Proteolytic Processing Mechanisms of a Miniprecursor of the Aspartic Protease of Human Immunodeficiency Virus Type 1†

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ABSTRACT: The infectivity of the human immunodeficiency virus (HIV) depends upon correct proteolytic processing of viral polyprotein precursors, the Pr55^{gag} and Pr160$^{gag\text{-}pol}$ polyproteins. The processing is mediated spontaneously by the viral protease unit (PR) contained within the Pr160$^{gag\text{-}pol}$ precursor. However, little is known about the mechanism of this process. The expression in *Escherichia coli* and the isolation of a 14-kDa HIV-1 PR "miniprecursor" with Ala^{28} mutated to serine has permitted study of the mechanism for cleavage at the N-terminus of the protease. The miniprecursor is active against a synthetic peptide substrate, and its specific activity is near that of the mutant mature protease. The rate of conversion of radiolabeled precursor to mature protease is quantitated by measuring the amounts of the two radiolabeled proteins separated by SDS–PAGE. The apparent first-order conversion rate constant, k_{app}, is dependent on miniprecursor concentration indicating a second-order reaction and suggesting an interdimeric processing mechanism. A significant first-order rate constant is observed when the plot of k_{app} versus initial precursor concentration is extrapolated to zero. This observation suggests the presence of an alternative processing mechanism involving a single active precursor dimer. The presence of both mechanisms is an advantage for the virus to ensure processing under various conditions.

The aspartic protease of human immunodeficiency virus (HIV)[1] is synthesized as a part of the Pr160$^{gag\text{-}pol}$ polyprotein precursor. The single chain precursor contains the viral protein units matrix (MA), capsid (CA), nucleocapsid (NC), the 11-kDa protease monomer (PR), reverse transcriptase (RT), and integrase (IN) in the order mentioned (Jacks et al., 1988). Active site mutation demonstrates that the protease is responsible for cleavage of the Pr160$^{gag\text{-}pol}$ precursor to yield the structural proteins and enzymes required for replication of the virus (Debouck et al., 1987; LeGrice et al., 1988). Since the HIV protease is essential for the maturation of infectious virions (Kohl et al., 1988; Gottlinger et al., 1989; Ross et al., 1991), it is a target for inhibitory drugs designed to treat acquired immunodeficiency syndrome (Petteway et al., 1991; Tang et al., 1992).

While the *pol* gene of HIV codes for the protease as a single chain polypeptide which contains 99 amino acids, the protease functional unit is a homodimer (Meek et al., 1989; Cheng et al., 1990; DiIanni et al., 1990; Babe et al., 1991; Grant et al., 1992). Crystal structure determination has shown the dimeric HIV protease to be structurally homologous to members of the aspartic protease family which includes, among others, pepsin, renin, and cathepsin D. The active site of these proteases contains two aspartic acid residues and a highly conserved fold of the backbone chain adjacent to the active aspartyls (Davies, 1990). Most members of the aspartic protease family of enzymes are synthesized as precursors with an N-terminal extension of about 40 residues. Detailed studies of pepsinogen, the pepsin precursor, have shown that zymogen conversion can be catalyzed by an intramolecular cleavage of the propeptide by the nascent active site (Bustin & Conway-Jacobs, 1971; Al-Janabi et al., 1972). As a result of this activation, the N-terminal strand of pepsin replaces the N-terminal strand of pepsinogen in a six-chain β-sheet present in both structures (Davies, 1990).

Detailed kinetic study of the lytic reactions that release the functional proteins from the HIV polyprotein precursor has been hampered by the alacrity of these reactions. An intact precursor has not been isolated (Debouck et al., 1987; Gottlinger et al., 1989; Strickler et al., 1989; Ido et al., 1991). Moreover, mutation at the N-terminus of the protease has demonstrated processing activity even though mature protease is not formed (Kotler et al., 1992). Most investigators have chosen to express mutant miniprecursors which cannot be processed because the cleavage site is made unacceptable to the protease. In several instances, for ease of purification, pseudoprecursors have been prepared. These pseudoprecursors result from the fusion of the HIV protease gene with the gene for β-galactosidase (Valverde et al., 1992), maltose binding protein (Louis et al., 1991), or an IGG binding domain (Boutelje et al., 1990). In these constructs, the HIV protease is active and, for the latter, the functional unit has been shown to be the dimeric form of the pseudoprecursor. Finally, Strickler et al. (1989) have developed an expression system from which a 25-kDa HIV-protease precursor can be isolated. Studies of the maturation of this molecule have not been reported.

The goal of the present study is to quantitate the rate of formation of 11-kDa HIV-protease monomer from a miniprecursor and to determine the kinetic mechanism of processing

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[1] Abbreviations: HIV, human immunodeficiency virus; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; k_{app}, apparent first-order processing rate constant.

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activity. In order to perform these experiments, isolation of stable precursor is required. A necessary retardation of activity to prevent processing during isolation of homogenous precursor is achieved by a point mutation A28S near the enzyme active site. This mutant HIV-protease is a less efficient enzyme but has pH dependence of its kinetic parameters very similar to the wild-type enzyme (Ido et al., 1991). The miniprecursor protein is the HIV-protease molecule with an N-terminal extension of 25 amino acids. As the miniprecursor is expressed with the correct mature C-terminus, there is only one possible cleavage site allowing a single reaction to be studied kinetically. The results reported here indicate the HIV-1 protease, expressed as a 14-kDa miniprecursor with a mutation to alter its catalytic efficiency, is capable of both unimolecular[2] and bimolecular processing.

