# The HIV-1 Protease as Enzyme and Substrate: Mutagenesis of Autolysis Sites and Generation of a Stable Mutant with Retained Kinetic Properties

Ana M. Mildner,[‡] Donna J. Rothrock,[‡] Joseph W. Leone,[‡] Carol A. Bannow,[‡] June M. Lull,[‡] Ilene M. Reardon,[‡] Jean L. Sarcich,[‡] W. Jeffrey Howe,[§] Che-Shen C. Tomich,[∥] Clark W. Smith,[‡] Robert L. Heinrikson,[‡] and Alfredo G. Tomasselli*,[‡]

*Biochemistry, Computational Chemistry, and Molecular Biology Units, Upjohn Laboratories, Kalamazoo, Michigan 49001*

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ABSTRACT: Site-directed mutagenesis of autolysis sites in the human immunodeficiency virus type 1 (HIV-1) protease was applied in an analysis of enzyme specificity; the protease served, therefore, as both enzyme and substrate in this study. Inspection of natural substrates of all retroviral proteases revealed the absence of β-branched amino acids at the $P_1$ site and of Lys anywhere from $P_2$ through $P_2'$. Accordingly, several mutants of the HIV-1 protease were engineered in which these excluded amino acids were substituted at their respective P positions at the three major sites of autolysis in the wild-type protease ($Leu_5$-$Trp_6$, $Leu_{33}$-$Glu_{34}$, and $Leu_{63}$-$Ile_{64}$), and the mutant enzymes were evaluated in terms of their resistance to autodegradation. All of the mutant HIV-1 proteases, expressed as inclusion bodies in *Escherichia coli*, were enzymatically active after refolding, and all showed greatly diminished rates of cleavage at the altered autolysis sites. Some, however, were not viable enzymatically because of poor physical characteristics. This was the case for mutants having Lys replacements of Glu residues at $P_2'$ and for another in which all three $P_1$ leucines were replaced by Ile. However, one of the mutant proteases, Q7K/L33I/L63I, was highly resistant to autolysis, while retaining the physical properties, specificity, and susceptibility to inhibition of the wild-type enzyme. Q7K/L33I/L63I should find useful application as a stable surrogate of the HIV-1 protease. Overall, our results can be interpreted relative to a model in which the active HIV-1 protease dimer is in equilibrium with monomeric, disordered species which serve as the substrates for autolysis.

In the span of a few years, the protease from human immunodeficiency virus (HIV), the causative agent in acquired immunodeficiency syndrome (AIDS), has become one of the best understood of enzymes, in terms of both its structure and its function [*cf.* Kay and Dunn (1990), Tomasselli *et al.* (1991a), Norbeck and Kempf (1991), Debouck (1992), Martin (1992), and Wlodawer and Erickson (1993) for reviews]. The viral protease is indispensable for processing of *gag* and *pol* gene polyprotein products during the final stage of maturation, and mutations which render the protease inactive, or inhibitors of the enzyme, prevent formation of infectious virus in cell culture (Kohl *et al.*, 1988; Ashorn *et al.*, 1990; Crawford & Goff, 1985; Katoh *et al.*, 1985; Seelmeier *et al.*, 1988; Gottlinger *et al.*, 1989). Accordingly, the HIV-1 protease has become a target for development of drugs against AIDS, and this objective has brought to bear a wide armamentarium of technologies to promote understanding of the enzyme as a target and its inhibitors as potential drugs. Some HIV-1 protease inhibitors are currently in clinical trials for treatment of AIDS.

Understanding the specificity of the HIV-1 protease remains a formidable challenge. To this end, we (Tomasselli *et al.*, 1990a,b, 1991b,c; Hui *et al.*, 1990) and others (Shoeman *et al.*, 1990; Wallin *et al.*, 1990; Rivière *et al.*, 1991; Oswald & von der Helm, 1991; Tomaszek *et al.*, 1992) have evaluated nonviral proteins as substrates of the enzyme, in part, to extend knowledge beyond that provided from the limited number of cleavage points documented in the viral polyproteins (Darke *et al.*, 1988; Henderson *et al.*, 1988; Tozser *et al.*, 1991). These analyses complement those carried out with peptide substrates modeled after processing sites in the viral polyproteins in which a variety of amino acids were substituted at $P_4$ through $P_4'$ (Konvalinka *et al.*, 1990; Margolin *et al.*, 1990; Phylip *et al.*, 1990; Griffiths *et al.*, 1992; Tozser *et al.*, 1992). To summarize a vast number of studies of HIV-1 protease specificity, it seems clear that the enzyme interacts with amino acid side chains from $P_4$ through $P_4'$ [notation according to Schechter and Berger (1967)] in its substrate and that, with notable exceptions, it can cleave almost any peptide bond, given that the surrounding amino acid residues can be accommodated productively in its extended binding pocket. The additive, or "cumulative" (Poorman *et al.*, 1991), nature of this specificity makes it unlikely that a single amino acid replacement at any given site in the substrate from $P_4$ through $P_4'$ would be sufficient to block, substantially, the cleavage at $P_1$–$P_1'$. However, inspection of 64 octapeptide protease substrates of the HIV-1 protease (Tomasselli *et al.*, 1993), and several more for other retroviral proteases (Pettit *et al.*, 1991), revealed absences of certain amino acids at particular P-sites, suggesting that these residues might have a negative impact on catalysis. For example, not seen in these substrates were β-branched amino acids at $P_1$ or Lys anywhere between $P_2$ and $P_2'$. A primary objective of the present work was to test whether such substitutions are able to block cleavage of peptide and protein substrates of the HIV-1 protease.

