Limited proteolysis of reverse transcriptase from human immunodeficiency virus and separation of polypeptide fragments containing RNase H activity

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LIMTED PROTEOLYSIS OF REVERSE TRANSCRIPTASE FROM HUMAN IMMUNODEFICIENCY VIRUS AND SEPARATION OF POLYPEPTIDE FRAGMENTS CONTAINING RNase H ACTIVITY

by

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B.S., Eastern Montana College, 1989

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Approved by

Chairman, Board of Examiners

Dean, Graduate School

Date
The reverse transcriptase from human immunodeficiency virus type-I (HIV-RT) is a bifunctional enzyme, displaying DNA polymerase and ribonuclease H (RNase H) activities. In order to better characterize the molecular domains responsible for these activities, limited proteolysis on the 66 kilodalton form of HIV-RT (p66) was performed. The treatment of p66 with subtilisin-Carlsberg resulted in a characteristic breakdown pattern which depended upon the extent of proteolysis as well as the concentration of sodium dodecyl sulphate (SDS). The products of proteolysis (fragments) had apparent molecular masses of 51, 42, 33, 27, and 15 kilodaltons (kDa) as determined from SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Through Western blot analysis of the fragments, both the 51 and 42kDa fragments were found to be recognized by a monoclonal antibody (mAb) specific for a region near the C-terminus of p66, while the 27 and 15kDa fragments were found to be recognized by a mAb specific for a region near the N-terminus. In contrast, the 33kDa fragment was recognized by a mAb specific for a region in the middle of p66. It was proposed that HIV-RT, when denatured, is susceptible to proteolytic cleavage in at least two areas near the amino terminus. Cleavage at one site produces the C-terminal 51 and N-terminal 15kDa fragments, while cleavage at the other site produces the C-terminal 42 and N-terminal 27kDa fragments. An RNase H activity gel analysis was performed with the fragments to determine if any of these fragments contained RNase H activity. Only the 51 and 42kDa fragments were found to contain RNase H activity. Attempts were also made, through dialysis, to renature the fragment mixture in solution for RNase H kinetic analysis. However, only very low levels of RNase H activity were observed with the fragment mixture.

This study shows that proteolytic elimination of N-terminal portions of p66 results in C-terminal fragments which, under specific conditions, still demonstrate RNase H activity. These unique RNase H-active fragments may help elucidate mechanisms of RNase H catalysis, substrate binding, inhibition by RNase H specific inhibitors, and may aid in the determination of the crystal structure of the p66 RNase H domain.


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CHAPTER I: INTRODUCTION

Human immunodeficiency virus type-1 (HIV-1)\(^1\), a retrovirus, is known to be the causative agent of the acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983). All retrovirus particles contain a reverse transcriptase (RT) which is responsible for conversion of viral RNA into double-stranded DNA provirus, which is then integrated into host DNA (Varmus and Swanstrom, 1984). Retroviral RT catalyzes several enzymatic reactions, including RNA- and DNA-directed DNA polymerization as well as hydrolysis of the RNA component of RNA•DNA hybrids (Varmus and Swanstrom, 1984). The latter activity is referred to as ribonuclease H (RNase H). The function of the RNase H in retroviruses is not well defined, however, several functions have been suggested. It has been shown that, with the avian sarcoma virus reverse transcriptase associated RNase H, the RNase H is capable of not only generating oligomeric RNA primers for plus-strand DNA synthesis, but also removing RNA primers of both minus- and plus-strand DNA (Omer and Faras, 1982; Resnick et al., 1984;). Furthermore, it has been shown that both the DNA polymerase and RNase H activities of the reverse transcriptase enzyme from human immunodeficiency virus (Hansen et al., 1988; Starnes and Cheng, 1989; Mizrahi et al., 1989), avian myeloblastosis virus (Grandgenet et al., 1973), and murine lekemia virus (Moelling, 1974) reside on a single polypeptide. Studies with HIV-RT have shown that the RNase H activity is
associated with the carboxy terminus of the protein (Johnson et al., 1986; Tisdale et al., 1989; Hansen et al., 1988) and the DNA polymerase activity with the amino terminus (Johnson et al., 1986; Starnes and Cheng, 1989; Tisdale et al., 1988; Mizrahi et al., 1989).

The HIV-RT gene encodes a 66kDa protein. However, two reverse transcriptase polypeptides, with apparent molecular masses of 66 and 51kDa, have been isolated from HIV virions (Di Marzo Veronese et al., 1986). While both share the same amino termini, the larger form has an additional stretch of amino acids present in the carboxy terminus (Lightfoot et al., 1986). In vitro, the 66kDa protein (p66), alone, exists as a homodimer, whereas an equal mixture of p66 and the 51kDa protein (p51) exists as a p66/p51 heterodimer (Lowe, et al., 1988). In vivo, p66 is thought to be processed to p51 by cleavage of a 15kDa, C-terminal portion of p66 (Di Marzo Veronese et al., 1986; Lightfoote et al., 1986; Ferris et al., 1990). It is not known for sure whether the endogenous HIV-RT exists as a homodimer, heterodimer, or p66 monomer.

Homogeneous preparations of the p66 have been prepared by cloning in E. coli (Hizi et al., 1988; Larder et al., 1987). The p66 has been shown to contain RNA-and DNA-directed DNA polymerase activities (Starnes et al., 1988) as well as an RNase H activity (Starnes and Cheng, 1989). While low levels of DNA polymerase activity have been observed with the p51 form (Starnes and Cheng, 1989; Tisdale et al., 1988), this
form contains no detectable RNase H activity (Mizrahi et al., 1989; Tisdale et al., 1989).

The HIV-RT is essential for virus replication. Without this enzyme, the HIV genome could not be replicated, and thus, could not be expressed. Because of the crucial role of the HIV-RT in virus replication, this enzyme is an important target for AIDS chemotherapy. Several compounds have been tested for their ability to inhibit HIV replication. Most of these compounds are nucleoside analogues. So far, the most promising compound is the thymidine analogue, 3'-azido-3'-deoxythymidine (AZT). The structure of AZT is essentially identical to that of thymidine, with the exception that the 3'-hydroxyl group of thymidine is replaced by an azido (N₃) group. AZT has been shown to be a potent inhibitor of HIV replication in vitro (Mitsuya et al., 1985). Furthermore, AZT has been shown to not only significantly improve the condition of AIDS patients, but also increase their survival rates (Fischl et al., 1987). The proposed mechanism of action of AZT involves both inhibition of the HIV-RT and termination of the viral DNA chain (Langtry and Campoli-Richards, 1989). Before AZT can be active, however, it must be converted to the 5'-triphosphate form. This phosphorylation is performed by cellular kinases (Furman et al., 1986). AZT-triphosphate has been shown to competitively inhibit the binding of thymidine-triphosphate to HIV-RT (St. Clair et al., 1987). Once AZT-triphosphate has become bound to HIV-RT, a DNA chain
elongation reaction occurs in which AZT-monophosphate is incorporated into the DNA chain, accompanied by the release of pyrophosphate. It has been shown that, following AZT incorporation, DNA chain elongation is terminated because AZT is lacking the 3'-hydroxyl group which is essential for chain elongation (St. Clair et al., 1987). Studies, such as these, demonstrate the importance of HIV-RT as a target for AIDS chemotherapy. Although most HIV-RT inhibitors (such as AZT) are targeted to the reverse transcriptase activity of HIV-RT, it has been suggested that the RNase H activity of HIV-RT may also be a possible target for inhibition of HIV replication (Starnes and Cheng, 1989).