EXPERIMENTAL PROCEDURES

Materials

Expression vector pET3b was a gift from Dr. F. W. Studier, Brookhaven National Laboratory. PCR reagents were obtained from Perkin-Elmer-Cetus Corp. DNase I was purchased from Sigma. DEAE-Sephacel was purchased from Pharmacia. ^{14}C amino acid hydrolysate was obtained from Amersham. Solvable gel solubilizer and Formula 989 scintillation cocktail were purchased from NEN Research Products. Peptide substrate Lys-Ala-Arg-Val-Leu-Nph-Glu-Ala-Met (Nph, *p*-nitrophenylalanine) was synthesized at the Molecular Biology Resource Center at the University of Oklahoma Health Sciences Center. Inhibitor U71038 was a gift from Dr. Robert Heinrikson, The Upjohn Company. All other reagents were of the highest grade available commercially.

Methods

Preparation of Radiolabeled HIV-1 Miniprecursor. The expression vector pET-HIVPRA28S encoded HIV-1 protease with the A28S mutation and a 25 amino acid extension at the N-terminus; positions −10 to −1 were the upstream *pol* sequence and positions −25 to −11 were derived from the vector (Ido et al., 1991). *Escherichia coli* strain BL21(DE3)pLysS harboring the expression vector was grown overnight at 37 °C in ZB media (10 g of N-Z-amine and 5 g of NaCl/L) with 60 μg/mL ampicillin and 25 μg/mL chloramphenicol. The preculture media was diluted 100-fold in LB media (Luria broth) containing 60 μg/mL ampicillin and 25 μg/mL chloramphenicol and grown for at least 3 h at 37 °C with constant shaking at 225 rpm in a water bath. When the OD of the culture had reached 0.4–0.5 at 600 nm, 100 μCi of ^{14}C amino acid hydrolysate was added. After 15 min isopropyl β-D-thiogalactopyranoside was added to a final concentration of 400 mM. Growth of the culture was continued for another 3 h. The inclusion bodies of radiolabeled miniprecursor were recovered, dissolved in 8 M urea, and purified through a DEAE-Sephacel column as previously described (Ido et al., 1991). The refolding of A28S HIV-1 protease miniprecursor was accomplished by changing the pH of the breakthrough peak from the DEAE column to pH 3.5 with 10% trifluoroacetic acid and dialyzing for 4 h at 4 °C against four changes of 10 mM sodium acetate, pH 3.5, containing 1 mM DTT. The extinction coefficient of 1.0 at 280 nm for a 0.1% solution was determined previously and used to determine precursor concentration (Ido et al., 1991). At the miniprecursor concentration employed herein, dimer dissociation should be negligible if K_d is similar to that of the mature protease (Zhang et al., 1991).

Assay for Proteolytic Activities of A28S Miniprecursor or A28S HIV-1 Protease. The HIV-1 protease activity was assayed using synthetic substrate, Lys-Ala-Arg-Val-Leu-Nph-Glu-Ala-Met (Nph, *p*-nitrophenylalanine) (Richards et al., 1990). A typical reaction mixture consisted of a 1 mL total volume which was 10 mM in sodium acetate (pH range 3.5–6), 1 mM in DTT, and approximately 0.1 mM in miniprecursor. The reaction was initiated by the addition of 60 μL of 8.48 mM substrate. The rate of hydrolysis was monitored at A_{300} in a Hewlett Packard 8452A Diode Array Spectrophotometer using the HP 89531A UV/VIS operating software. For measuring the catalytic activity of the miniprecursor, the hydrolysis of peptide substrate was recorded for 150 s. During this time period, the mature protease concentration was not significantly altered by conversion of precursor to mature protease.

Quantitation of the number of active sites in an enzyme sample was accomplished by titration using the method of Tomasselli et al. (1990). A stock solution of inhibitor U71038, 5.7 mM in 50% acetic acid, was diluted 10-fold into water and used for all determinations.

Measurement of Conversion of A28S Miniprecursor to A28S HIV-1 Protease. To an aliquot of 2 mL of the dialyzed miniprecursor solution, 100 μL of 1 M sodium acetate, pH 4.5, was added. The change in pH from 3.5 to 4.5 initiated the conversion of the precursor to the A28S HIV-1 protease. The solution was incubated at 26 °C up to 6 h. At various time intervals, 100-μL aliquots were removed, mixed with a 10-fold excess of electrophoresis sample buffer (containing 4% SDS), and subjected to electrophoresis under reducing conditions in 15% polyacrylamide gels. The bands representing the 14-kDa miniprecursor and 11-kDa protease were cut out from the gel and solubilized in separate scintillation vials with 1 mL of gel solubilizer at 55 °C overnight. After the addition of 10 mL of scintillation fluid, the radioactivity was determined in a scintillation counter. The radioactivity determined by this procedure was linearly related to miniprecursor concentration (data not shown). In the precursor processing experiments, the time course demonstrated a fraction of the precursor which could not be converted to the 11-kDa protease. Since this inactive 14-kDa precursor was probably incorrectly folded, an unsuccessful search for more productive folding conditions was made. The percentage of mature 11-kDa protease as a function of time was fitted to a first-order model with nonlinear regression using SAS 6.06 (SAS Institute, Inc., 1985). The pseudo-first-order rate constant, k_{app}, and the amount of 14-kDa miniprecursor which could not be processed were obtained from the regression. The precursor concentrations used in further analyses were corrected to the quantity of processable precursor in each preparation. To examine the concentration dependence of the 14-kDa processing rate, the apparent first-order rate constants (k_{app}) as a function of concentration of 14-kDa miniprecursor were fit to a linear model weighted with the inverse error for k_{app} using SAS 6.06. Each regression included processing experiments from a given preparation of 14-kDa miniprecursor to obtain independent parameter estimates.