Another obstacle with regard to the retroviral proteases concerns autolysis. For instance, the HIV-1 protease is known to undergo autodigestion at three major sites in its polypeptide chain of 99 amino acids: $Leu_5$-$Trp_6$, $Leu_{33}$-$Glu_{34}$, and $Leu_{63}$-$Ile_{64}$ [or $Pro_{63}$-$Val_{64}$ in another enzyme variant (Rosé *et al.*, 1993)] (Strickler *et al.*, 1989; Rosé *et al.*, 1993; Hui *et al.*,

* To whom correspondence should be addressed: 7240-267-118, The Upjohn Co., 301 Henrietta St., Kalamazoo, MI 49001 (telephone 616-384-9464; Fax 616-385-5488).
‡ Biochemistry Unit.
§ Computational Chemistry Unit.
∥ Molecular Biology Unit.
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1993). This continual self-degradation of the HIV proteases poses problems for structural and kinetic analysis. To address this issue, Rosé *et al.* (1993) substituted the $Gln_7$ residue in the HIV-1 protease with a lysine, a $P_2'$ substitution, and greatly diminished autolysis at $Leu_5$-$Trp_6$. Moreover, since $Leu_5$ was identified to be a primary site of autolysis, this single change in the Q7K mutant led to general stabilization against autodigestion.

In this paper, we apply site-directed mutagenesis to test whether replacement of $P_1$ or $P_2'$ residues by apparently excluded amino acids can block cleavage at the three major sites of autodigestion in the HIV-1 protease and of peptides modeled after the cleavage sites. The three-dimensional structure of the HIV-1 protease homodimer has been well established [reviewed by Wlodawer and Erickson (1993)] and serves as a reference for evaluating accessibility of regions of the polypeptide backbone corresponding to the three autolysis sites. Accordingly, inferences about protein folding and dissociation can be derived from the patterns of autolysis in the mutant proteases. The results establish that introduction of β-branched amino acids at $P_1$ or Lys at $P_2'$ in the three autolysis sites greatly diminishes hydrolysis at corresponding $P_1$–$P_1'$ positions. Moreover, these results are consistent with a model in which the HIV-1 protease dimer is resistant to autolysis but is in equilibrium with its monomeric units and presumed unfolded species which serve as substrates for autodegradation. Finally, one of the HIV-1 protease mutants generated in this study, Q7K/L33I/L63I, retains the specificity and kinetic properties of the wild-type enzyme but is highly stabilized against autolysis. Q7K/L33I/L63I may be valuable in studies of the structure and function of the HIV-1 protease and, perhaps, as a tool in protein chemistry.

## MATERIALS AND METHODS

*Materials.* Restriction endonucleases, T4 polynucleotide kinase, and T4 ligase were obtained from Boehringer Mannhein or New England Biolabs. Taq DNA polymerase was from Perkin-Elmer and Sequenase version 2.0 from U.S. Biochemicals. Media and antibiotics for cell culture were from BRL. All reactions were performed following manufacturer's guidelines. Oligonucleotides were purchased from Genosys. Reagents for peptide synthesis were purchased from Applied Biosystems or Advanced ChemTech. Solvents for protein sequence analysis, and HPLC, and other routine laboratory chemicals were of the highest grade commercially available.

*Site-Directed Mutagenesis and Protein Expression.* Mutant K7/V33 was constructed by PCR using two long (165 nucleotides) and two short (36 nucleotides) overlapping oligonucleotides (Ciccarelli *et al.*, 1991). Other mutants were derived by replacement of segments of the gene using conveniently engineered restriction sites or by the PCR method described by Higuchi (1990). The nucleotide sequence coding for the 99 amino acid residues of the HIV-1 protease was preceded by a methionine codon for protein initiation and followed by two translation termination codons. The entire sequence was contained in a *Cla*I–*Hin*dIII fragment. For expression of the protease and mutants, the sequences were placed behind the phage λ leftward promoter $P_L$ and an AT-rich ribosome binding site (constructed by John Mott, The Upjohn Co.) in the pUC-vector background. Recombinant plasmids were constructed and screened in *Escherichia coli* MC1061λ+ (wild-type λ lysogen) and transformed into *E. coli* 1458 (λ lysogen with tscI857, a temperature-sensitive mutation of the repressor cI) to allow the induction of the PL promoter by high temperature. Constructs were analyzed with restriction endonucleases and by sequencing the double-stranded DNA by the dideoxy chain termination method.

Cell cultures containing recombinant plasmids were grown at 30 °C with aeration in Luria broth supplemented with 100 μg/mL ampicillin. Expression of protease mutants was induced by dilution of an overnight culture 100-fold into Luria broth with 100 μg/mL ampicillin; growth was continued at 30 °C until the $A_{550}$ reached 0.6–0.8, at which point the culture was rapidly switched to 40 °C for about 4 h. Refractile inclusion bodies (aggregates of the recombinant proteases) can be observed by phase contrast microscopy about 2 h after induction and reach a maximum in about 4 h. Cell extracts were prepared by resuspending cells in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA to a cell density of $A_{550} = 20$ and sonicating with a Branson sonifier. The inclusion bodies and other insoluble matter were collected by centrifugation and washed in the same buffer. Sonication and centrifugation were done at 4 °C, and the inclusion bodies were stored at −20 °C. Expression of the HIV-1 protease and its mutants was analyzed by SDS–PAGE (Laemmli, 1970). A prominent band of approximately 11 kDa was found in induced cell extracts but not in the uninduced control; this 11-kDa protein routinely accounts for greater than 50% of the total protein in the inclusion bodies.

