Purification and Characterization of Heterodimeric Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase Produced by in Vitro Processing of p66 with Recombinant HIV-1 Protease*

(Received for publication, February 19, 1992)

Debasish Chattopadhyay, David B. Evans, Martin R. Deibel, Jr., Anne F. Vosters, Frances M. Eckenrode, Howard M. Eispahr, John O. Hui, Alfredo G. Tomasselli, Heidi A. Zurcher-Neely, Robert L. Heinrikson, and Satish K. Sharma†

From The Upjohn Company, Kalamazoo, Michigan 49001

Active recombinant reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) with an amino-terminal extension containing a hexa-histidine sequence has been prepared in milligram quantities in a pure heterodimeric (p66/p51) form by coordinated applications of immobilized metal affinity chromatography (IMAC) and HIV-1 protease treatment. The precursor protein, isolated from extracts of recombinant Escherichia coli by IMAC in a predominantly unprocessed form (p66), migrated on sodium dodecyl sulfate-polyacrylamide gels as a 66-kDa band with minor heterogeneity at lower relative molecular mass. Incubation of this protein with recombinant HIV-1 protease produced a stable heterodimeric RT that was purified in a single step by IMAC. The purified protein retained both RT and RNase H activity, and kinetic parameters ($K_{cat}$ and $V_{max}$) were measured with both RNA-dependent DNA polymerization and RNase H activity assays. Carboxyl-terminal sequencing of purified heterodimeric RT indicated that one subunit is intact p66, whereas the other, p51, is a truncated form of p66 that terminates at residue Phe$^{640}$. Analysis of the HIV-1 protease digest revealed two cleavage sites, at Tyr$^{365}$-Leu$^{368}$ and Tyr$^{382}$-Leu$^{385}$, in addition to the site at Phe$^{640}$-Tyr$^{641}$ that is cleaved to produce p51.

Reverse transcriptase (RT) is an essential enzyme in the replication of human immunodeficiency virus type 1 (HIV-1) (1). The enzyme purified from virions is found to be composed of 66-kDa (p66) and 51-kDa (p51) polypeptide chains that have identical amino-terminal sequences (2). Although the exact nature of the cleavage that creates p51 from p66 is not clear, it is assumed that the smaller subunit must be generated from the larger one by the action of viral protease.

1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: The Upjohn Company, 7240-267-117, 301 Henrietta St., Kalamazoo, MI 49001. Tel.: 616-384-9413; Fax: 616-385-7373.

The abbreviations used are: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; RNase H, ribonuclease H; IMAC, immobilized metal affinity chromatography; Temed, N,N,N',N'-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; IDA, iminodiacetic acid; BBOT, 2,5-bis(5'-tertbutylbenzoxazolyl[2'])thiophene.

* The exact nature of the cleavage that creates p51 from p66 is not clear, it is assumed that the smaller subunit must be generated from the larger one by the action of viral protease.

** In studies of recombinant versions of HIV-1 RT, the protein recovered from the host expression system is invariably processed to heterodimeric (p66/p51) form by nonviral enzymes during isolation and purification (4). As a result, it has proved difficult to prepare heterodimeric HIV-1 RT free from microheterogeneity in quantities sufficient to support a variety of crystallographic and biochemical studies.

** Recently, we reported (5) preparation of a recombinant p66 form of HIV-1 RT with an amino-terminal extension containing a hexa-histidine sequence engineered to facilitate purification by immobilized metal affinity chromatography (IMAC). Here we describe the production, purification, and characterization of a pure heterodimeric HIV-1 RT that was generated by the action of HIV-1 protease on the active p66 form of RT.