RESULTS

Proteolytic Activities of Miniprecursor and Mature HIV-1 Protease. Specific activity for the hydrolysis of synthetic

[2] Unimolecular refers to a single functional protease dimer or miniprecursor dimer. Bimolecular refers to interaction of one functional protease or miniprecursor dimer with a second protease or miniprecursor dimer.

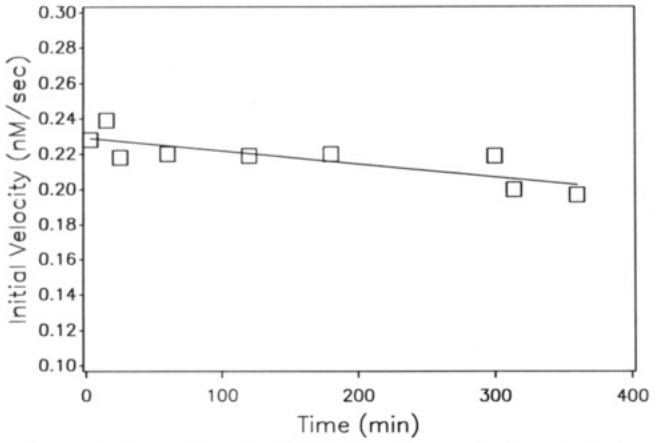

FIGURE 1: Rate of synthetic peptide substrate hydrolysis during processing of A28S miniprecursor. At different time intervals, synthetic substrate was added to an aliquot of the processing solution, and the rate of peptide hydrolysis was followed spectrophotometrically. The total precursor concentration was 1 mg/mL, and the processing pH was 4.5.

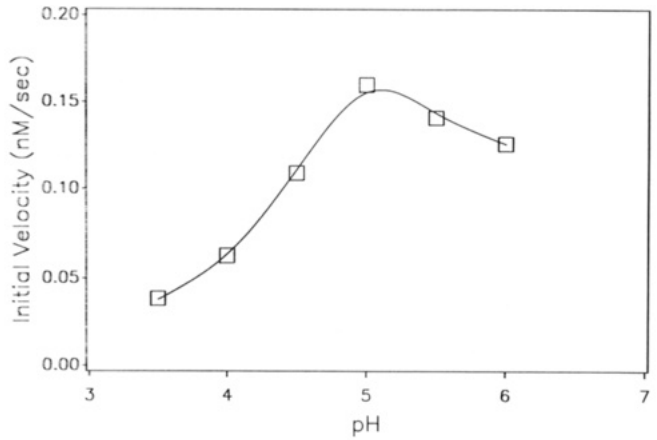

FIGURE 2: pH dependence of the activity of A28S miniprecursor for the cleavage of synthetic peptide substrate. The optimum pH for activity is near that of the active A28S mature protease using the same substrate.

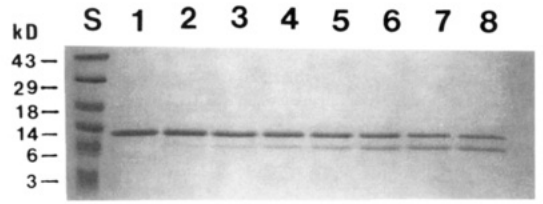

FIGURE 3: SDS–PAGE pattern of the processing of the 14-kDa A28S miniprecursor to the 11-kDa A28S HIV-1 protease during the course of the reaction. Lane S, molecular weight markers; lanes 1–8, processing of A28S miniprecursor halted at times noted (0, 30, 60, 120, 180, 240, 300, and 360 min, respectively).

peptide substrate was measured at different time points during miniprecursor processing (Figure 1). The activity at time zero was that of the miniprecursor since no mature protease was present. After 6 h, a time sufficient to convert nearly all miniprecursor to mature enzyme, only a 10% decrease in activity was observed, which was likely due to autodigestion. (In some experiments a small amount of 6-kDa autodigestion product was observed in SDS–PAGE: data not shown.) In another experiment, titrations of active sites showed identical concentrations of active sites (0.0017 ± 0.0003 mM for each) before and after processing. Likewise nearly identical specific activities of 62 ± 4 units/mg and 70 ± 4 units/mg were observed. These results indicated that the miniprecursor has