*Purification, Refolding, and Activation of Wild-Type and Mutant HIV-1 Proteases.* The procedures followed here were essentially as described recently by Hui *et al.* (1993) and in earlier references from our laboratory (Tomasselli *et al.*, 1990c). Inclusion bodies prepared as described in the preceding section were dissolved in 50% acetic acid. The solution was centrifuged (20 min) in an Eppendorf benchtop centrifuge, and at an analytical scale (0.3–0.5 mg), the supernatant was submitted to RP-HPLC on a Vydac $C_4$ or $C_{18}$ column (0.46 × 25 cm) eluted over a period of 60 min at a flow rate of 1.0 mL/min with a linear gradient of increasing acetonitrile (0–100%) in 0.15% trifluoroacetic acid (TFA). The single, prominent peak, thus separated from minor contaminants, was identified as inactive protease by compositional and sequence analysis. Fractions containing the protease were lyophilized and stored at −80 °C. Gel filtration in 50% acetic acid was employed to provide milligram quantities of the protease or mutants (Hui *et al.*, 1993). The level of expression in *E. coli* of the HIV-1 protease, and all of the mutants generated in the present work, varied from 20 to 50 mg/L of cell culture.

In a typical refolding and activation experiment, 0.3–0.5 mg of purified, lyophilized HIV-1 protease was dissolved in 100–150 μL of 50% $CH_3COOH$. To this solution was then added a 30-fold excess of refolding buffer (0.1 M sodium acetate, pH 5.5, containing 5% ethylene glycol, 10% glycerol, and 2–3 mM DTT), and the pH was adjusted to 4.0 or 5.5. Folding and activation were allowed to proceed over a period of 6 h at 0 °C; at this temperature autolysis is minimized (*cf.* Figure 2b). The folded and activated enzyme was mixed gently, and an aliquot was assayed for catalytic activity.

*Enzymatic Assay of Wild-Type and Mutant HIV-1 Proteases.* The synthetic peptide H-Val-Ser-Gln-Asn-Tyr-↓-Pro-Ile-Val-OH (GSP) served as the substrate for the measurement of HIV-1 protease activity in the wild-type and mutant enzymes (Tomasselli *et al.*, 1990c). This peptide corresponds to the sequence from residue 128 to residue 135 in the HIV *gag* protein. Cleavage of the synthetic peptide, as well as the *gag* protein, takes place at the Tyr–Pro bond, as indicated by the arrow in the sequence shown above. HIV-1

Table 1: Kinetics of Hydrolysis of VSQNYPIV and Peptides Corresponding to the Three Autolysis Sites in the HIV-1 Protease and Mutants Thereof by the Wild-Type Enzyme and by the Q7K/L33I/L63I Mutant[a]

| peptide model | peptide sequence | protease | $V_{max}$ ($\mu$mol mg$^{-1}$ min$^{-1}$) | $k_{cat}$ (min$^{-1}$) | $K_m$ ($\mu$M) | $k_{cat}/K_m$ (min$^{-1}$ $\mu$M$^{-1}$) |
|---|---|---|---|---|---|---|
| gag | VSQNY-PIV | wild type | 22.4 | 241 | 2000 | 0.12 |
| | | Q7K/L33I/L63I | 24.1 | 259 | 2020 | 0.13 |
| $Leu_5$-$Trp_6$ | PQITL$_5$-WQRP | wild type | 0.54 | 5.8 | 2200 | 0.0026 |
| | | Q7K/L33I/L63I | 0.58 | 6.2 | 2130 | 0.0030 |
| | PQITL$_5$-WKRP | wild type | $<10^{-4}$ | $<10^{-3}$ | ND | $<10^{-6}$ |
| | | Q7K/L33I/L63I | $<10^{-4}$ | $<10^{-3}$ | ND | $<10^{-6}$ [b] |
| $Leu_{33}$-$Glu_{34}$ | DTVL$_{33}$-EEMS | wild type | 24.3 | 261 | 1020 | 0.26 |
| | | Q7K/L33I/L63I | 25.5 | 275 | 1110 | 0.25 |
| | DTVV$_{33}$-EEMS | wild type | 0.73 | 7.8 | 950 | 0.0082 |
| | | Q7K/L33I/L63I | 0.70 | 7.5 | 870 | 0.0085 |
| | DTVI$_{33}$-EEMS | wild type | 0.27 | 2.9 | 810 | 0.0036 |
| | | Q7K/L33I/L63I | 0.17 | 2.4 | 714 | 0.0034 |
| | DTVV$_{33}$-EKMS | wild type | $<10^{-4}$ | $<10^{-3}$ | ND | $<10^{-6}$ |
| | | Q7K/L33I/L63I | $<10^{-4}$ | $<10^{-3}$ | ND | $<10^{-6}$ [b] |
| $Leu_{63}$-$Ile_{64}$ | REYDQIL$_{63}$-IEVS[c] | wild type | 15.9 | 171 | 215 | 0.80 |
| | | Q7K/L33I/L63I | 17.1 | 184 | 202 | 0.92 |
| | REYDQIL$_{63}$-IKVS[c] | wild type | 0.005 | 0.05 | 135 | 0.0004 |
| | | Q7K/L33I/L63I | 0.006 | 0.06 | 125 | 0.0005 |
| | REYDQII$_{63}$-IEVS[c] | wild type | 0.14 | 1.5 | 152 | 0.0099 |
| | | Q7K/L33I/L63I | 0.15 | 1.6 | 143 | 0.0112 |

[a] Bold letters equal mutations. Enzyme concentrations ranged from 84 to 1700 ng/50 $\mu$L of assay mixture (160–3200 nM enzyme monomer) in 0.2 M sodium acetate, pH 5.5, 10% glycerol, 5% ethylene glycol, and 1 mM DTT at 30 °C. The concentration of substrates varied from 0.2 to 2.1 mM for the first seven substrates and from 0.02 to 0.75 mM for the last three substrates. Active site titration with U-75875 (see Table 3) indicated that both wild-type and mutant proteases were about 85 ± 5% folded. [b] The value is valid if $K_m$ is assumed in the low millimolar range like the other substrates of the same group. [c] Peptide has S in place of $C_{67}$ and V in place of $I_{66}$ (as in the HIV-2 protease).