In several studies, limited proteolysis has been used to physically separate two or more enzymatic activities that are contained within the same polypeptide. Such studies have been performed with both retroviral and non-retroviral DNA polymerases. The alpha subunit of avian myeloblastosis virus RT has been shown to contain both DNA polymerase and RNase H activities (Grandgnett et al., 1973). Limited proteolysis of this RT with chymotrypsin showed that the enzyme could be broken down into a number of polypeptide fragments, one of which contained RNase H activity (Lai and Verma, 1978). However, under the conditions used, a fragment containing only DNA polymerase activity was not isolated. Furthermore, limited proteolysis of E. coli DNA polymerase I with subtilisin results in the production of the Klenow fragment
which contains DNA polymerase activity and 3'-5'exonuclease (degradative) activity but is devoid of 5'-3' exonuclease activity (Klenow and Overgaard-Hansen, 1970; Brutlag et al., 1969). Under the conditions used, the polymerase activity of the fragment was significantly greater than that of the intact enzyme.

Domain specific studies such as these are important in the physical characterization of enzymes and may be important in the development of inhibitors of enzymes from pathogens. Such analyses may answer questions pertaining to the number and arrangement of functional sites present on these enzymes and the effect of conformational changes (via proteolytic cleavage) on enzyme activity. In the work reported here, the SDS-dependent proteolysis of HIV-RT by subtilisin was examined and the cleavage fragments containing RNase H activity were identified.

Due to the significance of the HIV-RT as a target for AIDS chemotherapy, there is a need to further develop HIV-RT inhibitors of not only the reverse transcriptase domain, but also the RNase H domain. However, this development will depend upon a better understanding of these two domains. The purpose of this research was to further characterize these two domains with the view that the results obtained might facilitate the development of HIV-RT targeted AIDS chemotherapy. Initially, the goal of this research was to physically separate the reverse transcriptase domain from the RNase H domain of HIV-RT
by proteolytically cutting the enzyme at the domain interface. Under the proteolysis conditions used in this research, however, it was shown that, rather than cutting between the two domains, cuts were made near the amino terminus of HIV-RT (in the reverse transcriptase domain). However, these cuts resulted in the generation of carboxy terminal fragments which still contained RNase H activity. The new goal of this research was then to identify these RNase H active fragments and examine the proteolysis reaction which produced them.
CHAPTER TWO:
MATERIALS AND METHODS

Materials

Recombinant p66 and p66 specific monoclonal antibodies (mAb) were kindly provided by Dr. Stephen H. Hughes (National Cancer Institute, Frederick, MD). The cloning, expression, and purification of p66 have been described (Hizi et al., 1988; Clark et al., 1990) as has the production and mapping of the mAb's (Ferris et al., 1990). The recombinant p66/p51 heterodimer was provided by Burroughs Wellcome Company. Subtilisin Carlsberg, guanosine 5'-triphosphate, phenylmethanesulfonyl fluoride (PMSF), and alkaline phosphatase conjugated goat anti-mouse IgG were purchased from Sigma Chemical Company. The alkaline phosphatase chromogenic substrates, 5-bromo-4-chloro-3-indolylphosphate p-Toluidine Salt (BCIP) and nitroblue tetrazolium chloride (NBT), were purchased from Bethesda Research Laboratory. RNA polymerase and Ribonuclease H, both from E. coli, were purchased from Pharmacia LKB Biotechnology. The [α-32P]GTP and [3H]GTP were purchased from Dupont New England Nuclear. Materials for polyacrylamide gel silver staining were purchased from Sigma Chemical Company (kit AG-5 or AG-25). All other materials were reagent grade or better.

Limited Proteolysis

Reaction mixtures (110μl) contained 86mM sodium phosphate
buffer, pH 6.5, 8.6mM 2-mercaptoethanol, 5μg of p66 or p66/p51 (as indicated), 0.05μg subtilisin (11.3 Units/mg) and SDS as indicated. Phosphate buffer containing 2-mercaptoethanol and SDS were prewarmed to 37°C, then p66 was added. This mixture was incubated at 37°C for another minute and proteolysis was initiated by the addition of subtilisin. These reactions proceeded at 37°C for the indicated times and were terminated by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2.4mM.

**Termination of proteolysis**

Two different compounds were tested for their ability to terminate subtilisin mediated proteolysis. These compounds were PMSF and Indole.

**PMSF**—Standard cleavage reaction mixtures were prepared as described above, except 5μg of BSA was used as the protease substrate instead of p66. Prior to subtilisin addition, PMSF was added to final concentrations of 0.001, 0.1, 1, and 10mM. Subtilisin was then added and samples were incubated for 5 min at 37°C and analyzed by SDS-PAGE.

**Indole**—The procedure was the same as described for the PMSF termination, except Indole, instead of PMSF, was added prior to subtilisin addition.

**SDS-polyacrylamide gel electrophoresis**

Polyacrylamide gels, 12.5 or 10%, were prepared as
previously described (Laemmli, 1970). Proteolysis reaction mixtures were diluted in SDS-solubilization buffer (62.5 mM Tris-HCl, pH 6.8, 5% glycerol, 1% SDS, 50 mM 2-mercaptoethanol, and 0.025% bromophenol blue) and boiled for 5 min. Approximately 0.7 μg total protein from either cleavage reactions or control (without protease) reaction mixtures were loaded onto the gel. Low molecular weight standards (BIORAD) were run with each gel. These standards have molecular masses of 97.4 (phosphorylase b), 66.2 (bovine serum albumin), 45.0 (ovalbumin), 31.0 (carbonic anhydrase), 21.5 (soybean trypsin inhibitor), and 14.4 kDa (lysozyme). All samples were electrophoresed at 50 mA constant current for approximately two hours. Proteins in gels were silver-stained using the silver stain kit (number AG-25) purchased from Sigma Chemical Company.

**Western blotting and Immunostaining**

Following SDS-PAGE, solubilized cleavage reaction samples were electroblotted to nitrocellulose paper as previously described (Judd, 1988). Immediately following electrophoresis, the appropriate gel lanes were cut from the gel and overlaid with strips of nitrocellulose paper (NCP). Proteins were electroblotted from the gel to NCP at 0.6 A, 20 V for 16 h in 20 mM dibasic sodium phosphate. After electroblotting, the NCP strips were blocked for 1 h with 0.05% Tween-20 in Dulbecco's phosphate buffered saline (DPBS/tween) (Batteiger et al.,
1982), followed by incubation with a 1:50 to 1:400 dilution of the appropriate mAb (Ferris et al., 1990) for 16 to 24 h. The NCP strips were then washed for 2 h in 2 changes of DPBS/Tween, to remove unbound mAb. This was followed by incubation of the NCP strip for 1 h with 1μg of alkaline phosphatase conjugated goat anti-mouse IgG. After this, strips were washed for 1 h in 2 changes of DPBS/Tween to remove unbound IgG. Following this, strips were washed for 15 min in the alkaline phosphatase reaction buffer (0.1M Tris-HCl, pH 9.5, 0.1M NaCl, and 50mM MgCl₂), then incubated in 20ml of the above buffer containing the NBT (88μl) and BCIP (66μl) alkaline phosphatase substrates. Alkaline phosphatase activity was observed by the appearance of purple bands on NCP strips and the reaction was terminated by transfer of the NCP to tap water.