MATERIALS AND METHODS

Chemicals

General laboratory chemicals were purchased from Sigma. Protein assay reagents, SDS, Tris base, Coomassie Brilliant Blue G-250, Temed, ammonium persulfate, acrylamide, and bisacrylamide were obtained from Bio-Rad. Poly(rA):oligo(dT) and deoxythymidine triphosphate (dTTP) were obtained from Pharmacia LKB Biotechnol-
Pro-Ile-(His)$_2$-Pro-Phe-His-Leu-Val-Ile-His-HIV-1 RT

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis was carried out with 0.75-mm-thick 12 or 18% polyacrylamide gels as described by Laemmli (6) and stained with Coomassie G-250 or silver stain.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out as described by Chattopadhyay et al. (7). Briefly, electrophoresis in the first dimension was performed on a nonreducing non-SDS gel system by adaptation of the method of Panyim and Chalkley (8). The RT heterodimer was electrophoresed at 4 °C in 6% polyacrylamide gel (1 mm thick) containing 6.25 M urea at pH 3.2. The electrophoresis buffer was 0.9 M acetic acid. In order to reduce the oxidative substances from the gel, each gel was run prior to loading the protein sample. For the second dimension, the lane containing RT was cut and equilibrated with SDS reservoir buffer for 15 min and then added to the top of a 1.5-mm-thick 12% polyacrylamide gel containing 0.1% SDS.

Protein Concentration

The Bradford protein assay was used to determine unknown protein concentration of samples with bovine serum albumin as the standard protein. In some cases, protein concentration was established for further accuracy by amino acid analysis on a Beckman 6300 analyzer.

Recombinant HIV-1 Protease

Purification of the well characterized HIV-1 protease from Escherichia coli inclusion bodies has been described elsewhere (9).

RNA-dependent DNA Polymerase RT Activity Assay

Reverse transcriptase activity was assayed by using a standard method (6, 7, 10). Assay solution contained 0.5 A$_{260}$ units/ml of poly(rA):oligo(dT), 0.25 mM [H]dTMP (138,000 cpm/nmoll, 5 mM MgCl$_2$, 10 mM Tris-HCl, 50 mM KCl, 50 mM Tris-HCl, pH 8.0, and 0.25 mM DTT. Typically, 25 μl of the enzyme solution was incubated with 100 μl of assay solution at 37 °C. The incubation mixture (25 μl) was spotted on glass fiber papers at 15, 30, 45, and 60 s. Reaction was stopped by freezing the reaction mixture. Papers were then washed in cold 0.2 M acetic acid. In order to reduce the oxidative substances from the gel, each gel was run prior to loading the protein sample. For the second dimension, the lane containing RT was cut and equilibrated with SDS reservoir buffer for 15 min and then added to the top of a 1.5-mm-thick 12% polyacrylamide gel containing 0.1% SDS.

RNase H Activity Assay

The RNase H assay is based on the following reaction, in which only RNA (represented by poly(rA)) hybridized to DNA (represented by poly(dT)), is hydrolyzed as follows.

\[
{[H]Poly(rA),poly(dT)} + RNase H \rightarrow n[H]AMP
\]

where \(m\) represents the original chain length of poly(rA) and \(n\) represents the number of residues of AMP removed as monomers or oligomers. Single-stranded DNA or RNA is not a suitable substrate for this reaction. The assays were performed essentially as described elsewhere (11). The assay solution contained 2.5-6 μM of [H]poly(rA):poly(dT), 50 mM Tris-HCl, pH 8.5, 5 mM MgCl$_2$, 3% glycerol, 0.1 mg/ml bovine serum albumin, and 0.02% Nonidet P-40. 100 μl of assay solution was incubated with 25 μl of enzyme at 37 °C. Reaction mixture was spotted on GF/C papers at various times and treated as described for the RNA-dependent DNA polymerase activity assay. The RNase H activity is expressed either as a decrease in counts/min/h at 37 °C or in units where 1 unit is defined as 1 pmol of trichloroacetic acid-soluble radiolabeled adenylate released in 1 h at 37 °C.

Preparation of E. coli Extract

The cell paste (12 g) was suspended in extraction buffer (5 ml/g of cell) and stirred at 4 °C for at least 1 h. The extraction buffer was composed of 20 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamide hydrochloride, 10 mg/liter leupeptin, 10 mg/liter aprotinin and 10 mg/liter bestatin hydrochloride. The suspension was passed through a French Press three times to insure breakage of the cells. The suspension was cooled for 5 min in an ice bath between successive applications of pressure. The cell lysate was stirred for another hour at 4 °C followed by centrifugation at 12,000 rpm for 1 h in the cold. The pellet was discarded.