protease activity was measured at 30 °C in 100–200 mM sodium acetate, pH 5.5, containing 10% glycerol and 5% ethylene glycol, in a total volume of 50 $\mu$L. After 30 min of incubation, the reaction was stopped by addition of 75 $\mu$L of 1% TFA or 30 $\mu$L of 8 M guanidinium chloride, and a 20-$\mu$L sample was subjected to HPLC analysis. HPLC was carried out with a Vydac $C_{18}$ column (0.46 × 15 cm), eluting with a linear gradient of 0–30% acetonitrile in 0.15% TFA over a period of 25 min at a flow rate of 1.0 mL/min. In the case of peptides that cleaved slowly, longer periods were allowed for hydrolysis, and higher concentrations of enzyme were employed (Table 1).

*Stability Experiments.* In these experiments with wild-type enzyme, time 0 was defined as that time following 6-h activation at 0 °C (see above). Wild-type and mutant HIV-1 proteases were incubated at pH 5.5 (or, in some cases, pH 4.0) for up to 11 days at 25 ± 1 °C. Samples contained protein (0.1–0.2 mg/mL) in 1.0–1.5 mL of 0.1 M refolding buffer (see above). Aliquots (200–250 $\mu$L) were withdrawn at 0 h, 24 h, 4 days, and 11 days: 5-$\mu$L portions were taken for amino acid analysis, and 150–180-$\mu$L aliquots were submitted to RP-HPLC on a Vydac $C_4$ column (0.46 × 25 cm) eluted as described in the purification procedure. Peaks generated by autoproteolysis were collected manually and subjected to amino acid analysis and/or sequencing.

*Peptide Synthesis.* Peptides were synthesized by solid-phase technology employing a Model 430-A peptide synthesizer from Applied Biosystems Inc.; purity was assessed by HPLC analysis. Chemical authenticity of each peptide substrate and inhibitor was established by amino acid analysis, FAB-MS, and sequence analysis.

*Amino Acid Analysis.* Quantitation of the wild-type and mutant HIV-1 proteases was afforded by amino acid compositional analysis as determined by automated ion-exchange chromatography on a Beckman amino acid analyzer (Model 6300). Samples were hydrolyzed in 6 N HCl for 24 h at 110 °C in evacuated sealed tubes. Dried hydrolysates were dissolved in buffer at pH 2.2 (NaS; Beckman) prior to application to the analyzer.

*Protein/Peptide Sequence Analysis.* Automated Edman degradation of protein and peptide samples was performed in an Applied Biosystems sequencer (Model 470) fitted with an on-line HPLC analyzer (Model 120A) for phenylthiohydantoin amino acids. Quantitation of the latter was afforded by a Nelson Analytical 3000 series chromatography data system connected in parallel with the recorder to the output of the HPLC system.

*SDS-PAGE.* Laemmli (1970) gels (12%) were run in the presence of reducing agents and stained with Coomassie Blue for detection of proteins.

## RESULTS

The strategy we adopted to block autolysis in the HIV-1 protease and, thereby, to study the specificity of the enzyme was to replace $P_1$ leucines with isoleucine or valine and to replace $P_2'$ residues, whatever they might be, with lysine. Before embarking on a mutagenesis program, we tested the idea on model peptides corresponding to the autolysis sites.

*HIV-1 Protease Hydrolysis of Peptide Substrates.* Kinetic parameters for the HIV-1 protease-catalyzed hydrolysis of a variety of peptides modeled after the three major sites of autolysis in the enzyme ($L_5$-$W_6$, $L_{33}$-$E_{34}$, $L_{63}$-$I_{64}$) are given in Table 1. Also included are corresponding data obtained with one of the mutant proteases, Q7K/L33I/L63I, engineered during the course of this work (*vide infra*). In each of the three sets, the wild-type peptide is compared as a substrate relative to derivatives having $P_1$ Ile (or Val) or $P_2'$ Lys. Several interesting facts emerge from this comparison. First, the peptide representing the $Leu_5$-$Trp_6$ autolysis site, the primary autolysis site in the enzyme (Rosé *et al.*, 1993), is by far the worst of the three model peptide substrates; its $k_{cat}/K_M$ value is 100–300 less than those for the wild-type representatives of the $Leu_{33}$-$Glu_{34}$ and $Leu_{63}$-$Ile_{64}$ sites. Second, Ile or Val substitutions at $P_1$ or Lys at $P_2'$ all greatly diminish the rate of hydrolysis, and the order of effectiveness of the substitutions in blocking catalysis appears to be Lys > Ile > Val. Ile at $P_1$ is about 3 times more effective than Val in blocking hydrolysis, and a $P_2'$ Lys is another 10-fold more effective

Table 2: Description of Mutants of the Wild-Type HIV-1 Protease Designed in the Present Study and Some Stability Data for Select Mutants following an 11-Day Period of Incubation at 25 °C

| protease | sites of mutagenesis | | | | | | recovery[a] | |
|---|---|---|---|---|---|---|---|---|
| | $P_1$ | $P_2'$ | $P_1$ | $P_2'$ | $P_1$ | $P_2'$ | protein | activity |
| wild type | $L_5$ | $Q_7$ | $L_{33}$ | $E_{35}$ | $L_{63}$ | $E_{65}$ | 25 | 60 |
| Q7K | | K | | | | | 68 | 88 |
| L33I/L63I | | | I | | I | | | |
| Q7K/L33V/L63I | | K | V | | I | | 93 | 105 |
| Q7K/L33I/L63I | | K | I | | I | | 96 | 102 |
| Q7K/L33V/E35K/L63I | | K | V | K | I | | | |
| Q7K/L33V/L63I/E65K | | K | V | | I | K | | |
| Q7K/L33V/E65K | | K | V | | | K | | |
| Q7K/L33V/E35K/E65K | | K | V | K | | K | | |
| L5I/L33I/L63I | I | | I | | I | | | |