RNase H Substrate Preparation

Poly(dC)·[α-32P]poly(rG)- This substrate was used for the RNase H activity gel analysis and was prepared as already described (Starnes and Cheng, 1989). The reaction mixtures (50μl) contained 50mM Tris-HCl, pH 7.8, 100mM KCl, 5mM MgCl₂, 1mM MnCl₂, 5mM dithiothreitol (DTT), 5% glycerol, 0.2 A₉₆₀ units of poly(dC) and 500μM [α-32P]GTP (10 Ci/mmol). This mixture was warmed to 37°C and hybrid synthesis was initiated with 40U of E. coli RNA polymerase. The reaction proceeded for 10 min and was terminated by addition of EDTA to a final concentration of
83 mM. The hybrid was separated from unreacted GTP by chromatography on a 1.5 ml Sephadex G-50 column. Void volume fractions were pooled and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (1:1:0.04). The aqueous phase was precipitated with two volumes of 100% ethanol and the precipitate, containing the hybrid, was resuspended in sterile water.

Poly(dC)·[^H]poly(rG)- This substrate was used in the solution assay for RNase H and was prepared as described above except the reaction volume was 1 ml and contained 4 A₂₆₀ units of poly(dC) and 500 μM[^H]GTP (0.5 Ci/mmol). The hybrid was separated from free nucleotides by chromatography on Sephadex G-50 (9 ml column).

**RNase H activity gel**

The RNase H activity gel was prepared as previously described (Starnes and Cheng, 1989). A 10% polyacrylamide gel was prepared as described above, except that 10⁶ cpm of poly(dC)·[^32P]poly(rG) was added to the running gel mixture prior to polymerization. Samples to be analyzed on these gels (40 μl), were solubilized by incubation of the protein mixtures at 37°C for 5 min in a buffer containing 50 mM Tris-HCl, pH 7.5, 5% glycerol, 2 mM EDTA, 1% SDS, 1 mg/ml nuclease free bovine serum albumin (BSA), and 0.5 mM 2-mercaptoethanol. Electrophoresis was performed at room temperature for 3 h at 30 mA constant current. After electrophoresis, proteins were
renatured as already described (Starnes and Cheng, 1989). This involved washing the gel by shaking at room temperature for 1 h in 2 changes (1 liter) of 50 mM Tris-HCl, pH 8.0, 2mM DTT, 20% glycerol, followed by washing for 16 h in 2 changes (1 liter) of the above buffer also containing 50mM KCl and 8mM MgCl₂. The gel was then washed for another 8 h in 2 changes (1 liter) of the same buffer without glycerol. For the RNase H reaction, the gel was incubated for 20 h at 37° in the same buffer without shaking. This was followed by washing (with shaking) at 4°C for 4h in 4 (1 liter) changes of cold 5% trichloroacetic acid, 10mM pyrophosphate, followed by 30 min at room temperature in 5% glycerol. The gel was dried under vacuum and autoradiography was performed using Kodak X-OMAT AR film.

Protein renaturation and solution RNase H analysis

Renaturation- These experiments were performed with preparations which had undergone proteolysis in 0.95% SDS for 50 s. Four attempts were made to renature the polypeptides in the reaction mixtures. In the first attempt to renature the polypeptides, following PMSF termination, guanidine hydrochloride was added to the reaction mixtures, to a final concentration of 3M. These mixtures were then incubated for 2 h at 37°C and transferred to prewashed (see below) dialysis tubing (molecular weight cut off 3500). Samples were then dialyzed (with shaking) at room temperature for 24 h in 4, 1
liter changes of a renaturation buffer which consisted of 50mM Tris-HCl, pH 8.0, 20% glycerol, and 2mM DTT. This was followed by dialysis for 3 h in 1 liter of the above buffer with 50mM KCl, followed by dialysis for 14 h in 2, 1 liter changes of the same buffer without KCl. Samples were then put on ice for 1 h and 15μl aliquots were taken and assayed for RNase H activity using the solution RNase H assay procedure (below). Nuclease free BSA was then added (40μg/ml) to the mixtures, which were then concentrated by ultrafiltration, using a Centricon-3 microconcentration apparatus (molecular weight cut off 3000) (Amicon). This involved centrifugation of the samples at 5000 x g for 6 h at 0°C. Aliquots (15μl) of each concentrated sample were then assayed for RNase H activity.

In the second trial, samples were transferred, immediately after PMSF termination, to dialysis tubing (3500 molecular weight cut off) and dialyzed (with shaking) at room temperature for 40 h in 8, 1 liter changes of a renaturation buffer consisting of 50mM Tris-HCl, pH 8.0, 20% glycerol, and 2mM DTT. Samples were put on ice for 1 h, then 15μl aliquots were assayed for RNase H activity. The remainder of the samples were then concentrated by ultrafiltration as described in trial one. An aliquot (15μl) of each concentrated sample was then assayed for RNase H activity.

In the third attempt to renature the fragments, the procedure was essentially identical to that used in the first attempt, however, the renaturation buffer also contained 1mM
PMSF, which was added to inhibit any subtilisin which renatured with the p66 cleavage fragments.

In the fourth and final attempt, immediately after termination of proteolysis, cleavage or control reaction mixtures (113μl, see methods) were added to 1200μl of ice cold BSA (20mg/ml). Samples were then dialyzed with the renaturation buffer described in the second trial, and concentrated (4 h centrifugation at room temperature) as described above. 15μl aliquots of concentrated dialyzed samples were assayed for RNase H activity.

The dialysis tubing used in the four above renaturation procedures was prepared by heating the tubing at 80°C for 1 h in a buffer containing 5.4mM Na₂EDTA, 14.0mM NaHCO₃, and 7.0mM 2-mercaptoethanol. This was followed by rinsing the tubing overnight in dH₂O.

