma. Photolysis was conducted using a UVP Inc. lamp (Model UVGL-58) which has a maximum intensity output at a wavelength of 366 nm.

DNA Templates and Primer. Single-stranded synthetic DNA templates are ST18, 3′d[GCGCGGGGCGCGGTGGTA]5′; ST19, 3′d[GCGCGGGGCGCGGTGTGTA]5′; ST20, 3′d[GCGCGGGGCGCGGTGTTGTA]5′; and ST21, 3′d[GCGCGGGGCGCGGTTGTTGTA]5′. The sequence of primer P12 is 5′d[CGCGCCCCGCGC]. When the primer (P12) was used to label product, the ^{32}P was introduced into the 5′-terminal position according to Davis et al. (1986).

Synthesis of Plus (+) Strand DNA. A DNA synthesis reaction mixture (total volume 20 μL) was 20 mM Tris-HCl,

[1] Abbreviations: AzTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; N_3-dUTP, 3-azidodeoxyuridine 5′-triphosphate; S^4-dUTP, 4-thiodeoxyuridine 5′-triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; DTT, dithiothreitol; dNTP, deoxynucleotide triphosphate; EDTA, ethylenediaminetetraacetic acid; DNase I, deoxyribonuclease I.

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pH 7.9, and 6 mM $MgCl_2$ and contained 1.7 μg of HIV-1 RT heterodimer (M_r = 117 000) and 0.15 nmol each of DNA template (ST) and primer (P12). Nonradioactive substrates (dATP and TTP) were added to a final concentration of 132 μM, and the radioactive substrate [α-^{32}P]dCTP (150 Ci/mmol) was added to a final concentration of 13.2 μM. First, the primer was annealed to the template in 10 μL of reaction buffer (20 mM Tris-HCl, pH 7.9, and 6 mM $MgCl_2$) by heating to 100 °C for 2 min and slowly cooling to room temperature. The HIV-1 RT was then added, and the mixture was incubated at 37 °C for 5 min. After the substrates were added, the incubation was continued at 37 °C for 15 min. To terminate the reaction, EDTA was added to a final concentration of 14 mM. In some experiments the label was inserted into the product by using 5′-^{32}P-labeled primer P12.

Product Analysis of DNA Synthesis. A 5-μL aliquot of the DNA synthesis reaction mixture was used for product analysis on a 20% polyacrylamide–7 M urea gel. Prior to the sample being loaded, the gel was preelectrophoresed at 800 V for 30–45 min. A 10-μL aliquot of loading solution (0.01% bromphenol blue in deionized formamide) was added to the sample, and electrophoresis was performed at 800 V until the dye reached the bottom of the gel. The product was visualized by autoradiography of the wet gel.

Preparation of 4-Thio-dUTP. 4-Thio-dUTP was prepared from 4-thio-2′-deoxyuridine according to the method of Ruth and Cheng (1981).

Photoaffinity Labeling of HIV-1 RT. A reaction mixture (20 μL) for photoaffinity labeling was 20 mM Tris-HCl, pH 7.9, and 6 mM $MgCl_2$ and contained 1.7 μg of HIV-1 RT heterodimer (M_r = 117 000) and 0.15 nmol each of DNA template (ST) and primer (P12). The nonradioactive substrate dATP and photoprobe were added (as indicated) to a final concentration of 132 μM. The radioactive substrate [α-^{32}P]-dCTP (150 Ci/mmol) was added to a final concentration of 13.2 μM. The primer was annealed to the template as described above; then HIV-1 RT was added and the mixture was incubated at 37 °C for 5 min. Immediately after the addition of substrate, the reaction mixture was placed in an individual well of a microtiter plate and irradiated from the top for 15 min at 37 °C with UV light as indicated. A time study conducted with S^4-dUTP showed linear incorporation of radiolabel into the RT enzyme over a 60-min period (data not shown).

Samples to be digested with DNase I were transferred to a 1.5-mL Eppendorf tube, and DNase I was added to a final concentration of 0.9 mg/mL. After incubation at 37 °C for 30 min, the reaction was stopped by heating to 100 °C.