[a] Recovery of protein was measured by RP-HPLC as noted in the text; enzyme activity was also monitored by assay with the GSP substrate (Table 1). The time course of the breakdown of protein and loss of activity is shown in Figure 4. Mutants for which no data are given were active initially but lost activity over the time of incubation because of instability; no meaningful data could be collected for them relative to autolysis.

than a $P_1$ Ile. Finally, the peptides representing autolysis sites at residues 33 and 63 are better substrates than GSP (Table 1), the peptide used routinely in our laboratory for HIV-1 protease assay. The results in Table 1 support, therefore, a strategy for site-directed mutagenesis of the protease polypeptide based upon replacement of $P_1$ and/or $P_2'$ residues at the three sites of autolysis with Ile/Val and Lys, respectively.

*Stability of Recombinant Wild-Type and Mutant HIV-1 Proteases.* An overview of the mutants generated in this work and of stabilities for some of them at pH 5.5 during several days of incubation at 25 °C is given in Table 2. The following narrative is restricted to the wild-type HIV-1 protease and mutants of particular interest.

*(a) Wild-Type HIV-1 Protease.* The baseline for the present work was established with respect to autolysis in the parent wild-type HIV-1 protease. Autolysis was followed by HPLC analysis of samples taken at various times of incubation; peaks arising by autodegradation were submitted to compositional and sequence analysis to confirm their N-terminal residues and total length. A typical elution profile is given in Figure 1. Peptides noted on the chromatogram correspond to those expected on the basis of the known sites of autolysis in the wild-type HIV-1 protease at $Leu_5$-$Trp_6$, $Leu_{33}$-$Glu_{34}$, and $Leu_{63}$-$Ile_{64}$ (Strickler *et al.*, 1989; Rosé *et al.*, 1993; Hui *et al.*, 1993). Recoveries of protein and enzymatic activity were greatly *enhanced* by DTT, and all studies of autolysis in the mutant proteases were done in the presence of this reducing agent. The protease shows substantial loss of activity and protein over the period of 11 days (Table 2; Figure 2). Analysis of individual peaks isolated by HPLC gave results that were in accord with earlier identifications of autolysis products as being generated by autodigestion at positions 5, 33, and 63.

*(b) Mutant Q7K.* In a recent paper, Rosé *et al.* (1993) described this same mutant, and they demonstrated a high level of protection against cleavage at $Leu_5$-$Trp_6$. This is in keeping with the principle that Lys cannot be tolerated at $P_2'$ in protease substrates. Moreover, the HIV-2 protease has Lys at position 7 and does not undergo autodigestion at $Leu_5$ (Rosé *et al.*, 1993). Autolysis at positions 33 and 63 was also reduced in this active mutant. In our hands as well, the Q7K mutant showed markedly enhanced stability over the wild-type protease. Therefore, although the peptide corresponding

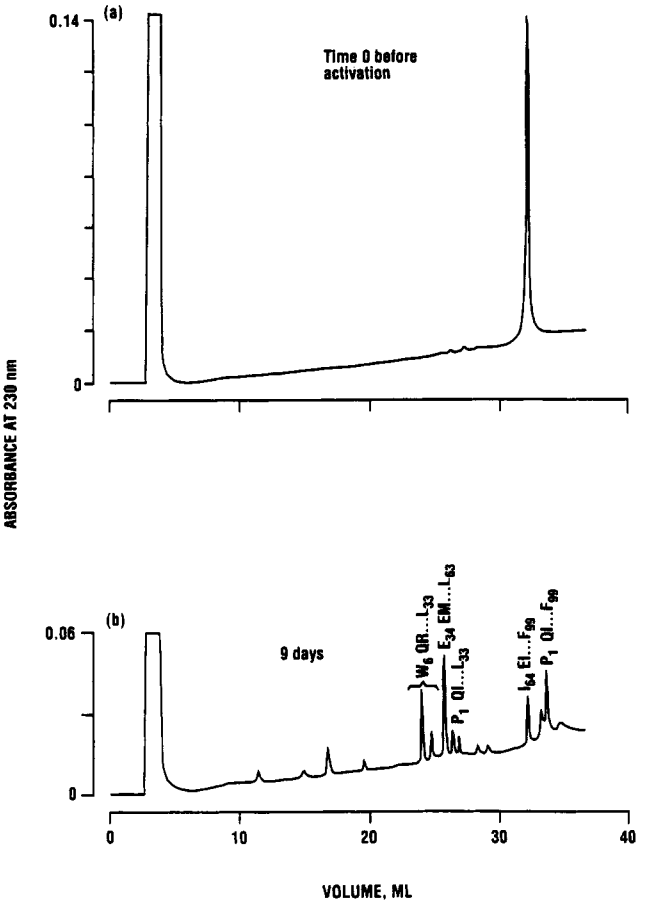


FIGURE 1: Elution profiles obtained by reverse-phase HPLC of (a) intact wild-type HIV-1 protease and (b) fragments obtained therefrom by autodigestion. A Vydac C4 column (0.46 × 25 cm) was eluted over a period of 60 min at a flow rate of 1.0 mL/min with a linear gradient of increasing acetonitrile concentration (0–100%) in 0.15% TFA. Peaks noted in (b) were collected manually and subjected to compositional and sequence analysis. Panel a shows the HPLC profile of the protease purified from inclusion bodies but not yet activated; the pH was maintained at about 2 throughout all procedures. This material was diluted into refolding buffer (Materials and Methods) and allowed to activate for 6 h at 0 °C. Autolysis is reduced under these conditions (*cf.* Figure 2b), and time 0 is defined as that following the 6-h activation period.