**Solution RNase H assays**—Conditions for assays were identical to those described by Starns and Cheng (1989). Reaction mixtures (50μl) contained 50mM Tris-HCl, pH 8.0, 2mM DTT, 100μg/ml nuclease free BSA, 50mM KCl, 8mM MgCl₂, and 1.5 x 10⁵ cpm of ³H-labeled substrate (poly(dC)·[³H]poly(rG)). Reactions were initiated with 15μl of dialyzed cleavage or control samples and incubated at 37°C for 2 h. Reactions were terminated by transfer to ice followed by addition of 50μl of ice cold 7% perchloric acid. After 30 min on ice, precipitates (poly(dC)·[³H]poly(rG)) were pelleted by centrifugation at
10,000 rpm (Savant, model HSC10K) for 15 min. An aliquot (75µl) of each supernatant was added to 5 ml of scintillation mixture which was then counted for radioactivity. One unit of RNase H is defined as the amount of enzyme which produces 1 nmol of acid-soluble ribonucleotide/h at 37°C.
CHAPTER III:

Results

Limited Proteolysis of p66—In preliminary experiments (see SDS-requirement below) it was observed that, under the reaction conditions used, proteolysis of p66 by subtilisin required SDS. Here, p66 was treated with subtilisin in the presence of SDS, and both the reaction time and the concentration of SDS were varied. As shown by SDS-PAGE (fig. 1A), no significant proteolysis occurred unless SDS was present. However, in 0.095% or 0.95% SDS, subtilisin produced several distinct polypeptide fragments. In a 25 s reaction with either concentration of SDS, fragments with apparent molecular masses of 51, 42, 33, 27, and 15kDa were generated (lanes 7 and 8). The breakdown patterns were essentially identical when proteolysis took place for 25 s in 0.95% (lane 7) and 0.095% SDS (lane 8). However, the intensity of the 33kDa cleavage fragment was much less from the 0.95% SDS reaction than that from the 0.095% SDS reaction. A less intense 40kDa band was also observed in lane 8, but was not readily detectable in lane 7. Intact p66 was still present in both reactions as seen from the 66kDa band in lanes 7 and 8. When cleavage reactions were run for 70 s, the breakdown products were similar to those of the 25 s reactions, except, p66 was almost completely degraded in reactions containing 0.095% SDS (lane 5). Extensive proteolysis was observed in the 5 min reactions with either 0.95% or 0.095% SDS (lanes 1 and
Figure 1A: Silver stained gel of SDS-PAGE analysis of p66 digestions. Proteolysis was performed for different time intervals in the presence or absence of SDS. Terminated reaction mixtures were solubilized in SDS sample buffer and 0.7μg total protein was loaded onto each lane. The major breakdown fragments are shown by arrows in lane 8. Lanes: 1, 5 min digestion in 0.95% SDS; 2, 5 min digestion in 0.095% SDS; 3, 5 min digestion without SDS; 4, 70 s digestion in 0.95% SDS; 5, 70 s digestion in 0.095% SDS; 6, 70 s digestion without SDS; 7, 25 s digestion in 0.95% SDS; 8, 25 s digestion in 0.095% SDS; 9, 25 s digestion without SDS; 10, molecular weight markers.
2 respectively). After these treatments, neither p66 nor the 51kDa polypeptide were detected. The 0.095% SDS reaction showed a faint 42kDa band whereas the same band in the 0.95% SDS reaction was clearly visible. Both reactions generated strong 33 and 15kDa bands, as well as a number of less intense bands between the 33 and 15kDa bands. Even after 5 min, there was no significant proteolysis of p66 in reactions without SDS (lane 3).

In order to further define the generation of these cleavage fragments, 0.95% SDS proteolysis reactions were carried out with shorter reaction times, and again visualized by SDS-PAGE (fig. 1B). In 30 s, 20 s, and 15 s cleavage reactions (lanes 2-4, respectively), all five of the above breakdown products were generated. In contrast, the 10 s cleavage reaction (lane 5) contained all of the breakdown products except the 33kDa fragment, suggesting that this fragment may be generated by further proteolysis of the 51 or 42kDa fragment.

SDS-requirement—In preliminary experiments, proteolysis of p66 by subtilisin was assumed to be stopped by addition of SDS-sample buffer followed by boiling. Later, it was found that proteolysis did not actually start until the SDS-sample buffer was added. To show that this was true, two identical p66, 5 min cleavage reactions were set up as described (see methods), except SDS was not present in the reaction mixtures. One cleavage reaction was terminated by adding PMSF (see below) to a final concentration of 2mM followed by
Figure 1B: Silver stained gel from shorter duration p66 proteolysis reactions. Proteolysis was performed in 0.95% SDS for the indicated times. The cleavage fragments are shown by arrows. Lanes: 1, p66 control; 2, 30 s cleavage reaction; 3, 20 s reaction; 4, 15 s reaction; 5, 10 s reaction; 6, molecular weight markers.
solubilization with SDS-sample buffer and boiling. The other reaction was terminated with only SDS-sample buffer followed by boiling. Aliquots of each reaction mixture were analyzed by SDS-PAGE (fig. 2). The reaction terminated with PMSF (lane 3) showed only minor proteolysis as compared to the single 66kDa band seen in the p66 control reaction (lane 1). In contrast, the reaction terminated with SDS-sample buffer (lane 2) contained the 5 breakdown products which have already been described suggesting that something in the SDS-sample buffer was facilitating proteolysis. SDS seemed to be the most likely component. To show that SDS was responsible for the observed proteolysis, digestion of p66 with subtilisin was performed both in the presence or absence of SDS. Two concentrations of SDS were used, 0.95% (approximately the same as that present in the SDS-sample buffer) and 0.095% (a 10 fold decrease of that present in the SDS-sample buffer). The results of this experiment have already been discussed above (fig. 1A) in which it was confirmed that, under the conditions used, SDS was essential for proteolysis to take place.

Termination- Two different compounds were separately tested for their ability to terminate proteolysis. The two compounds chosen were PMSF and Indole. Indole was chosen because it is known to be a potent competitive inhibitor of subtilisin (Smith et al., 1966). PMSF was also chosen because it has been used by others to terminate proteolytic reactions mediated by serine proteases (Lai and Verma, 1978). Due to a limited
Figure 2: Silver stained gel showing the SDS requirement of subtilisin. Proteolysis of p66 was performed for 5 min and reactions were terminated with SDS-sample buffer or PMSF. Lanes: 1, p66 control; 2, cleavage reaction terminated with SDS-sample buffer; 3, cleavage reaction terminated with PMSF; 4, molecular weight markers.
availability of p66, BSA was used as the protease substrate in these experiments instead of p66. In order to insure that these compounds would inhibit proteolysis, either PMSF or Indole was added to the reaction mixtures prior to the addition of subtilisin. As shown by SDS-PAGE analysis of the PMSF terminations (fig. 3A), when PMSF was added to final concentrations of 0.1, 1, and 10mM (lanes 4-6, respectively), no proteolysis was observed when compared to the BSA control sample (lane 1), which contained no subtilisin. In contrast, when PMSF was only present at concentrations of 0.001 and 0.01mM (lanes 2 and 3), proteolysis was observed as seen by the breakdown bands which were not present in the BSA control sample. In reactions which contained no PMSF, a ladder of breakdown products was generated (lanes 7 and 8), which was similar to the breakdown seen in the 0.001 and 0.01mM PMSF reactions. It should be noted, however, that the 0.1, 1, and 10mM reactions (lanes 4-6) only contained one 66.2kDa BSA band, whereas the BSA control reaction (lane 1) contained several bands. This was more than likely due to incomplete silver staining in lanes 4-6, however, the results still clearly indicate that there is no significant proteolysis occurring in these reactions.