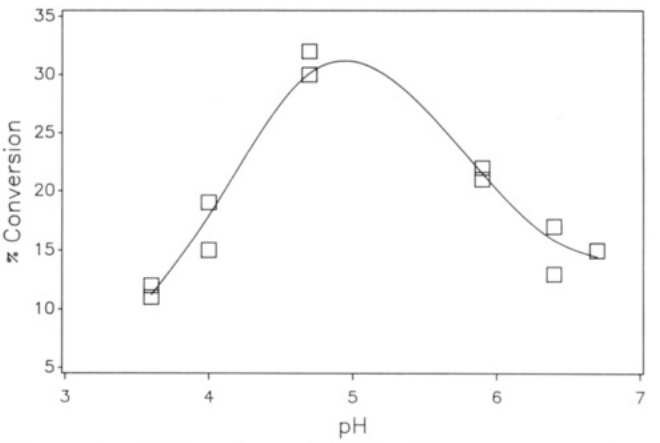

FIGURE 4: pH dependence of A28S miniprecursor processing. Fractional conversion measured the ratio of the 11-kDa moiety to the sum of the 14-kDa and 11-kDa species. Quantitation was determined from radioactivity on SDS–PAGE. Processing had been terminated after 1 h.

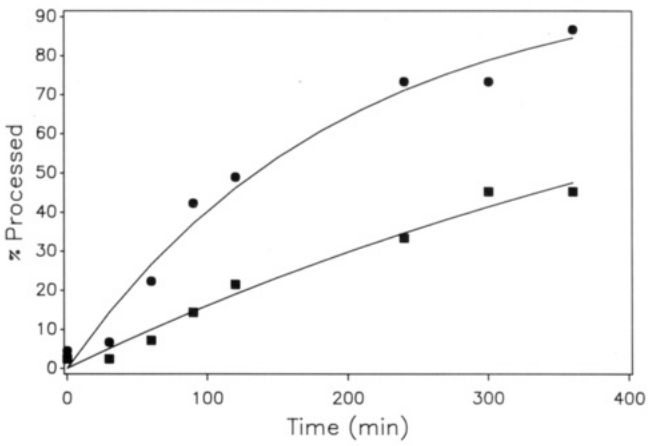

FIGURE 5: Plot of conversion of A28S HIV-1 protease miniprecursor. Two concentrations of 14-kDa miniprecursor used were 0.03 mg/mL (■) and 0.34 mg/mL (●). Data were fit to a first-order processing model to determine k_{app} for each concentration. Processing completion is more rapid for the higher concentration suggesting concentration dependence of conversion.

nearly the same specific activity as the mature A28S HIV-1 protease. Also, under these conditions, the proteolytic capability does not change during processing. The pH dependence of peptide hydrolyzing activity of miniprecursor was measured between pH 3.5 and 6 (Figure 2). Maximal activity was observed between pH 4.5 and 5.5.

Conversion of A28S Miniprecursor to A28S HIV-1 Protease. Incubation of A28S miniprecursor at pH 4.5 caused a spontaneous conversion of the 14-kDa precursor to the 11-kDa mature enzyme. This phenomenon was clearly seen in the SDS–polyacrylamide gel electrophoresis (Figure 3). The electrophoretic pattern showed that in the zero time sample almost no 11-kDa species was present. Processing was prevented by the presence of equimolar amounts of HIV protease inhibitor U71038 (data not shown). The pH dependence of the processing rate revealed a maximum near pH 5 (Figure 4). Therefore, peptide hydrolysis and processing had similar pH optima.

Sets of gels of which Figure 3 is typical were used to quantitate the conversion of miniprecursor to mature protease during incubation times of up to 6 h. Five independent preparations of A28S miniprecursor were studied. Each preparation was diluted to prepare five to ten samples covering a 5–10-fold range of concentration. Each sample was subjected