to this sequence is the poorest of those modeled after the three sites of autolysis (Table 1), blocking the cleavage at $Leu_5$-$Trp_6$ is a major deterrent to further breakdown of the protease. As pointed out earlier by Rosé *et al.* (1993), the $Leu_5$–$Trp_6$ bond appears to be a *primary* site for autolysis. Nevertheless, after 11 days of incubation there is still about 30% breakdown to peptides with an average loss of 12% in protease activity.

*(c) Mutants with Lys at Position 35 or 65.* Since substitution of $Gln_7$ by Lys was so successful in blocking cleavage at $Leu_5$-$Trp_6$, we tested Lys substitutions at positions 35 and 65 as stabilizers of cleavages at $Leu_{33}$-$Glu_{34}$ and $Leu_{63}$-$Ile_{64}$, respectively. In both cases, substitution at $P_2'$ involved replacement of Glu residues 35 and 65, thereby causing charge reversals at these sites. As shown in Table 2, several mutants were designed, expressed, and purified. The $K_{35}/K_{65}$ mutants all showed enzymatic activity immediately upon refolding, but these mutants were not stable, and we could not include them in a time–course study of autolysis (Table 2). For example, mutant Q7K/L33V/E35K/E65K lost about 50% of its activity over 11 days as a result of precipitation out of solution, rather than autolysis. This mutant also lost activity after freeze–thawing and was difficult to study relative to the other mutants. These findings suggested that charge reversal

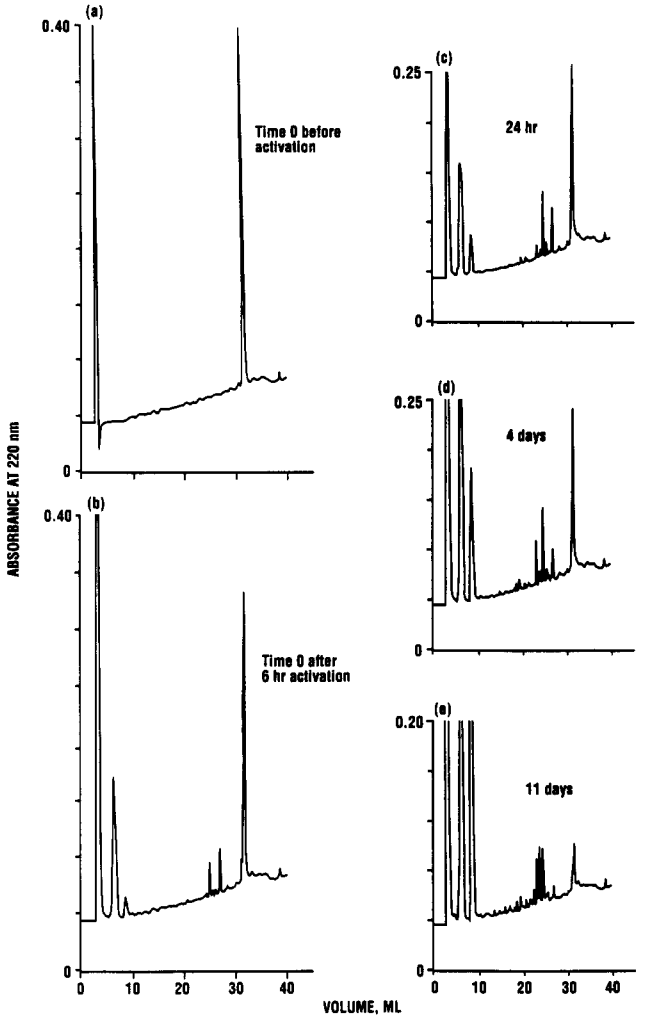


FIGURE 2: Time course of wild-type HIV-1 protease autodigestion over a period of 11 days at 25 °C, pH 5.5, in the presence of DTT, measured by reverse-phase HPLC (see legend to Figure 1). Panel a corresponds to Figure 1a. Panel b shows the extent of autolysis following 6-h activation at 0 °C, and this is defined as time 0 for all subsequent measurements (c–e).