In contrast to the above results, it was found that Indole, at the concentrations used, was not an effective terminator of subtilisin mediated proteolysis of BSA. SDS-PAGE analysis of
Figure 3: Silver stained gels of PMSF (A) and Indole (B) termination reactions. BSA was treated with subtilisin for 5 min in the presence of PMSF or Indole at the indicated concentrations. (A) PMSF termination; Lanes: 1, BSA control; 2, 0.001mM PMSF; 3, 0.01mM PMSF; 4, 0.1mM PMSF; 5, 1.0mM PMSF; 6, 10mM PMSF; 7 and 8, contained no PMSF; 9, molecular weight markers. (B) Indole termination; Lanes: 1, BSA control; 2, 0.001mM Indole; 3, 0.01mM Indole; 4, 0.1mM Indole; 5, 1.0mM Indole; 6, 10mM Indole; 7, no Indole; 8, molecular weight markers.
the Indole termination reactions (fig. 3B) showed that when Indole was present during protolysis at concentrations of 0.001, 0.01, 0.1, 1, and 10mM, proteolysis of BSA was observed (lanes 2-6) when compared to the BSA control sample (lane 1). In proteolysis reactions which contained no Indole (lane 7), the observed breakdown was identical to that seen when Indole was present at the above concentrations. After analyzing the abilities of PMSF and Indole to terminate proteolysis by subtilisin, it was evident that only PMSF effectively stops the proteolysis of BSA by subtilisin. Because of this, PMSF was chosen to terminate the subtilisin digestion of p66.

Localization of Fragments—In order to determine the approximate location of the cleavage fragments within p66, the fragments were mapped by Western blotting with mAb's specific for defined regions of the HIV-RT (Ferris et al., 1990) (fig. 4A). The 51 and 42kDa fragments, along with p66, were recognized by mAb-50 which is specific for a region near the C-terminus of HIV-RT (fig. 4B). The fragments were also probed with mAb-42, which is specific for a region near the N-terminus. The 51, 27, and 15kDa fragments, along with intact p66, were recognized by this mAb (fig. 4D). In contrast, none of the fragments were recognized by mAb-19, which is specific for a smaller region on p66 near the N-terminus (fig. 4C). Only p66 was recognized by mAb-19. The 33kDa fragment, along with the 51, 42, and 40kDa fragments, was recognized by mAb-21, which is specific for a region in the middle of p66 (fig. 
Figure 4: Western blot analysis of p66 breakdown fragments. Solubilized cleavage reaction mixtures (0.7 µg total protein) and control mixtures (0.7 µg intact p66) were subjected to SDS-PAGE followed by protein transfer to nitrocellulose paper. Transfers were probed with mAb's and binding was observed by alkaline phosphatase reaction. (A) A map showing p66 binding regions of mAb-19, mAb-42, mAb-21, and mAb-50. (B) mAb-50 blot containing cleavage (CLV) or control (CT) samples. (C) mAb-19 blot. (D) mAb-42 blot. (E) mAb-21 blot.
The 33kDa fragment was not recognized by mAb's specific for the N or C terminus (mAb-42 and mAb-50, respectively). These data indicated that the 51 and 42kDa fragments are associated with the C-terminus, the 27 and 15kDa fragments with the N-terminus, and the 33kDa fragment with a central portion of p66.

Antibody titration- Prior to the above blotting experiments, it was first necessary to determine the appropriate mAb concentrations to be used for probing the fragments. This was done in order to insure that recognition of cleavage fragments by p66 specific mAb's was a result of specific binding of the mAb's to defined regions on p66 rather than to non-specific binding. This was performed by Western blotting of NCP strips with SDS-PAGE protein standards side by side with the cleavage fragments. Since the mAb's were specific for only p66, or defined regions of p66, any binding of the mAb's to the protein standards would be non-specific. The concentrations of the primary (mAb) and secondary (IgG) antibodies were both varied. Initially, mAb-50 blots were probed with 1:50, 1:100, 1:200, and 1:400 dilutions of mAb-50 followed by 20μg of the IgG conjugate (fig. 5A). When blots were probed with a 1:50 dilution (lanes 1 and 2), three of the protein standards (97.4, 66.2, and 21.5kDa) (lane 2), most noticeably the 21.5kDa standard (Soybean trypsin inhibitor), were bound by mAb-50, indicating that non-specific binding was occurring. Similar binding to the standards was seen with the 1:100 (lane
Figure 5A: mAb-50 probed Western blots of p66 cleavage fragments and protein standards. Cleavage fragments and protein standards were electroblotted from the polyacrylamide gel and probed with the indicated mAb-50 dilutions. Lanes: 1, cleavage fragments probed with 1:50 mAb-50 dilution; 2, protein standards probed with 1:50 mAb-50 dilution; 3, cleavage fragments probed with 1:100 dilution; 4, protein standards probed with 1:100 dilution; 5, cleavage fragments probed with 1:200 dilution; 6, protein standards probed with 1:200 dilution; 7, cleavage fragments probed with 1:400 dilution; 8, protein standards probed with 1:400 dilution.
4), 1:200 (lane 6), and 1:400 dilutions (lane 8). As a result of this non-specific binding, the observed mAb-50 recognition of the cleavage fragments (51 and 42kDa) in these blots (odd numbered lanes) was not valid. The non-specific binding still present in blotting with the 1:400 dilution of mAb-50 suggested that the IgG (secondary antibody) might be the cause of this non-specific binding. Another set of blots were run again with the fragments and standards, side by side. This time, however, the dilution of mAb-50 remained constant (1:100), while the amount of IgG was varied (fig. 5B). Two different amounts of IgG were used, 5 and 2.5μg. Although non-specific binding of the standards (97.4, 66.0, and 21.5) was observed in the 5μg IgG blot (lane 2), nonspecific binding to standards was negligible in the 2.5 μg IgG blot (lane 4). These results established that binding of mAb-50 to the fragments (lanes 1 and 3) was specific. The appropriate conditions for Western blotting with mAb-50 therefore were found to be a 1:100 dilution of mAb-50, followed by a maximum of 2.5μg IgG. Similar antibody titration experiments were run with the other mAb's used. The results were similar to the mAb-50 results, and it was determined that a minimum 1:100 dilution of each of these mAb's followed by a maximum of 2.5μg of IgG were appropriate Western Blotting conditions for these antibodies.

**RNase H Activity of Fragments** - An RNase H activity gel analysis was performed to determine if any of the cleavage
Figure 5B: Western blots of p66 cleavage fragments and protein standards probed with a 1:100 dilution of mAb-50, followed by incubation with the indicated amounts of IgG. Lanes: 1, cleavage fragments incubated with 5 μg of IgG; 2, protein standards incubated with 5μg IgG; 3, cleavage fragments incubated with 2.5μg of IgG; 4, protein standards incubated with 2.5μg IgG.
fragments retained RNase H activity. Results of this analysis are shown in Figure 6A. Proteolysis of p66 generated two fragments with RNase H activity (lane 3). The apparent molecular masses of the fragments associated with these regions of activity (windows) were determined to be 51 and 42kDa. The first molecular mass determination of these windows yielded high apparent molecular masses of 57 and 46kDa for the 51 and 42kDa fragments, respectively. However, these were later determined (see below) to be from the 51 and 42kDa fragments respectively. As expected, a window in this lane corresponding to undigested p66 was not observed since p66 was completely degraded as seen by SDS-PAGE analysis of this reaction (fig. 6B). Unexpectedly, there was no window of RNase H activity observed with the p66 control reaction (lane 2). Activity windows with p66 have been observed when larger amounts (10U RNase H) are used (fig. 6C, lane 2). However, when this amount of intact p66 was analyzed in the gel assay, an intense 51kDa window was also observed. A faint 51kDa band was also observed in the SDS-PAGE analysis of this same sample (fig. 6D lane 1). Since this sample contained no subtilisin, the observed breakdown was not due to subtilisin proteolysis. The source of this breakdown is not known.