Isolation of p66 RT by Immobilized Metal Affinity Chromatography

The cell-free extract was applied to the pre-equilibrated (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) Ni$^{2+}$-charged IDA-Sepharose column (50-ml bed volume) at a flow rate of 1 ml/min. The column was then washed with equilibration buffer until all the unbound proteins were washed away. Washing was continued with 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 100 mM imidazole. After the absorbance of the eluted solution returned to base line, the column was washed further with 250 ml of equilibration buffer. The flow-through and washes were collected batchwise. A constant flow rate of 1.5 ml/min was maintained during the washes. Next, a linear gradient of imidazole (100-450 mM) was applied in a total volume of 200 ml of 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0. The flow rate was 1 ml/min, and fractions of 6 ml were collected. Each fraction was assayed for RT activity as described before.

Preparation and Purification of p66/p51 Heterodimer

The second peak eluted from the IMAC column with a linear gradient of imidazole concentration was dialyzed thoroughly against 10 mM Tris-HCl, 1 mM NaCl and DTT, 1 mM NaN$_3$, pH 8.0, overnight in the cold. The retentate was centrifuged at 12,000 rpm for 15 min at 4 °C. Ten ml of the clean supernatant containing 15 mg of RT protein was incubated with 0.25 mg of purified HIV-1 protease at 37 °C for 50 min. Part of the reaction mixture was subjected to SDS-PAGE. The rest was immediately brought to 4 °C and dialyzed against 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0, overnight in the cold. Retentate was centrifuged at 12,000 rpm for 15 min at 4 °C. The clean supernatant was applied to a Ni$^{2+}$-charged IDA-Sepharose column (10-ml bed volume) pre-equilibrated with 200 ml of 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0. The column was then washed with 50 ml of the above buffer. A peak of protein was eluted when the column was further washed with 100 mM imidazole in the same buffer. After the absorbance of the eluted fluid reached base line, RT bound to the column was eluted with 450 mM imidazole in the above buffer. All washes were collected batchwise and analyzed by SDS PAGE and RT assay.

Gel Filtration Chromatography of Heterodimeric RT

An aliquot (0.25 ml) of the pool eluted with 450 mM imidazole was dialyzed against 0.2 M Tris-HCl, 1 mM DTT, pH 8.0. The retentate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was then chromatographed on a Superose 12 sizing column (1.0 × 25 cm) equilibrated with 0.2 M Tris-HCl, 1 mM DTT, pH 8.0. The column was eluted with the same buffer isocratically at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. Each fraction was analyzed by SDS-PAGE and by RT and RNase H assays.

Amino- and Carboxyl-terminal Analyses of HIV-1 RT Heterodimer

For carboxyl-terminal sequencing, purified heterodimeric RT was first buffer exchanged with a Centricron 30 filter into digestion buffer of 50 mM sodium phosphate, pH 6.0, containing 4 μM hydroxypoline and 0.05% Brij 35. Hydroxypoline was included as an internal standard for amino acid analysis. HIV-1 RT heterodimer (450 μg) in the above buffer was digested with carboxypeptidase Y at an enzyme-to-substrate ratio of 1:25 (w/w). Aliquots taken at various times were quenched with 10% trichloroacetic acid. The time 0 sample was removed prior to enzyme addition and used as a blank. Amino acid analysis was made by the PTC method (12). Results were expressed as pmol of amino acids as a function of time.

For analysis of the products of cleavage by HIV-1 protease, 180 μl of the p66 RT was incubated with 8 μg of purified HIV-1 protease in 50 mM Tris-HCl, pH 7.2, 1 M NaCl. After 60 min the reaction mixture was passed through a Superdex 75 sieving column (HR10/30). The column was eluted with 0.1 M ammonium bicarbonate, pH 7.0 and dialyzed against 0.1 M ammonium bicarbonate buffer prior to analysis. The reaction mixture was spotted on a Superose 12 gel filtration column equilibrated with 0.1 M ammonium bicarbonate, pH 7.0. The column was eluted with the same buffer isocratically at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. Each fraction was analyzed by SDS-PAGE, and gel filtration, and amino acid analysis.
Kinetic Analyses