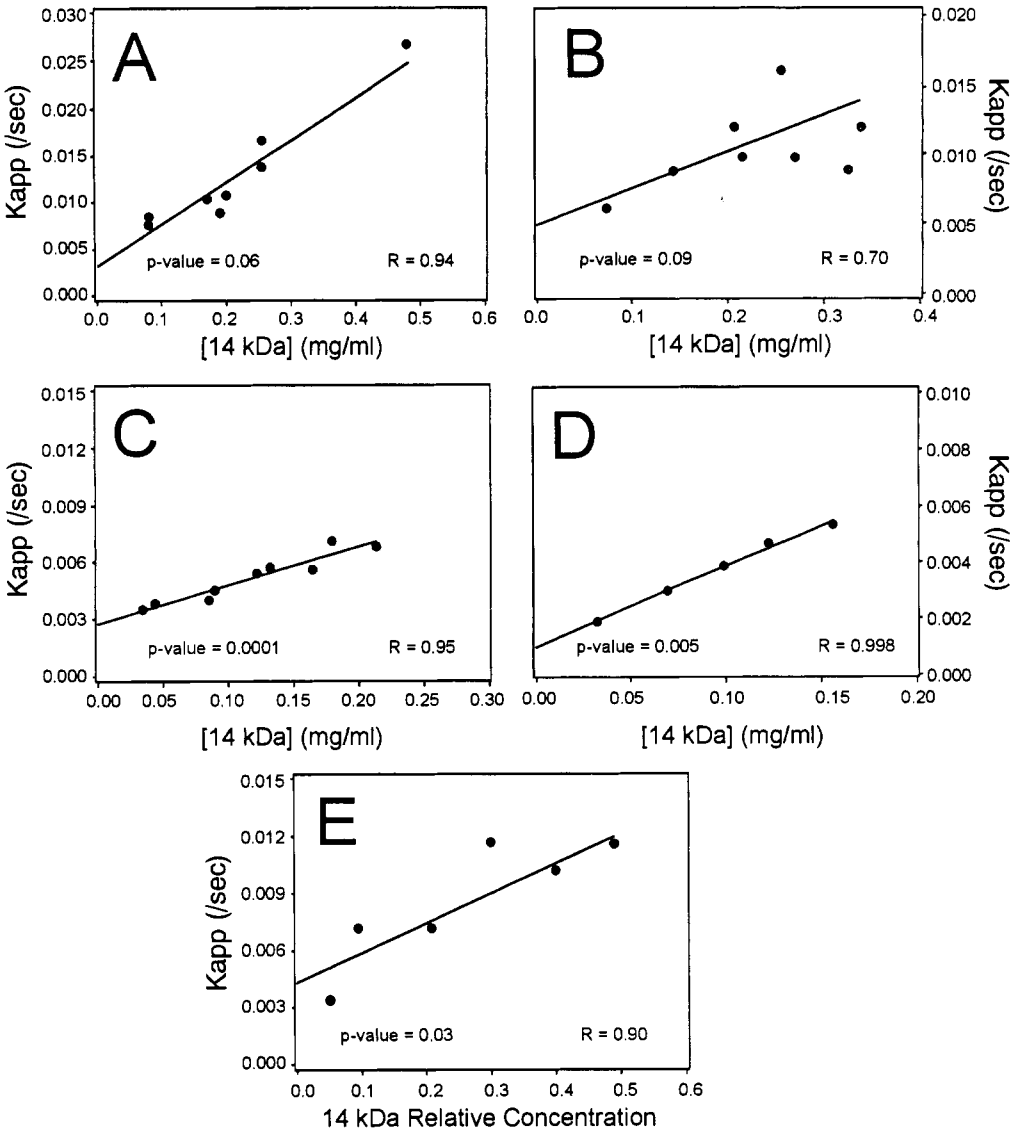

FIGURE 6: Dependence of k_{app} on the concentration of A28S miniprecursor. Data in each panel are from different miniprecursor preparations and correspond to a row in Table 1. The significance of the Y-intercept (p-value for H0: intercept = 0) and the correlation coefficient (R) are indicated on each plot.

to processing incubation, and aliquots were withdrawn at various times. Time courses for two such samples are shown in Figure 5. These time courses were exponential and could be fit with a first-order kinetic model. This fit allowed determination of k_{app}, the apparent first-order rate constant, and percentage of miniprecursor which could be processed. Although the fraction of 14-kDa miniprecursor which could be processed varies between preparations, it is in good agreement within each preparation as determined from independent nonlinear regressions (data not shown). In a control experiment, the percentage of processable 14-kDa miniprecursor was determined from active site titration. Relative to the concentration determined from absorbance at 280 nm, 44% of the active sites were intact. In this experiment, a nearly identical amount of processable precursor (46%) was quantitated from radioactive counts of 11-kDa and 14-kDa bands in SDS–PAGE after processing for 36 h.

Treatment of Data. For each of the five independent preparations, a plot of k_{app} as a function of processable miniprecursor concentration is shown in Figure 6. In no case is there visual evidence for systematic deviation from linearity. The concentration dependence of k_{app} suggests the presence of a second-order conversion reaction. In addition, since the extrapolated value of k_{app} in each treatment does not approach zero at zero concentration of precursor, the reaction has a first-order component. A kinetic scheme which accommodates the observed processing data includes three possible modes of conversion:

$$P \xrightarrow{k_1} M \qquad (1)$$

Table 1: Conversion Rate Constants for the Processing of 14-kDa Miniprecursor to 11-kDa Mature HIV A28S Protease

prep	concn (mg/mL)	% processable	N	k_1 (min^{-1})	k_2 [min^{-1}·(mg/mL)$^{-1}$]
A	2.50	20	8	0.003 (0.001)[a]	0.045 (0.006)
B	0.65	53	8	0.005 (0.002)	0.03 (0.01)
C	0.35	60	8	0.0027 (0.0003)	0.021 (0.003)
D	0.30	54	5	0.0009 (0.0001)	0.029 (0.005)
E	NA[b]	50	6	0.004 (0.001)	NA
average				0.003 (0.001)[c,d]	0.03 (0.01)[d]

[a] Standard deviation in parentheses. [b] NA, not available. [c] The k_1 was averaged from five experiments as noted under Methods. The k_1 is significant (p-value = 0.007 for H0: mean = 0, Student's T test). [d] Reported constants are averages weighted by the product of the correlation coefficient and the inverse of standard error from linear regression.