Due to the high molecular masses, 57 and 46kDa, associated with the two regions of activity in the gel assay, it was necessary to confirm that these observed windows were generated by the 51 and 42kDa fragments and not from fragment
Figure 6A: Autoradiogram of RNase H activity gel analysis of RNase H enzymes and cleavage fragments. The gel was run as described under Materials and Methods. Lanes: 1, *E. coli* RNase H (10 Units); 2, HIV-RT control (1.5μg p66 (3 Units RNase H, initially)); 3, cleavage reaction mixture (1.5μg total protein (3 Units RNase H, initially)).
Figure 6B: Silver stained gel from SDS-PAGE analysis of cleavage and control samples ran in the RNase H activity gel. Lanes 1 and 2 contain the p66 control and cleavage sample, respectively.
Figure 6C: Autoradiogram of RNase H activity gel performed with p66. Lanes 1 and 2 contain *E. coli* RNase H (10 Units) and p66 (10 Units).
Figure 6D: Silver stained gel from SDS-PAGE analysis of the p66 sample used in the above activity gel. Lanes: 1, p66; 2, molecular weight markers.
aggregation or complexes of the fragments with BSA. Normally in SDS-PAGE, protein aggregation is not a problem because 2-mercaptoethanol (reducing agent) is present in excess amounts (50mM) in the SDS-solubilization buffer. However, in the modified (see methods) SDS-sample buffer used in the activity gel assay, the concentration of 2-mercaptoethanol is significantly reduced (0.5mM). Furthermore, this modified sample buffer also contains BSA at 1mg/ml, whereas the standard sample buffer contains no BSA. Hence the potential for protein aggregation is greatly increased. In order to show that the observed 57 and 46kDa windows were generated from the 51 and 42kDa fragments, respectively, the cleavage mixture analyzed in the above activity gel assay and a p66 cleavage mixture in standard SDS-sample buffer (containing the five breakdown fragments), were Western blotted side by side and probed with mAb-50 (fig. 7). As seen from the Western blots of these samples, the migration of the C-terminal 51 and 42kDa bands from the standard cleavage mixture (lane 4) was the same as that of the two C-terminal fragments contained in the mixture analyzed from the activity gel analysis (lane 3). Furthermore, these were the only two bands recognized by mAb-50 in lane 3. Since the RNase H domain of p66 is associated with the C-terminus of the polypeptide, these results indicated that the two windows of RNase H activity were produced by the C-terminal 51 and 42kDa fragments.

p66 and Fragment Renaturation- In order to regain enzymatic
Figure 7: mAb-50 probed Western blots of cleavage or control samples in the activity gel SDS-sample buffer or standard SDS-sample buffer. Lanes: 1, p66 control in activity gel sample buffer; 2, p66 control in standard sample buffer; 3, Cleavage fragments in activity gel sample buffer; 4, cleavage fragments in standard SDS-sample buffer.
activity, in solution, with the SDS-denatured cleavage fragments (or p66), it was necessary to remove the SDS present in cleavage and control samples. For further analysis of the RNase H activity associated with the 51 and 42kDa fragments, attempts were made to renature these cleavage fragments in solution so that enzyme kinetic analysis could be performed. Such analysis are not possible with the activity gel assay. Four attempts were made to renature the fragments (or intact p66) following treatment with SDS in cleavage reaction conditions. For the first trial, after proteolysis, cleavage or control samples were immediately treated with guanidine hydrochloride (G-HCl) (see methods) and then transferred to dialysis tubing. The samples were then dialyzed for a total of 41 h in renaturation buffers (see methods). In the second trial, samples were not treated with G-HCL. The renaturation procedure for the third trial was essentially identical to that of the second trial with the exception that PMSF was added to the renaturation buffer. In the fourth trial, immediately following PMSF termination, cleavage or control samples were added to ice cold, 20mg/ml BSA, prior to dialysis. The purpose of the BSA was to act as an alternate substrate for subtilisin with the view that subtilisin might possibly renature simultaneously with the p66 cleavage fragments during dialysis. In all of the above trials, after dialysis, aliquots of the samples were analyzed for RNase H activity by a liquid RNase H assay (see methods). The
remainder of the samples in trials one and two were concentrated, by ultrafiltration (see methods) and again analyzed for RNase H activity. Table 1 shows the results of the four trials.

In the first attempt, cleavage and renaturation was performed with the p66/p51 HIV-RT heterodimer (see introduction). In contrast to procedures two, three, and four, guanidine hydrochloride was added prior to dialysis to ensure that renaturation would start from completely denatured (unfolded) polypeptides. However, it should be noted that treatment with G-HCl resulted in the formation of precipitates in several steps of the renaturation procedure. Initially, 1710 Units of RNase H (from p66/p51) were present in cleavage and control reactions. After dialyzing these samples to remove SDS and G-HCl so that protein refolding would occur, the control reaction regained 25.4 Units (1.5% of total) of RNase H. However, there was still no detectable RNase H activity after identical treatment of the cleavage sample. Following a 3-fold increase in concentration, the control contained 11.2 units of activity (0.6% of total), whereas the cleavage sample still showed no detectable RNase H activity.

In the second attempt to renature the proteins, the samples were not treated with G-HCl. Control and cleavage samples initially contained 102 Units of RNase H (from p66). After dialysis, 2.1 Units (2.1% of total) of RNase H was recovered in the control sample and no detected activity was recovered
Table 1: Table showing RNase H activity recovered with p66 or p66/p51 control (CT) or cleavage reactions (CLV) after the dialysis renaturation procedure (see methods).
with the cleavage sample. After a 15-fold increase in concentration, the control sample contained only 0.2 Units (0.2% of total), while the concentrated cleavage sample still showed no detectable RNase H activity. Due to the lack of RNase H activity with the cleavage sample in this trial and the above trial, concentrated cleavage and control samples from the second trial were analyzed by SDS-PAGE to confirm that the fragments were still present. The results are shown in figure 8A. As seen from the gel, there are no cleavage fragments present in the concentrated cleavage sample (lane 4). These results suggested that subtilisin may have reactivated, during dialysis, in the cleavage sample and completely degraded the fragments.