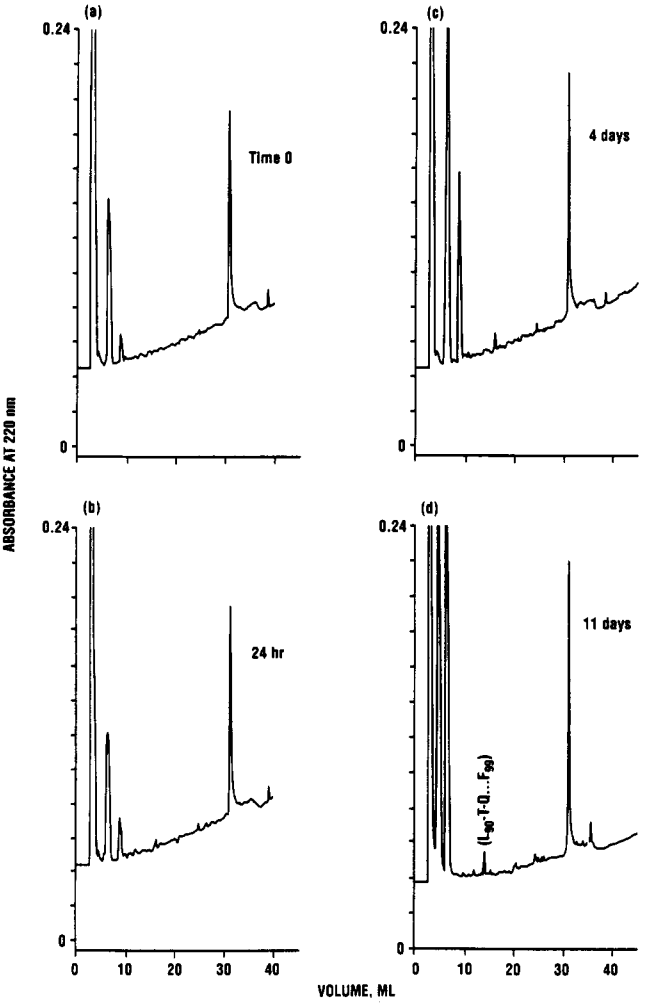


FIGURE 3: Time course of autolysis of mutant Q7K/L33I/L63I at pH 5.5. Elution profiles were obtained by reverse-phase HPLC (legend to Figure 1) of samples taken at various times of incubation at 25 °C in the presence of DTT. Peptides noted in (d) at 11 days of incubation were collected manually and subjected to sequence analysis. Because this mutant is stabilized against autolysis, there is no measurable breakdown of the protease during the 6-h activation period.

at residues 35 and/or 65 was structurally unfavorable and that more conservative replacements might be necessary to produce viable mutant proteases.

*(d) Mutant L5I/L33I/L63I.* The mutant with L → I at all three $P_1$ positions seemed an obvious choice as the most conservative of those mentioned thus far. In fact, although it was expressed in high yield, and activity was observed after refolding from acid extracts of *E. coli* inclusion bodies, the mutant lost activity rapidly overnight. This loss was not due to fragmentation but, most likely, to instability of the protease dimer; similar results were obtained with the L33I/L63I mutant (Table 2). The defect in this mutant is still not understood, but it appears that this approach is not the answer to creating a stable and useful mutant enzyme.

*(e) Mutant Q7K/L33I/L63I.* The best of the mutant HIV-1 proteases made in this study is that which incorporates features of those mentioned above. The stability of this protein over a period of 11 days at pH 5.5 is illustrated in Figure 3; very little loss of protein and no loss of activity was encountered (Figure 4). The fact that one can lose some protein but no activity seems illogical, but we believe this phenomenon is a consequence of the kinetics of protein folding from the acid extracts of inclusion bodies in the initial step of activation. Even after hours, especially at low pH, there is a significant amount of protein that has not refolded properly. Given time, that material will continue to sample equilibrium intermediates, and if it is not digested by active species present in the mixture, it will eventually generate more active dimer. Therefore, the >100% values seen for the mutant at pH 4 (Figure 4) are accounted for by these slow-folding species which are not destroyed before they fold correctly. Over the time of incubation at pH 4.0, there is continual autolysis, principally at positions 33 and 63. This lower pH most likely favors dissociation of the dimer to monomeric species that are subject to autolysis. Autolysis goes on even though the sites are protected in the mutant, at low rates reflected by those seen in model peptides (Table 1). At the higher pH of 5.5, it appears that there is much less opportunity for autolysis since there is little loss of protein or activity (Figure 4). All of the mutagenized positions in Q7K/L33I/L63I remain essentially intact after 11 days of incubation at pH 5.5, but interestingly, a new position of autodigestion becomes evident (Figure 3). This minor cleavage is at $Leu_{89}$-$Leu_{90}$ and very nearly coincides with one of the sites of autolysis in the HIV-2 protease at $Leu_{90}$-$Thr_{91}$ (Rosé *et al.*, 1993).

In order to test whether Val would work as well as Ile as a $P_1$ blocker of hydrolysis, we inserted Val at position 33 in the Q7K/L33I/L63I mutant. The stability of Q7K/L33V/L63I was similar at pH 5.5 but less at pH 4 (data not shown).

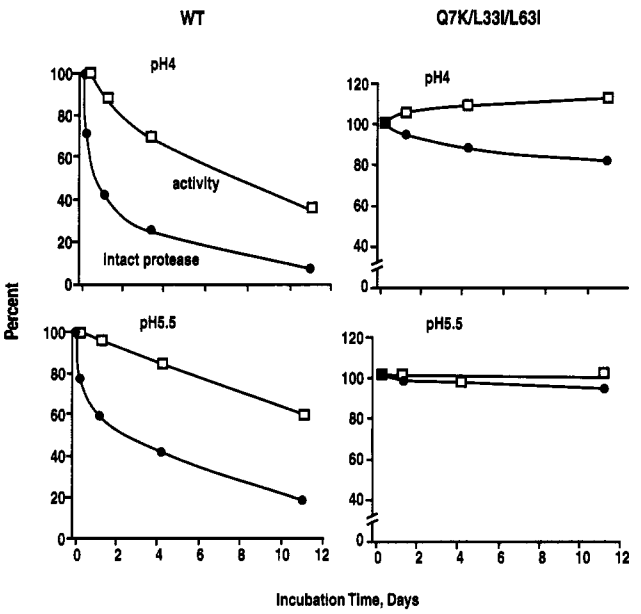


FIGURE 4: Time course of loss of protein and enzyme activity with wild-type HIV-1 protease (Figure 2) and mutant Q7K/L33I/L63I (Figure 3 and Table 2). Incubation was at 25 °C, at pH 4.0 or 5.5, in the presence of DTT. The first measurement of activity was after 6-h incubation in ice, the period allotted for folding from acid. For the wild-type protease, loss of enzyme activity is nearly linear over the 11 days, while protein breakdown is biphasic. This is because of the presence, initially, of unfolded, inactive species which are rapidly degraded by folded and active protease dimer. It is necessary, therefore, to extrapolate back to time 0 in order to estimate the amount of starting, active dimer. Since the mutant autolysis sites are protected during refolding, activity continues to increase at pH 4.0; at pH 5.5, folding is essentially complete after 6 h, and there is minimal change over the 11-day period.