Photoaffinity-labeling reaction samples were separated by 10% SDS–PAGE (Laemmli, 1970) and detected by autoradiography. For quantitative analysis, the radioactive bands of interest were excised for Cerenkov counting.

Proteolytic Digestion of the Labeled Subunit and Amino Acid Sequencing of the Labeled Proteolysis Product. The photoaffinity-labeling reaction [total volume 10 mL containing 10 nmol of HIV-1 RT and 100 nmol each of DNA template (ST18) and primer (P12)] was performed similarly as described above. After UV irradiation, the reaction mixture was heated to 100 °C for 5 min, quickly chilled in ice, and incubated in ice overnight. The precipitated protein was isolated by centrifugation at 13000g/min for 10 min, dissolved in 200 μL of protein denaturation buffer (25 mM Tris-HCl, pH 7.9, 2% SDS, and 5% 2-mercaptoethanol), and heated to 95 °C for 20 min. After denaturation, the reaction was allowed to cool, and buffer (25 mM Tris-HCl, pH, 7.9, and 1 mM

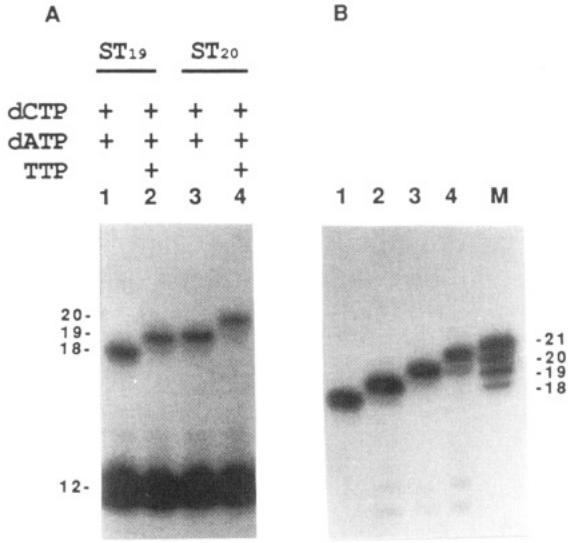

FIGURE 1: Autoradiogram of electrophoretically separated plus-strand products derived from primer elongation by HIV-RT in the presence of different templates. In panel A the standard reaction mixture of 20 μL contained HIV-RT heterodimer (14 pmol), 5′-^{32}P-labeled 12-mer primer (150 pmol, 86 nCi/pmol), and 150 pmol of the specific DNA template, and each substrate dNTP was added to give a final concentration of 132 μM. In panel B the label was introduced by adding [α-^{32}P]dCTP (150 Ci/mmol, final concentration 13.2 μM). The reactions were assembled and incubated as described under Experimental Procedures. In panel A, the products from two different templates (ST19 and ST20), using different combinations of substrate dNTP's, are shown. In panel B the products from four different templates (ST18–21) are shown, where TTP was replaced by S^4-dUTP at the same final concentration.

EDTA) was added until the concentration of SDS was 0.1%. Endoproteinase Lys-C was added at a protein:protease ratio of 27:1, and the mixture was incubated at 37 °C for 20 h. The proteolysis products were analyzed by 15% SDS–PAGE and detected by autoradiography. The gel slice containing the smallest radioactive peptide (~16 kDa) was excised, and the peptide was eluted into 1.3 mL of buffer (10 mM Tris-HCl, pH 7.9, and 0.01% SDS) at 25 °C for 20 h. After centrifugation the supernatant was removed, reduced in volume to 300 μL, and then digested with DNase I (final concentration: 0.9 mg/mL) at 37 °C for 60 min. The sample was analyzed on a second 15% SDS–polyacrylamide gel, and the radioactive peptides were electrotransferred to a 0.45-μm Immobilon P (PVDF) membrane in 10 mM CAPS and 10% methanol, pH 11.0. The peptides were visualized by autoradiography and Coomassie brilliant blue staining. The radioactive peptide of interest was subjected to amino acid sequencing analysis.