In the third attempt to renature the proteins, PMSF was added to the renaturation buffer in order to insure that subtilisin would remain inactivated during the renaturation process. In the above trials, PMSF was readily removed from the samples by dialysis. Hence in those trials subtilisin could potentially have been reactivated. Once again, 102 Units of RNase H was initially present in cleavage and control reactions. However, after assaying the dialyzed samples for RNase H activity, it was found that neither the control sample nor the cleavage sample contained any detectable RNase H activity. The lack of RNase H activity with the control sample could only have been due to the presence of PMSF, since the control sample in trial two demonstrated RNase H activity in
Figure 8A: Silver stained gel from SDS-PAGE analysis of the p66 control and cleavage samples used in the trial two renaturation experiment. Lanes: 1, p66 control prior to dialysis; 2, cleavage sample prior to dialysis; 3, p66 control after dialysis and concentration; 4, cleavage sample after dialysis and concentration; 5, molecular weight markers.
the absence of PMSF. To verify this, native (active) p66 was assayed for RNase H activity in the presence of PMSF and it was found that PMSF abolishes RNase H activity. The dialyzed control and cleavage samples were then analyzed by SDS-PAGE (fig. 8B). As expected, intact p66 was present in the control sample (lane 3). However, the only bands present in the dialyzed cleavage sample were the 15kDa fragment and two other fragments with molecular masses of less than 15kDa (lane 4). The fact that all five breakdown fragments were present in the cleavage sample, prior to dialysis (lane 2), suggests that even in the presence of PMSF, subtilisin is still significantly functional, if given sufficient reaction time (41 h).

In the fourth attempt to renature p66 cleavage or control samples, BSA was present in the samples during dialysis in order to act as an alternate substrate for the reactivated subtilisin so as to prevent the subtilisin from completely degrading the fragments in the cleavage sample. Control and cleavage samples initially contained 5.44 Units of RNase H. After dialysis and concentration (see methods), 0.22 Units (4.0% of total) of RNase H activity was recovered in the control sample and 0.07 Units (1.2% of total) of RNase H activity was recovered in the cleavage sample. Although RNase H activity was observed with the cleavage sample, the specific source of this activity has not been determined. This activity could be from undigested p66, or the 51 or 42kDa fragments or
Figure 8B: Silver stained gel from SDS-PAGE analysis of p66 cleavage and control samples from the trial three renaturation experiment. Lanes: 1, p66 control prior to dialysis; 2, cleavage sample prior to dialysis; 3, p66 control after dialysis; 4, cleavage sample after dialysis; 5, molecular weight markers.
aggregates of these. These results, however, did indicate that the BSA sufficiently acted as an alternate substrate for subtilisin so as to protect the fragment mixture from further proteolytic degradation.

Proteolysis of the 66/51 heterodimer—In order to further examine the proteolysis of HIV-RT, the 66/51 heterodimer was treated with subtilisin, in the presence of 0.95% SDS. The reaction time varied from 15 s to 5 min, and the cleavage fragments were separated by SDS-PAGE (fig. 9). In 15 s, 30 s, 45 s, 1 min, and 2 min cleavage reactions (lanes 2-6, respectively), four definite breakdown fragments, which were not present in the 66/51 control sample (lane 1), were generated. The molecular masses of these fragments were determined to be 42, 33, 27, and 15 kDa. A 51 kDa polypeptide was also observed in each of these samples, however, it was not certain whether this polypeptide was a product of proteolysis or if it was the initial N-terminal p51 subunit of the heterodimer, or both. While the intensities of the p66, p51 (or a 51 kDa cleavage fragment), 42 kDa, and 27 kDa bands decreased with time, the intensities of the 33 and 15 kDa cleavage bands remained constant. Hence these results were consistent with the p66 cleavage results, mentioned earlier. In order to directly compare the heterodimer cleavage fragments with the p66 cleavage fragments, a p66 cleavage sample, from a previous study, was also run on this same gel (lane 8). Since the migration of each of the heterodimer
Figure 9: Silver stained gel from SDS-PAGE analysis of limited proteolysis of the p66/p51 heterodimer. Proteolysis took place in 0.95% SDS for the indicated times. Lanes: 1, p66/p51 control; 2, 15 s cleavage reaction; 3, 30 s cleavage reaction; 4, 45 s cleavage reaction; 5, 1 min cleavage reaction; 6, 2 min cleavage reaction; 7, 5 min cleavage reaction; 8, p66 cleavage sample containing the five already described cleavage fragments (see figures 1A and B); 9, molecular weight markers.
cleavage fragments was the same as the migration of each of the corresponding p66 cleavage fragments, it appeared that the two sets of cleavage fragments were identical.

These apparently identical sets of fragments were further examined by Western blot analysis. The heterodimer cleavage fragments and p66 cleavage fragments were transferred from the gel to NCP and probed with mAb-50 (fig. 10). As seen from this blot, mAb-50 recognized the 51 and 42kDa fragments from the heterodimer cleavage sample (lane 2). These C-terminal fragments appeared to be identical to the C-terminal 51 and 42kDa cleavage fragments in the p66 cleavage sample (lane 3). The only major band recognized by mAb-50 in the heterodimer control sample (lane 1) was a 66kDa band, which corresponded to the p66 subunit. There was a faint 51kDa band recognized by mAb-50 in the control sample. This polypeptide must have been a naturally occurring, C-terminal breakdown product, rather than the N-terminal p51 subunit, since the latter is missing the mAb-50 recognition site.
Figure 10: mAb-50 probed Western blots of cleavage or control samples from the p66/p51 heterodimer. Lanes: 1, p66/p51 control; 2, cleavage fragments from heterodimer cleavage; 3, cleavage fragments from p66 cleavage.
CHAPTER IV:
DISCUSSION/CONCLUSIONS

Limited proteolysis of p66 with subtilisin resulted in the production of distinct polypeptide fragments with apparent molecular masses of 51, 42, 33, 27, and 15 kDa. The presence of SDS is essential for generating these fragments. Of the five breakdown fragments generated, the 33 and 15kDa fragments appeared to be the most stable. While the intensities of the other fragments were significantly reduced with increasing reaction time, the intensities of the 33 and 15kDa fragments appeared to remain constant over 5 min. However, the fact that all of the fragments, including the 33 and 15kDa fragments, were completely degraded during the trial two solution renaturation procedure indicates that there are subtilisin sensitive sites on the 33 and 15kDa fragments.

Proteolysis was more extensive in 0.095% SDS than in 0.95% SDS as demonstrated by complete degradation of p66 in the 70 sec, 0.095% SDS reaction but not in the 70 s, 0.95% SDS reaction. Similarly the 42kDa fragment was completely degraded in the 5 min, 0.095% SDS reaction but not in the 5 min, 0.95% SDS reaction. The higher level of SDS appears to be slightly reducing the rate of proteolysis, such that complete degradation of p66 and the 42kDa fragment in these reactions has not occurred. This proposed rate reduction could be due to: (1) SDS-induced denaturing of subtilisin or (2) stabilization of the fragments by SDS via a change in
localized charge on p66, preventing recognition of the cleavage sites by subtilisin. It has been shown that, under the conditions used, no significant proteolysis occurs unless SDS is present. This suggests that the subtilisin sensitive sites of p66 are accessible only when p66 is in a denatured state and inaccessible when it is in the native conformation.