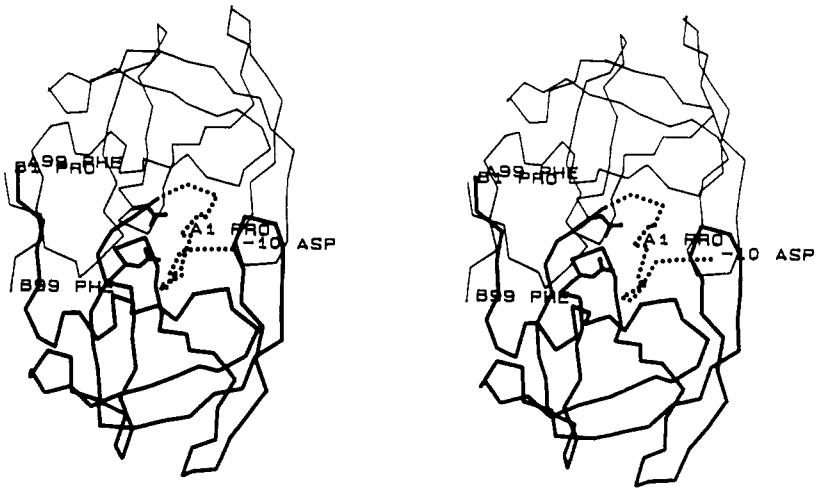

FIGURE 7: Stereoview of a computer graphics model of the HIV-1 protease miniprecursor engaging in intradimeric processing. (Dotted line) Region of the protease which is relocated from the N-terminal β-strand, including residue 6 (A6) of the A chain of the homodimer through 10 amino acid residues of the upstream *pol* sequence. (Heavy line) Residues A6–A99; (light line) residues B1–B99 of the B chain. Both catalytic aspartic acids are illustrated and in this model are adjacent to the scissile bond between proline A1 (labeled) and phenylalanine −1 of the A chain. The model was based upon the structure of the HIV-1 protease–inhibitor complex of Miller et al. (1989), entry 4HVP obtained from the Protein Data Bank (Bernstein et al., 1977). Graphic modeling was done using PSFRODO (Jones, 1978) on an Evans and Sutherland PS390.

$$P + P \xrightarrow{k_2} M + P \quad (2)$$

$$P + M \xrightarrow{k_3} M + M \quad (3)$$

where P is the 14-kDa miniprecursor dimer, M is the 11-kDa mature protease dimer, and k_1, k_2, and k_3 are the catalytic rate constants for the reactions. Apparently, misfolded miniprecursor is not a substrate for lysis by either P or M since the 14-kDa band persists on the gels and since the above scheme fits the data. The appearance of M as a function of time is

$$\frac{dM}{dt} = k_1P + k_2P^2 + k_3PM \quad (4)$$

Since the 14- kDa miniprecursor has nearly the same specific activity as the mature 11-kDa protease, it may be assumed that $k_2 = k_3$:

$$\frac{dM}{dt} = [k_1 + k_2(P + M)]P = k_{app}P \quad (5)$$

Consequently the first-order nature of k_{app} is justified. The k_{app} is linearly related to the concentration of (P + M). The slope of the linear plot is k_2, and its intercept is k_1. Table 1 shows the results from five independent determinations (k_1 = 0.003 ± 0.001 min^{-1}, and k_2 = 0.03 ± 0.01 min^{-1}·mg^{-1}·mL).

DISCUSSION

The quantitative kinetic studies reported herein have allowed, for the first time, determination of the processing mechanism for an HIV protease precursor and values of the associated rate constants. At the pH of maximum enzymic activity, the HIV-1 protease miniprecursor is capable of unimolecular autoprocessing and bimolecular or interdimeric processing. In a typical experiment (0.034 mg/mL), when half of the miniprecursor is processed, 47% occurs by the unimolecular pathway (eq 1) and 53% by the bimolecular pathways (eqs 2 and 3). For a higher protein concentration (0.21 mg/mL), 36% of processing occurs by the unimolecular and 64% the bimolecular mechanism. For even higher protein concentrations within a nascent virion, as estimated from its fine structure (Gelderblom et al., 1987), the importance of unimolecular processing is diminished, and the overall rate of conversion increases. Our results require that a protease precursor dimer be capable of accepting and processing its own N-terminal processing sites within its own active site. The possibility of unimolecular processing excludes the requirement for mature protease in a nascent virion to initiate processing and would preclude the need for host protease involvement in the initiation of precursor processing.

In the HIV protease crystal structures (Wlodawer et al., 1989; Lapatto et al., 1989) there is a four-stranded β-sheet composed of the N- and C-terminal strands from each monomer. The two C-terminal strands are interior in the sheet. For both interdimeric and intradimeric processing of the precursor, the N-terminal strand to be processed must dissociate from the described β-sheet and bind into an active site for cleavage. It is important to note that this dissociation of the N-terminal strand is no more severe a conformational alteration in intradimeric processing than in an interdimeric event. Unimolecular processing of a precursor dimer requires a conformation where the N-terminal peptide strand containing the processing site of the precursor is inserted into the active site of the same molecule while maintaining a conformation conducive to activity. The peptide strand containing the processing site is expected to adopt an extended conformation when in the active site as suggested by the conformation of inhibitors bound the HIV-1 protease (Jaskolski et al., 1991). Computer graphics modeling of a miniprecursor intermediate indicates that placement of an N-terminal strand of the precursor into its own active site is conformationally possible and does not necessitate other alterations in the protease structure (Figure 7). This model was constructed to show that our structural interpretation of the results presented herein is geometrically feasible; no refinement of the model was performed. In the model, the residues in the N-terminal strand and four upstream residues form regular interactions with the substrate-binding subsites in the active site while the Phe-Pro scissile bond is properly aligned with the pair of catalytic aspartates. Maintenance of an active conformation with the removal of an outer N-terminal strand of the β-sheet is feasible as mutagenesis results show that the residues in the region are not essential for activity (Loeb et al., 1989). Indeed, deletion of the N-terminal β-strands of the human T-cell leukemia virus type 1 protease (HTLV-I protease) results in an active