RESULTS

Plus (+) Strand DNA Synthesis. The synthesis of positive-strand DNA by HIV-RT was examined using single-stranded DNA templates, a 5′-^{32}P-labeled 12-mer DNA primer, and appropriate dNTP's as substrate. The products were separated by electrophoresis on a urea-containing polyacrylamide gel and analyzed by autoradiography. Synthesis of the template-directed extension of the primer to produce the full-length product (n) requires that dCTP, dATP, and TTP be provided as substrates, whereas synthesis in the absence of TTP produces the $n - 1$ product. This $n - 1$ product is extended to the full-length product (n) by the inclusion of either TTP or one of the photoprobes (e.g., S^4-dUTP). In Figure 1, panel A, the product formed from the 5′-^{32}P-labeled 12-mer primer in the presence of various dNTP substrates is presented for several

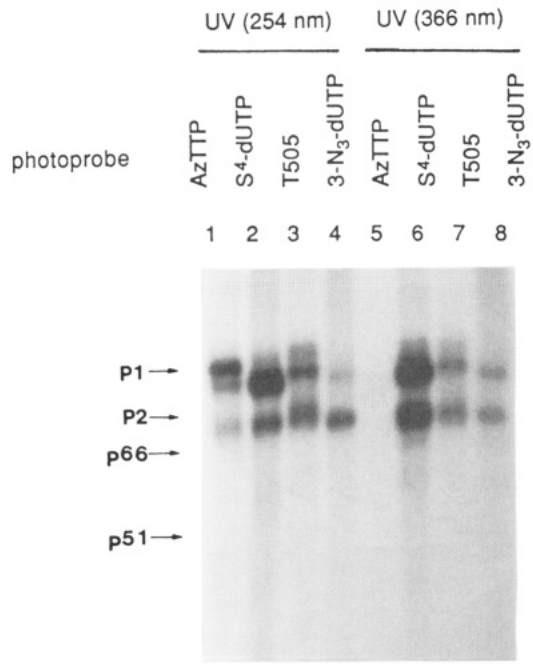

FIGURE 2: Autoradiogram of the electrophoretically separated photoaffinity-labeled (^{32}P-containing) protein subunits (P_1 and P_2) of the HIV-RT, using various photoprobes at two different wavelengths. The standard reaction mixture of 20 μL contained HIV-RT heterodimer (14 pmol), 12-mer primer (150 pmol), and ST19 DNA template (150 pmol), and each unlabeled substrate dNTP was added to give a final concentration of 132 μM. The substrate [α-^{32}P]dCTP (150 Ci/mmol) was added to give a final concentration of 13.2 μM. The reaction mixtures were assembled, incubated, and irradiated for 15 min (for photolysis details, see Experimental Procedures). The locations of the p66 and p51 subunits are indicated.

different templates. In panel A (lane 1 and lane 3) the $n-1$ product for two different length templates is formed when TTP is omitted from the reaction mixture, whereas the n product is formed (lane 2 and lane 4) when TTP is included. In panel B the labeled product contains [α-^{32}P]CMP; the full-length products (n) for each of a set of four templates differing in length by one base are shown (panel B, lanes 1–4) along with a mixture of 5′-^{32}P end-labeled DNA markers as indicated (lane 5). The positive-strand DNA products were sequenced and found to correspond to their specific template (data not shown).

Photoaffinity Labeling of HIV-RT with Various Photoprobes. In Figure 2, the photoaffinity labeling of the HIV-RT polymerization complex was analyzed by autoradiography of the components separated by electrophoresis on 10% SDS–polyacrylamide gel. The reaction mixtures were assembled and exposed to irradiation with either 254- or 366-nm UV light. The HIV-RT enzyme was derivatized in all cases except for the case when the AzTTP probe was irradiated with 366-nm UV light (lane 5). The migration of the radioactive bands (P_1 and P_2) was retarded compared to the migration position of the monomers of RT (p66 and p51 positions are indicated from silver-stained control markers), presumably due to the attached DNA polynucleotide. The efficiency of photoaffinity labeling with each photoprobe under standard conditions was determined by excising the P_1 and P_2 bands and assaying for radioactivity by Cerenkov counting. The values for femtomoles of the p66 subunit labeled are presented for lanes 1–8, respectively: 139, 261, 168, 101, 21, 360, 108, and 88. The highest value was obtained for photolysis of S^4-dUTP by using a 366-nm maximum-intensity UV light source.