Characterization of p66 proteolysis products with mAb's specific for defined regions of HIV-RT led to the approximate localization of at least two major protease sensitive sites. The Western blot data indicate that both these sites are located towards the N-terminus of p66 (fig. 11). Cleavage at the most N-terminal site produces the C-terminal 51 and N-terminal 15kDa fragments, while cleavage at the other site produces the C-terminal 42 and N-terminal 27kDa fragments. The sum of the molecular masses of the 51 and 15kDa fragments is 66kDa, the molecular mass of p66. In contrast, the sum of the molecular masses of the 42 and 27kDa fragments is 69kDa, 3kDa greater than the molecular mass of p66. However, this error lies within the limits of accuracy of SDS-PAGE. Since the 51kDa fragment was recognized by both mAb-50 and mAb-42, a portion of the mAb-42 recognition site may overlap onto this fragment resulting in recognition by this mAb. The 51kDa fragment, however, does contain RNase H activity, confirming that it is a C-terminal fragment. The possibility does exist, however, that there may be two different 51kDa fragments, one containing the C-terminus and the other the N-terminus. Since
Figure 11: Schematic showing the approximate location of the subtilisin cutting sites (arrows) of p66.
the 33 and 40kDa fragments were not recognized by either mAb-50 or mAb-42, but were recognized by mAb-21, these fragments are probably generated from an area in the middle of p66. It is not known whether these fragments are generated specifically from intact p66 or from the 51 or the 42kDa fragment (or both). In other subtilisin digestions of p66 in which proteolysis proceeded for shorter times (10 s), all breakdown fragments (51, 42, 27, and 15) were produced, with the exception of the 33kDa fragment. This suggests that the 33kDa fragment may not be generated until the 51 and 42kDa fragments have already been produced. Therefore, it is likely that the 51 or 42kDa fragment (or both), are precursors to the 33kDa fragment.

It is unclear as to why mAb-19 failed to recognize the 27 and 15kDa N-terminal fragments whereas, mAb-42 clearly bound these fragments. It has been shown that mAb-19 requires the first N-terminal 13 amino acids of p66 for recognition (Ferris et al., 1990). It is possible that during proteolysis, one or more of these terminal amino acids are trimmed from the N-terminal ends of these two fragments, or from p66 prior to cleavage into these fragments. Another possibility is that binding of mAb-19 to p66 requires a conformation induced by a C-terminal region of p66, which is not present in the 27 or 15kDa fragments.

Studies involving the proteolysis of p66 have been done with the retroviral protease (Ferris et al., 1990) and other non
retroviral proteases (Lowe et al., 1988). In contrast to the above results observed with subtilisin, these groups reported production of an N-terminal 51kDa fragment; the corresponding C-terminal 15kDa fragment was not detected. Hansen et al. (1988) have cloned a portion of the HIV-RT gene, encoding only the C-terminal 15kDa region of p66, into E. coli. The resulting polypeptide exhibited RNase H activity when precipitated as an immune complex by a mAb raised against a C-terminal portion of p66. The C-terminal 51 and 42kDa cleavage fragments we produced with subtilisin are unique in that they are large, rather than small, fragments which contain RNase H activity.

It was surprising that uncleaved p66 was less active than the 51 and 42kDa fragments in the RNase H activity gel assay. In order to detect activity of p66 in this gel assay, it was necessary to apply 10 Units of RNase H to the gel. In contrast, RNase H activity of the 51 and 42kDa fragments was readily detected in cleavage samples which initially contained 3 Units of RNase H. The reason for the low relative activity of p66 in the gel assay is not known. One possibility is that there may be an interaction of the reverse transcriptase domain of p66 with the RNase H substrate poly(rG)·poly(dC). This interaction could reduce RNase H activity by inducing a conformational change in p66 such that the RNase H domain is no longer functional. Loss of N-terminal regions from the reverse transcriptase domain by proteolysis may reduce or
eliminate interaction of the reverse transcriptase domain with the RNase H substrate, enhancing RNase H activity of the C-terminal fragments. Another possibility may be due to faster renaturation of the fragments as opposed to slower renaturation of p66, brought about by its increase in size. A final possibility may be that the 51 and 42kDa fragments are more active than p66. Intact p66/RNase H may be under certain constraints induced by the N-terminal domain, whereas, these constraints may not be present in the C-terminal fragments. In the RNase H activity gel, the 51 and 42kDa fragments migrated with high apparent molecular weights of 57 and 46kDa, respectively. This altered migration may have been due to interaction of these fragments with the RNase H substrate in the gel during electrophoresis.

In the solution renaturation experiments, RNase H activity was observed with p66 control samples in trials one, two, and four, but not with the control sample from trial three. The lack of RNase H activity in the control sample from trial three was a result of RNase H inhibition by PMSF. In trials one, two, and four, the RNase H activity recovered after dialysis of the p66 control samples was significantly less than the activity initially used. This decrease in RNase H activity may be a result of incomplete renaturation. The absence of RNase H activity with the cleavage samples from the first three renaturation trials is clearly a result of subtilisin reactivation and complete degradation of the
cleavage fragments by subtilisin. Even in the presence of PMSF (trial three), all of the breakdown fragments, except the 15kDa fragment, were completely degraded. This suggests that either subtilisin is not completely inactivated by PMSF, or PMSF and SDS act together to terminate proteolysis. SDS, however, is removed during dialysis and thus could not aid in keeping subtilisin inactive. The proteolysis termination experiments showed that 2mM PMSF prevents subtilisin from degrading BSA in a 5 min reaction. However, the fact that the p66 cleavage fragments were completely degraded during renaturation in the presence of PMSF suggests that there is still residual subtilisin activity present. Furthermore, after 41 h of renaturation, this residual subtilisin activity becomes significant. The results of the trial four solution renaturation procedure showed that when a large amount of BSA was added to the samples, RNase H activity was obtained with the fragment mixture, indicating that the subtilisin was no longer completely degrading the fragments. Although the recovered RNase H activity was significant, it was not high enough to examine by kinetic analysis.

The breakdown products generated by the proteolysis of the p66/p51 heterodimer were similar, if not identical, to those produced by p66 proteolysis. The five major cleavage fragments generated from the heterodimer had the same molecular masses as those from the p66 cleavage. Furthermore, the 51 and 42kDa fragments from the heterodimer cleavage sample were recognized
by mAb-50, indicating that these were also C-terminal fragments. These results suggest that the major subtilisin sensitive sites of the heterodimer are contained within the p66 subunit and these sites are the same sites as those on the p66 homodimer subunits. Because of the presence of SDS (1%) in the proteolysis reactions, question arises to whether or not p66 and p66/p51 exist in the homo and heterodimer forms, respectively, during proteolysis. This matter has not been addressed.

The data presented here show that elimination of N-terminal portions of the 66kDa form of HIV-RT results in C-terminal fragments which, under specific conditions, still contain RNase H activity. These fragments may help in elucidating mechanisms of catalysis and inhibition by RNase H specific inhibitors. These C-terminal fragments may also yield important structural information about HIV-RT. Due to the random ordering of HIV-RT crystals, the crystal structure of this enzyme has not been determined. Although a crystal structure for the 15kDa, C-terminal domain has recently been reported (Davies et al., 1991), this polypeptide alone contained no detectable RNase H activity (Hostomsky et al., 1991). In contrast, the larger C-terminal fragments described in this study do display RNase H activity. If these fragments can be crystallized, they might provide important information about the active conformation of RNase H, which would lead to the design of RNase H specific inhibitors.
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