Reaction kinetics for HIV-1 RT heterodimer were determined by standard methods. For RNA-directed DNA polymerase activity, one substrate was varied at subsaturation (limiting) concentrations, whereas the second substrate was held at saturating concentration. The concentration of Mg\(^{2+}\) ion was sufficient to complex with all available nucleotide even when both \(^{[3}H\)dTTP and poly(rA):oligo(dT) were at saturating concentration. Under these conditions, a series of time points was determined at each limiting substrate concentration and slopes, as a function of time, were determined by linear regression. These experimental lines, all with coefficients of correlation greater than 0.97, were then used to determine RT activity in units/ml. Data were then fit to a Michaelis-Menten equation with a least squares program ENZFITTER (Sigma). The program calculates \(K_m\) values and \(V_{max}\) values for the lines generated from each series of activity measurements at multiple substrate concentrations.

(a) Saturating \(^{[3}H\)dTTP, Limiting Template-Primer—The kinetic mechanism determined for RT polymerase activity has been described previously as an ordered bisubstrate pathway easily fit to the Michaelis-Menten equation when one substrate is saturating while the second substrate is at subsaturation levels. For this series of experiments, six different template-primer concentrations were used. To conduct these analyses, some changes in the reaction conditions were designed to maximize data collection. Final concentrations were 50 mM Tris-HCl, pH 8.0, 3 mM MgCl\(_2\), 50 mM KCl, 0.15 mM \(^{[3}H\)dTTP, and variable concentration of template-primer. Reactions were conducted at 37 °C in a final volume 100 pl. At 45- and 90-s intervals, 25-\(\mu\)l aliquots were spotted onto GF/C papers and immediately dropped into cold 5% trichloroacetic acid. Reaction velocities were calculated by linear regression analysis of the line generated from 0-, 45-, and 90-s time points for each substrate concentration. Velocities for each substrate concentration were then fit to the Michaelis-Menten equation with the program ENZFITTER which provided both \(K_m\) and \(V_{max}\) value with appropriate statistical errors. Line-weaver-Burk plots were constructed from Michaelis-Menten plots by programmed transformation.

(b) Saturating Template-Primer, Limiting \(^{[3}H\)dTTP—For this series of experiments, nine different \(^{[3}H\)dTTP concentrations were used. Final reaction conditions were 50 mM Tris-HCl, pH 8.0, 3 mM MgCl\(_2\), 50 mM KCl, poly(rA):oligo(dT), 1.25 A\(_{260}\) units/ml and varied concentration of \(^{[3}H\)dTTP. Reactions were conducted at 37 °C in a final volume of 100 \(\mu\)l. At 40-, 80-, and 120-s intervals, 25-\(\mu\)l aliquots were spotted onto GF/C papers. Data were obtained as described above.

(c) Limiting RNA:DNA Hybrid—To our knowledge, the kinetic mechanism determined for RNase H activity associated with HIV-1 RT has not been defined. However, in preliminary experiments we found that the velocities could easily be fit to a Michaelis-Menten equation (unisubstrate kinetics). For this series of experiments, five different concentrations of \(^{[3}H\)poly(rA):poly(dT) were used. Reactions were conducted at 37 °C in a final volume of 140 \(\mu\)l. At 0, 7, and 14 min, 25-\(\mu\)l aliquots were spotted onto GF/C papers for determining reaction velocities.

**RESULTS**

Isolation of Active p66 RT—Fig. 1a shows a typical chromatogram obtained by elution of p66 RT from an IMAC column with a linear gradient of increasing imidazole concentration. As shown in Fig. 1a, two peaks of protein were eluted under such conditions. Protein within peak 1 eluted at about 200 mM imidazole concentration and showed very little RT activity. When analyzed by SDS-PAGE, fractions in this peak were found to contain a number of shorter polypeptides along with a 66-kDa band (Fig. 1b). The second peak, which eluted at about 300 mM imidazole concentration, exhibited high RT activity. On SDS-PAGE, these fractions showed a 66-kDa polypeptide along with some contaminating bands of slightly lower molecular mass (Fig. 1b). On a Western blot, all of the bands in the second peak cross-reacted with antisera raised against recombinant HIV-1 RT (data not shown). These relatively smaller polypeptides, we think, arise due to minor proteolysis of p66 by bacterial enzymes. It is noteworthy that the 51-kDa band usually observed during purification of RT produced by bacterial expression of p66 was not observed in any of these fractions.