In order to identify the protein components of the radiolabeled bands resulting from photolysis of the polymerase

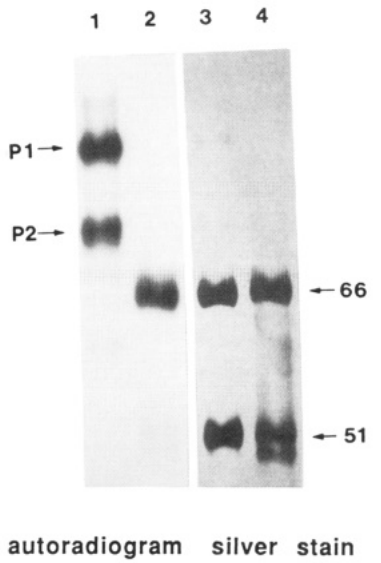

FIGURE 3: The electrophoretically separated, S^4-dUTP photoaffinity-labeled protein subunit (P_1 and P_2) of the HIV-RT with and without exposure to DNase I digestion. The control reaction is shown in lane 1 (autoradiogram) and lane 3 (silver-stained). The control reaction treated with DNase I is shown in lane 2 (autoradiogram) and lane 4 (silver-stained). The locations of the p66 and p51 subunits are indicated.

complex, a standard reaction was conducted using S^4-dUTP as the photoprobe. In Figure 3, the autoradiograms of the electrophoretically separated bands P_1 and P_2 are shown before (lane 1) and after controlled exposure to DNase I (lane 2). The corresponding set of silver-stained bands for the same native HIV-RT (lane 1 and lane 2) are shown in lane 3 and lane 4, respectively. We suggest that the removal of most of the ^{32}P-containing DNA polynucleotide from the P_1 and P_2 bands produces a single radiolabeled band which comigrates with the p66 subunit of the HIV-RT heterodimer. This suggestion is supported by the fact that the radioactive bands P_1 and P_2 are located at positions corresponding to molecular masses of 78.3 and 72.1 kDa, which would be the predicted positons for the p66 subunit having an increased mass of either 12.3 kDa (ST_{19} template and 19-mer transcript) or 6.1 kDa (19-mer transcript). The corresponding radioactive bands representing increased mass for the p51 subunit (57.1 and 63.3 kDa) were not observed.

Specificity of the Photoaffinity Labeling Procedure. The dependence of the photolabeling procedure on the photoprobe S^4-dUTP was investigated by comparing the amount of ^{32}P-labeled product bound to the reverse transcriptase enzyme as the mole ratio of S^4-dUTP/TTP was varied at constant total concentrations. In Figure 4 the results are shown where the amount of p66-labeled subunit increases progressively as the mole ratio of S^4-dUTP increases. The incorporation of the ^{32}P label into the p66 subunit is dependent on the incorporation of the nonradioactive photoprobe S^4-dUTP into the 3′-terminal position of the plus (+) strand DNA.

The dependence of the photolabeling procedure on the existence of a polymerase complex containing the nascent plus (+) strand DNA product was investigated by subjecting the reaction complex to various perturbations prior to or during irradiation. In Figure 5 the autoradiogram of the electrophoretically separated radioactive components is presented. In lane 1 the standard reaction mixture was incubated for 15 min and then irradiated for 15 min. In lane 2 the standard reaction was repeated except that 2 μL of 0.15 M EDTA was added to stop the polymerization prior to irradiation. The