protease (Hayakawa et al., 1992). An analogous relocation of N-terminal residues for eukaryotic aspartic protease zymogen maturation is exemplified by pepsinogen. In this case, the residues around the processing site are dissociated from their native conformation (Sielecki et al., 1991; Hartsuck et al., 1992) to be cleaved by the active site of the same zymogen molecule [for review, see Tang and Wong (1987)].

To accomplish the goal of determining by kinetic analysis a mechanism of autoprocessing, it was necessary to study a miniprecursor molecule. Processing quantitation and kinetic analysis were simplified by the existence of a single cleavage site. The choice of a miniprecursor construct with an N-terminal extension was made for several reasons. The N-terminal strands are the outer strands in the β-sheet composed of N- and C-terminal strands from each monomer in the HIV protease dimer. Moreover, there is evidence that removal of the C-terminal strand from the β-sheet of some retroviral proteases may compromise catalytic capability (Bennett et al., 1991; Hayakawa et al., 1992). In fact, in the Rous Sarcoma Virus protease a free C-terminus is required for the initiation of processing. The use of the A28S mutant form allowed purification of the miniprecursor whereas during the expression of the native miniprecursor spontaneous processing precludes its isolation. In the A28S mutant the k_{cat} is diminished by a factor of 200 while K_m is virtually unchanged (Ido et al., 1991). The pH dependence of the parameters is unchanged by the A28S mutation. Consequently the mutation is not expected to cause a change of processing mechanism.

Complete processing of the HIV protease from the $Pr160^{gag\text{-}pol}$ polypeptide requires cleavage at both termini. Whereas analogy of N-terminal processing exists with other aspartic protease zymogens, C-terminal processing is unique to retroviral proteases expressed as part of a polyprotein from the *pol* gene. The importance of the C-terminal region to activity (Bennett et al., 1991; Hayakawa et al., 1992) and dimer formation suggests unimolecular processing activity here is unlikely. Whether processing of other sites of the $Pr160^{gag\text{-}pol}$ polypeptide can be achieved before the HIV-1 PR is released remains to be determined.

Relevance of the present result to HIV virion maturation *invivo* depends upon the existence of an active precursor dimer. Activity of various precursor constructs has been demonstrated here and by others. The accessibility of the active site in the $Pr160^{gag\text{-}pol}$ precursor may be limited; the findings of Partin et al. (1989) demonstrate increased processing by the removal of the 68 residues expressed from *pol* immediately upstream of the protease. Boutelje et al. (1990) have determined that the dimeric form of their precursor construct is active. Consequently, the initial event in autoprocessing of $Pr160^{gag\text{-}pol}$ polyprotein precursors is likely the association of two protease molecules contained in the polyprotein to form a competent precursor dimer.

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REFERENCES

Al-Janabi, J., Hartsuck, J. A., & Tang, J. (1972) *J. Biol. Chem. 247*, 4628–4632.

Babe, L. M., Pichuantes, S., & Craik, C. S. (1991) *Biochemistry 30*, 106–111.

Bennett, R. P., Rhee, S., Craven, R. C., Hunter, E., & Wills, J. W. (1991) *J. Virol. 65*, 272–280.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Jr., Brice, M. D., Rodgers., J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Boutelje, J., Karlstrom, A. R, Hartmanis, M. G. N., Holmgren, E., Sjogren, A., & Levine, R. (1990) *Arch. Biochem. Biophys. 283*, 141–149.

Bustin, M., & Conway-Jacobs, A. (1971) *J. Biol. Chem. 246*, 615–620.

Cheng, Y. E., Yin, F. H., Foundling, S., Blomstrom, D., & Kettner, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9660–9664.

Darke, P., Leu, C.-T., Davis, L. J., Heimbach, J. C., Diehl, R. E., Hill, W. S., Dixon, R. A. F., & Sigal, I. S. (1989) *J. Biol. Chem. 264*, 2307–2312.

Debouck, C., Gorniak, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W., & Rosenberg, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8903–8906.

DiIanni, C. L., Davis, L. J., Holloway, M. K., Herber, W. K., Darke, P. L., Kohl, N. E., & Dixon, R. A. F. (1990) *J. Biol. Chem. 265*, 17348–17354.

Gelderblom, H. R., Hausmann, E. H. S., Ozel, M., Pauli, G., & Koch, M. A. (1987) *Virology 156*, 171–176.

Gottlinger, H. G., Sodroski, J. G., & Haseltine, W. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5781–5785.

Grant, S. K., Deckman, I. C., Culp, J. S., Minnich, M. D., Brooks, I. S., Hensley, P., Debouck, C., & Meek, T. D. (1992) *Biochemistry 31*, 9491–9501.

Graves, M. C., Lim, J. J., Heimer, E. P., & Kramer, R. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2449–2453.