Processing of p66 RT by HIV-1 Protease and Purification of Heterodimeric RT—Fractions within peak 2 (Fig. 1a) were pooled and dialyzed thoroughly against 10 mM Tris-HCl, 1 mM NaCl, 1 mM Na\(_2\)S, 1 mM DTT, pH 7.2. The dialyzed protein was incubated with purified HIV-1 protease at 37 °C. Fig. 2 shows an SDS-PAGE of the protein before and after proteo-
lysation for 30 min. Intensities of the bands corresponding to 66 and 51 kDa appear to be approximately the same; indeed, it appears that once half of the p66 is processed to p51, proteolysis ceases. The fact that no 15-kDa band appears on this gel may be explained by the occurrence of additional protease cleavage within the 15-kDa sequence, as described below.

The resulting HIV-1 RT heterodimer was purified in a single IMAC step by elution of the bound enzyme with 450 mM imidazole. When the eluted protein was further chromatographed on a Superose 12 sizing column, RT and RNase H activities were found to coelute with a protein peak of the molecular mass expected for p66/p51 heterodimer. On analysis by SDS-PAGE, active fractions from the sizing column showed a 1:1 distribution of two bands corresponding to relative molecular masses of 66 and 51 kDa; no other band was noted even on silver-stained gels (data not shown). Fig. 3 shows a two-dimensional polyacrylamide gel electrophoretic pattern of this purified heterodimer. Table I briefly describes specific activities of the enzyme at various stages of analytical scale purification.

Purified heterodimeric RT was subjected to carboxyl-terminal sequence analysis by digestion with carboxypeptidase Y. It was demonstrated previously that HIV-1 protease cleaves p66 RT at the Phe44'-Tyr44' bond to produce the p51 form (14). Carboxyl-terminal analysis of our heterodimeric RT supports a similar cleavage in the present case. Fig. 4 shows a plot of amino acids released as a function of time during digestion of HIV-1 heterodimeric RT with carboxypeptidase Y. As shown, Leu and Phe, the expected carboxyl-terminal amino acids (from p66 and p51, respectively) were released during the first 10 min of digestion. This was followed by detection of Thr and Ile, the expected C(-1) amino acids from the p51 and p66 subunits, respectively. The level of the 3rd predicted residue (Glu) from the carboxyl terminus of p51 was also increased, but the expected 3rd residue (Lys) from p66 increased only slightly after 20 min (not shown). This may be due to inefficient digestion of the Arg557-Lys558 bond in p66 by carboxypeptidase Y. In any case, our results are consistent with the expected carboxyl-terminal sequences, -Ala-Glu-Thr-Phe and -Arg-Lys-Ile-Leu, for p51 and p66, respectively.

In order to further characterize the cleavage by HIV-1 protease, size exclusion chromatography on a Superdex 75 sizing column was used to resolve the contents of the digest into a series of fragments (Fig. 5). The isolated peptides were

**TABLE I**

| Protein source                        | Specific RT activity |
|---------------------------------------|----------------------|
| Cell-free extract                     | 4251 ± 167           |
| IMAC-purified p66 HIV-1 RT            | 22,491 ± 1350        |
| IMAC-purified HIV-1 RT heterodimer    | 24,369 ± 1474        |

* One unit of activity is defined as one nmole of [3H]dTMP incorporated into poly rA:oligo dT in 1 h at 37°C. The data represent mean ± S.D. (n = 3).
HIV-1 RT from in Vitro Processing of p66

then subjected to amino-terminal sequence and compositional analyses. The p66/p51 heterodimer peak is indicated on the chromatogram, and the amino-terminal sequences are provided for individual fragments. The results clearly show that HIV-1 protease has cleaved only three peptide bonds in p66 during this digestion: at Phe440-Tyr441, giving rise to p51; and at Tyr482-Leu484 and Tyr532-Leu533, both within the carboxyl-terminal p15 sequence removed in processing to of p66 to p51. Compositions of peptide fragments fit exactly those expected from the known sequence of p66. The absence of any other peptide from this separation further confirms the identity of the carboxyl-terminal residue of p51 as Phe440.