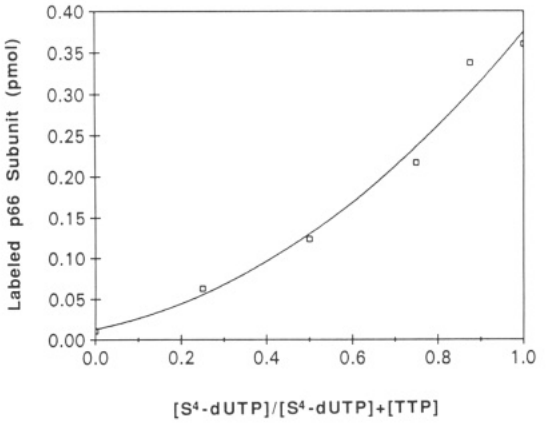

FIGURE 4: Relationship between the ^{32}P photoderivatized HIV-RT (p66) subunit and the mole ratio of S^4-dUTP to TTP. The standard reaction mixture, assembly, incubation, and irradiation were used. The gel bands of the p66 subunit (P_1 and P_2) were excised and assayed for radioactivity by Cerenkov counting.

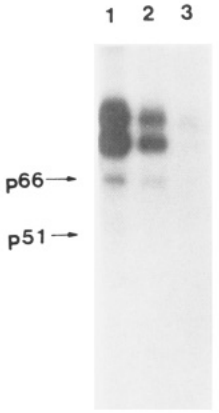

FIGURE 5: Dependency of the photoderivatization of HIV-RT on the existence of a polymerase complex. The standard reaction mixture was assembled and incubated for 15 min, followed by an additional 15 min of irradiation with light at λ 366 nm (lane 1). The standard reaction was assembled and incubated for 15 min; then 2 μL of 0.15 M EDTA was added 5 min prior to photolysis (lane 2). The standard reaction mixture was assembled and incubated for 15 min; then 2 μL of 10% SDS was added 5 min prior to photolysis (lane 3).

amount of labeled enzyme was reduced to 22% of the value obtained for lane 1 (cpm's of P_1 and P_2 bands are combined for the comparison). In lane 3 the standard reaction was repeated again except that 2 μL of 10% SDS was added to dissociate the polymerase complex prior to irradiation. The amount of labeled enzyme was reduced to 1% of the value obtained for lane 1.

These results suggest that the polymerase complex containing the bound product is required for photolytic derivatization of the active site subunit (p66). The polymerase reaction continues during the irradiation period, and an additional amount of enzyme is derivatized. The addition of SDS prior to irradiation disengages the formed product from the complex and reduces the photoderivatization to a negligible amount (~1%).

In order to investigate the possibility that the newly formed radioactive duplex DNA dissociates and reassociates prior to photolytic cross-linking to the catalytic site, the standard reaction was conducted in the presence of a 3-fold excess of the cognate nonradioactive DNA duplex [except that UMP replaced S^4-UMP at the 3′ end of the plus (+) strand product]. The amount of radioactive p66 subunit produced was unchanged. This result suggests that photoaffinity labeling by the S^4-UMP located at the 3′ end of the newly synthesized DNA plus (+) strand occurs on the p66 subunit without exchanging with unbound duplex DNA in solution.

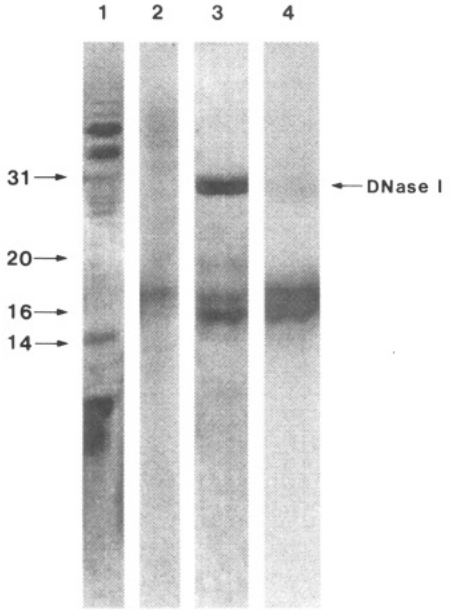

FIGURE 6: Peptide profile of the endoproteinase Lys-C digested p66 subunit. The peptides were produced and separated as described under Experimental Procedures. The profiles in lane 1 (Coomassie brilliant blue stained) and lane 2 (autoradiography) were obtained from the proteinase-digested photoaffinity-labeled reaction mixture. The p18 band was excised, treated with DNase, and rerun in the same gel. The p18 as well as a new band at p16 can be seen in lane 3 (Coomassie brilliant blue stained) and lane 4 (autoradiography).