Hansen, J., Billich, S., Schulze, T., Sukrow, S., & Moelling, K. (1988) *EMBO J. 7*, 1785–1791.

Hartsuck, J. A., Koelsch, G., & Remington, S. J. (1992) *Proteins: Struct., Funct., Genet. 13*, 1–25.

Hayakawa, T., Misumi, Y., Kobayashi, M., Yamamoto, Y., & Fujisawa, Y. (1992) *Eur. J. Biochem. 206*, 919–925.

Ido, E., Han, H. P., Kezdy, F. J., & Tang, J. (1991) *J. Biol. Chem. 266*, 24359–24366.

Jacks, T., Power, M. D., Masiarz, R. R., Luciw, P. A., Barr, P. J., & Varmus, H. E. (1988) *Nature 331*, 280–283.

Jaskolski, M., Tomasselli, A. G., Sawyer, T. K., Staples, D. G., Heinrikson, R. L., Schneider, J., Kent, S. B. H., & Wlodawer, A. (1991) *Biochemistry 30*, 1600–1609.

Jones, T. A. (1978) *J. Appl. Crystallogr. 11*, 268–272.

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., & Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4686–4690.

Kotler, M., Arad, G., & Hughes, S. H. (1992) *J. Virol. 66*, 6304–6313.

Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J. R., Whittle, P. J., Danley, D. E., Geoghegan, K. F., Hawrylik, S. J., Lee, S. E., Scheld, K. G., & Hobart, P. M. (1989) *Nature 342*, 299–301.

LeGrice, S. F. J., Mills, J., & Mous, J. (1988) *EMBO J. 7*, 2547–2553.

Leis, J., Baltimore, D., Bishop, J. M., Coffin, J., Fleissner, E., Goff, S. P., Oroszlan, S., Robinson, H., Skalka, A., Temin, H. M., & Vogt, V. (1988) *J. Virol. 62*, 1808–1811.

Loeb, D. D., Swanstrom, R., Everitt, L., Manchester, M., Stamper, S. E., & Hutchison, C. A., III (1989) *Nature 340*, 397–400.

Louis, J. M., McDonald, R. A., Nashed, N. T., Wondrak, E. M., Jerina, D. M., Oroszlan, S., & Mora, P. T. (1991) *Eur. J. Biochem. 199*, 361–369.

Meek, T. D., Dayton, B. D., Metcalf, B. W., Dreyer, G. B., Strickler, J. E., Gorniak, J. G., Rosenberg, M., Moore, M. L., Magaard, V. W., & Debouck, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1841–1845.

Miller, M., Schneider, J., Sathyanarayana, B. K., Toth, M. V., Marshal, G. R., Clawson, L., Selk, L., Kent, S. B. H., & Wlodawer, A. (1989) *Science 246*, 1149–1152.

Partin, K., Zybarth, G., Ehrlich, L., de Crombrugghe, M., Wimmer, E., & Carter, C. (1991) *Proc. Natl. Acad Sci. U.S.A. 88*, 4776–4780.

Petteway, S. R., Jr., Dreyer, G. B., Meek, T. D., Metcalf, B. W., & Lambert, D. M. (1991) in *AIDS Research Reviews* (Koff, W. C., Wong-Staal, F., & Kennedy, R. C., Eds.) pp 267–288, Marcel Dekker, Inc., New York.

Richards, A. D., Phylip, L. H., Farmerie, W. G., Scarborough, P. E., Alvarez, A., Dunn, B. M., Hirel, P.-H., Konvalinka, J., Strop, P., Pavlickova, L., Kostka, V., & Kay, J. (1990) *J. Biol. Chem. 265*, 7733–7736.

Ross, E. K., Ruerst, T. R., Orenstein, J. M., O'Neill, T., Martin, M. A., & Venkatesan, S. (1991) *AIDS Res. Hum. Retroviruses 7*, 475–483.

SAS Institute, Inc. (1985) *SAS User's Guide: Statistics*, SAS Institute, Inc., Cary, NC.

Sielecki, A. R., Fujinaga, M., Read, R. J., & James, M. N. G. (1991) *J. Mol. Biol. 219*, 671–692.

Strickler, J. E., Gorniak, J., Dayton, B., Meek, T., Moore, M., Magaard, V., Malinowski, J., & Debouck, C. (1989) *Proteins: Struct., Funct., Genet. 6*, 139–154.

Tang, J., & Wong, R. N. S. (1987) *J. Cell. Biochem. 33*, 53–63.

Tang, J., Lin, Y., Co, E., Hartsuck, J. A., & Lin, X. (1992) *Scand. J. Clin. Lab. Invest. 52*, 127–135.

Tomasselli, A. G., Olsen, M. K., Hui, J. O., Staples, D. J., Sawyer, T. K., Heinrikson, R. L., & Tomich, C.-S. C. (1990) *Biochemistry 29*, 264–269.

Valverde, V., Lemay, P., Masson, J.-M., Gay, B., & Boulanger, P. (1992) *J. Gen. Virol. 73*, 639–651.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science 245*, 616–621.

Zhang, Z.-Y., Poorman, R. A., Maggiora, L. L., Heinrikson, R. L., & Kezdy, F. J. (1991) *J. Biol. Chem. 266*, 15591–15594.