Kinetic Characterization of Heterodimeric HIV-1 RT—As shown in the inset of Fig. 6, for the case where dTTP concentration was varied and poly(rA):oligo(dT) concentration was saturating, the data fit the Michaelis-Menten equation as expected. A straight line was obtained when the data were transformed into a Lineweaver-Burk plot. These data provide no evidence of nonlinear behavior, substrate inhibition, or allosterism in the kinetics of heterodimeric RT. Values of $K_m$ and $V_{max}$ of 8 μM and 34,800 units/mg, respectively, were calculated under defined assay conditions. Similar kinetic analyses were performed for the case where poly(rA):oligo(dT) concentration was varied at subsaturation levels with dTTP concentration being held at saturation. A similar plot was obtained when the data were fit into a Michaelis-Menten equation (data not shown) with a $K_m$ and $V_{max}$ of 0.1 A260 units/ml and 25,680 units/mg, respectively.

Fig. 7 shows the measured RNase H activity at each substrate concentration and these data also follow the Michaelis-Menten equation (inset of Fig. 7) for a unisubstrate condition. $K_m$ and $V_{max}$ values of 10.2 μM and 307 units/mg, respectively, were calculated from these data. Under similar experimental conditions, a $V_{max}$ of 40 units/mg (data not shown) was determined for an E. coli-processed HIV-1 RT heterodimer sample purified by conventional methods (7). It is possible that specific cleavage by the HIV-1 protease may be responsible for the higher specific RNase H activity associated with the present heterodimer.

DISCUSSION

Isolation of an unprocessed p66 form of HIV-1 RT from recombinant expression systems by conventional methods has proved to be difficult due to the sensitivity of p66 to host proteases (4,15). In order to facilitate isolation from bacterial cells, the protein was genetically engineered with an additional peptide at the amino terminus designed for binding specific metal ions (5). When the crude bacterial lysate containing the modified RT was passed over the immobilized metal column, most of the host proteins did not bind, whereas RT remained bound to the column. The bacterial proteins retained on the column could be dissociated easily. This rapid one-step procedure effectively reduced the action of host proteases on RT. Isolation of partially purified p66 RT with a hexa-histidine peptide at the amino terminus by a pH reduction elution protocol has been reported previously (16).
HIV-1 RT from in Vitro Processing of p66

Based on our recent work (5), we have developed an imidazole gradient elution protocol for single-step purification of predominantly unprocessed p66 HIV-1 RT.

There are a number of factors that led us to produce active heterodimeric RT from purified p66 by the action of HIV-1 protease. The reasons were that (a) purified p66 could be easily prepared, (b) purified recombinant HIV-1 protease was readily available, (c) the heterodimeric RT produced by processing with HIV-1 protease could be easily purified by IMAC due to the presence of the amino-terminal linker, and (d) based on published reports (14, 17), we expected that the action of protease would produce a homogeneous heterodimer with a p51 subunit having as its carboxyl-terminal residue Phe\textsuperscript{469}.

It is difficult to deny the hypothesis that processing of p66 to p51 in virions must be mediated by the viral protease. Within the budded viral particles it seems likely that the newly activated viral protease, which is responsible for the processing of the gag-pol polyproteins and maturation and formation of infectious HIV-1, would also participate in the formation of the heterodimeric RT. In vitro HIV-1 protease cleavage of p66 at Phe\textsuperscript{469}-Tyr\textsuperscript{470} is demonstrated here by amino-terminal sequence and compositional analyses of the digestion products and by carboxyl-terminal analysis of the purified p66/p51 heterodimer with carboxypeptidase Y. Our studies also demonstrate the existence of two additional HIV-1 protease cleavage sites in the segment removed to form p51. These sites are as follows.

\[ \ldots \text{Gln}\text{-}\text{Ala}\text{-}\text{Ile}\text{-}\text{Tyr}^{498}\text{-}\text{Leu}^{499}\text{-}\text{Ala}\text{-}\text{Leu}\text{-}\text{Gin} \ldots \]
\[ \ldots \text{Glu}\text{-}\text{Lys}\text{-}\text{Val}\text{-}\text{Tyr}^{532}\text{-}\text{Leu}^{533}\text{-}\text{Ala}\text{-}\text{Trp}\text{-}\text{Val} \ldots \]

These bonds in the p66 subunit of heterodimer are refractive to hydrolysis. The clear homology between the two sequences suggests that both of these sites may be meant to undergo cleavage by HIV-1 protease. The cleavage at Tyr\textsuperscript{498}, Leu\textsuperscript{499} has been documented previously (18). The present communication is the first to describe the second site at Tyr\textsuperscript{532}-Leu\textsuperscript{533}.