In order to characterize the radioactive labeled domain of the p66 subunit, a large amount of the RT enzyme (10 nmol) was subjected to photoaffinity labeling, followed by endoproteinase Lys-C digestion. The resultant peptides were separated by SDS–PAGE, and the results are shown in Figure 6, lane 1 (Coomassie brilliant blue stained) and lane 2 (autoradiography). Essentially, only one peptide having a molecular weight of 18 kDa contained the radioactive label (lane 2). The radioactivity located in the p18 kDa peptide represents 30% of the total radioactivity incorporated into the RT heterodimer. In order to avoid contamination by any other unlabeled proteins which have the same mobility as the labeled peptide, the labeled peptide (in lane 2) was excised and eluted from the gel. The sample was then treated with DNase I to remove the attached DNA and analyzed by a second 15% SDS–PAGE (Figure 6, lane 3 and lane 4). If there was any contaminating peptide, it would have the same mobility in the second gel, while the labeled peptide fragment would show an increased mobility because the attached DNA was partially removed. The data showed that most of the protein was shifted to the lower position of the gel (lane 3). The autoradiograph of the second gel (lane 4) indicated that the shifted protein (~16 kDa) contained about one-third and the unshifted protein contained two-thirds of the total radioactivity, which was expected since the radioactive label dCMP was distributed along the DNA chain. The amino acid sequence of the p16 band confirmed that it was a single peptide beginning with alanine 288 (Table I). No radioactivity was found to be associated with any of the sequenced amino acids, suggesting that the labeled amino acid(s) is (are) beyond residue 313. On the basis of the size of the labeled peptide (16 kDa), we suggested that the catalytic site(s) of HIV-1 RT is (are) located on the subunit p66, between residues 314 and 423.

Table I: N-Terminal Amino Acid Sequence Analysis of the Radioactive Labeled (16-kDa) Peptide

cycle	residue	pmol	cpm	cycle	residue	pmol	cpm
1	Ala	21.74	23.0	15	Glu	9.17	25.0
2	Leu	15.70	23.5	16	Leu	8.88	17.0
3	Thr	10.69	27.0	17	Ala	7.86	19.5
4	Glu	12.87	58.5	18	Glu	7.45	21.5
5	Val	10.71	19.5	19	Asn	5.16	20.0
6	Ile	13.79	16.5	20	Arg	4.02	26.5
7	Pro	13.65	27.5	21	Glu	6.55	22.5
8	Leu	12.46	18.0	22	Ile	5.71	18.5
9	Thr	6.74	16.5	23	Leu	6.49	21.0
10	Glu	8.92	24.0	24	Lys	3.33	25.5
11	Glu	11.10	26.0	25	Glu	5.40	23.0
12	Ala	10.97	22.0	26	Pro	5.58	20.5
13	Glu	9.05	26.0	27		<1	19.0
14	Leu	9.74	21.5				

DISCUSSION

The p66 subunit of the HIV reverse transcriptase heterodimer involved in the catalysis of the elongation phase of the polymerization forming the plus (+) strand DNA product has been derivatized using the photoaffinity probe 4-thiodeoxyuridine triphosphate. Previous investigations of the HIV-RT enzyme have not positioned the probe uniquely at the catalytic site but have derivatized either the domain accessible to a bound primer [Cheng et al., 1991 (p66 subunit); Sobol et al., 1991 (peptide 195–300)] or a specific amino acid which directly or indirectly effected the kinetic behavior of the enzyme [Basu et al., 1989 (Lys_{262}); Mitchell & Cooperman 1992 (Arg_{277})]. Structure–function studies in progress with the HIV-RT enzyme might benefit from the use of a photoaffinity probe which is uniquely positioned at the 3′ terminus of the nascent product complex and therefore has a good possibility to derivatize the catalytic site of the enzyme. The derivatization target on the p66 subunit of the HIV-RT enzyme was located in a 16-kDa peptide between residues 314 and 423.

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