Our results are consistent with previous studies in which additions of amino acid residues at the amino terminus of HIV-1 RT were shown not to have any effect on the polymerase function (5, 16, 19). It is likely, therefore, that the protein structure is not disrupted by such extensions. Moreover, it is also evident that the amino-terminal extension does not affect the RNase H activity. In the light of our present results and previous observations, we conclude that the HIV-1 RT heterodimer preparation reported here is suitable for further structural and enzymological studies. We believe availability of such a well characterized preparation should aid in a great variety of studies aimed at understanding structure-function relationships of this key enzyme involved in the replicative machinery of HIV-1, the causative agent of AIDS.

Acknowledgments—We are thankful to C. M. Campbell and Dr. J. G. Hoogerheide for performing amino acid analyses in conjunction with carboxyl-terminal analysis.

REFERENCES
1. Chandra, P., Vogel, A., and Gerber, T. (1986) Cancer Res. 45, 4677-4684
2. Veronese, M. F., Copeland, T. D., DeSavo, A., Rahman, R., Oroszlan, S., Gallo, R. C., and Sarangaddaram, M. G. (1986) Science 231, 1289-1291
3. Goff, S. (1990) J. AIDS 3, 817-831
4. Lowe, D. M., Atken, A., Bradley, C., Darby, G. K., Larder, B. A., Powell, K. L., Pulfloy, D. J. M., Tisdale, M., and Stammers, D. K. (1988) Biochemistry 27, 8884-8889
5. Sharma, S. K., Evans, D. B., Vosters, A. F., McQuade, T. J., and Tarpley, W. G. (1991) Biotechnol. Appl. Biochem. 14, 9-81
6. Laemmli, U. K. (1970) Nature 227, 680-685
7. Chattopadhyay, D., Einapahr, H. M., Brunner, D. P., Strakalaitis, N. A., Tarpley, W. G., and Debel, M. B. (1993) Protein Expression and Purification 3, 151-159
8. Parysim, S., and Chalkley, R. (1989) Arch. Biochem. Biophys. 150, 337-349
9. Tomasselli, A. G., Olsen, M. K., Hui, J., Staples, D. J., Sawyer, T. K., Heinrikson, R. L., and Tomich, C. C. (1990) Biochemistry 29, 264-269
10. Larder, B., Pulfloy, D., Powell, K., and Darby, G. (1987) EMBO J. 6, 3133-3137
11. Evans, D. B., Brawn, K., Deibel, M. R., Jr., Tarpley, W. G., and Sharma, S. K. (1981) J. Biol. Chem. 266, 20583-20586
12. Hendrissler, M. (1985) Appl. Biosystems User Bull. 14, 1-27
13. Heinrikson, R. L., and Meredith, S. C. (1984) Anal. Biochem. 136, 65-74
14. Bathurst, J. C., Moen, L. K., Lujan, M. A., Gibson, H. L., Peuch, P. H., Pichaudes, S., Craak, C. S., Sasti, D. V., and Burt, P. J. (1990) Biochem. Biophys. Res. Commun. 171, 589-596
15. Barr, P. J., Power, M. D., Lee-Ng, C. T., Gibson, H. L., and Mos, B. (1988) Virology 166, 339-349
16. LeGrice, S. J., and Gruninger-Leitch, F. (1990) Eur. J. Biochem. 187, 307-314
17. Graves, M. C., Meidel, M. C., Pan, Y. C., Manneberg, M., Lahm, H. W., and Gruninger-Leitch, F. (1990) Biochem. Biophys. Res. Commun. 168, 30-36
18. Beegra, S. P., Claye, M. G., Greenblom, A. M., Karlestrom, A. R., Stahl, S. J., Wilson, S. M., and Wingfield, P. T. (1990) FEBS Lett. 270, 76-80
19. Prasad, V., and Goff, S. P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3104-3108