The model peptide containing Val at $P_1$ is somewhat less stable to hydrolysis than that having a $P_1$ Ile (Table 2). Consequently, if the protein is unfolding more at pH 4, then greater hydrolysis at $Val_{33}$ would be expected.

*(f) Kinetic Properties of Q7K/L33I/L63I.* It was of interest to know whether this mutant enzyme has altered properties *vis-a-vis* the wild-type HIV-1 protease. With the best of our mutants, we see higher specific enzyme activity than for the wild-type enzyme, due most likely to the fact that more of the recombinant material has an opportunity to fold properly before it is destroyed. For the most part, however, the mutants are indistinguishable in terms of $V_{max}$, $k_{cat}$, and $K_M$. This is demonstrated for mutant Q7K/L33I/L63I by data in Table 1, which show that it is identical in kinetic properties to the wild-type enzyme.

A test that has enabled us to distinguish easily between the HIV-1 and HIV-2 proteases is based upon the pattern of peptides obtained from these enzymes with calcium-free troponin C as substrate (Tomasselli *et al.*, 1991c). We found that the patterns of troponin C peptides produced by wild-type and mutant HIV-1 proteases on SDS–PAGE were identical and distinct from that generated by the HIV-2 enzyme. Another discriminating test depends upon the $K_i$ values for inhibition of these enzymes by a variety of inhibitors. From the series in Table 3, it is clear that the wild-type and mutant HIV-1 proteases are very similar, if not identical, in their inhibition profile but distinct from HIV-2 protease. Nevertheless, as shown in Figure 4, the wild-type and mutant HIV-1 proteases differ substantially in their resistance to autodegradation. With respect to the wild-type protease, there is a rapid loss of about 40% of the protein, in keeping with the fact that not all of the material is properly folded at the beginning of the experiment. After the initial destruction of these unfolded species, the loss of protein parallels loss of enzymatic activity (Figure 4). With the mutant, there is very little change in recovery of protein or enzymatic activity over the period of 11 days.

## DISCUSSION

The present paper addresses the specificity of the HIV-1 protease within the framework of an autolysis model. We have used the protease both as an enzyme and as its own substrate in an attempt to understand how single point mutations can override the cumulative specificity (Poorman *et al.*, 1991) of the protease. Analysis of protease specificity involves consideration of substrates with respect to their amino acid sequences and the nature of their folding and accessibility. Evidence from numerous laboratories has suggested that the HIV-1 protease engages in multiple interactions with an extended peptide substrate and that at least seven amino acid residues are recognized during catalysis. Specificity, then, may be defined as the product of cumulative contributions of amino acid side chains between $P_4$ and $P_4'$ (Poorman *et al.*, 1991). In this view, proper accommodation of an extended peptide segment is as important in specification of the peptide bond to be cleaved as is the nature of residues in the $P_1$–$P_1'$ positions. Thus, it would appear unlikely that a single amino acid replacement anywhere between $P_4$ and $P_4'$ of the substrate would have a major impact on hydrolysis at the scissile bond. Indeed, although the HIV-1 protease shows some preference for Glu or Gln at $P_2'$ in its substrates (Poorman *et al.*, 1991), it does not show an exact requirement for a particular amino acid in any of the positions from $P_4$ through $P_4'$.

There are, however, some amino acid residues that are excluded (Tomasselli & Heinrikson, 1994; Konvalinka *et al.*, 1990; Margolin *et al.*, 1990; Phylip *et al.*, 1990; Griffiths *et al.*, 1992; Tozser *et al.*, 1992), and the present paper shows that substitution at $P_1$ by $\beta$-branched amino acids or at $P_2'$ by Lys greatly diminishes catalysis at $P_1$–$P_1'$ in an otherwise good peptide substrate. This point was proven by studies of peptide substrates reflecting the sequences surrounding the three sites of autolysis in the HIV-1 protease (Table 1). Moreover, results thus obtained translated very well to the

Table 3: Structure–Activity Analysis of Inhibitors To Probe Selectivity toward HIV-1 Protease versus the HIV-1 Mutant Q7K/L33I/L63I and HIV-2 Protease

| U no. | structure | $K_i$ (nM) HIV-1 PR[a] | HIV-2 PR[a] | HIV-1 PR | HIV-1 mutant |
|---|---|---|---|---|---|
| U-81749E | Tba-Cha$\psi$[CH(OH)$CH_2$]Val-Ile-Amp | 70 | 1000 | 60 | 72 |
| U-79213 | Poa-His-Cha$\psi$[CH(OH)$CH_2$]Val-Ile-Amp | 7 | 105 | 9 | 15 |
| U-71017 | Poa-His-Leu$\psi$[CH(OH)$CH_2$]Val-Ile-Amp | 10 | 107 | 7 | 11 |
| U-71038 | Boc-Pro-Phe-N$^{\alpha}$MeHis-Leu$\psi$[CH(OH)$CH_2$]Val-Ile-Amp | 10 | 10000 | 11 | 16 |
| U-75875[b] | Noa-His-Cha[CH(OH)$CH_2$(OH)]Val-Ile-Amp | <1 | 30 | <1 | <1 |

[a] Tomasselli *et al.* (1991d). [b] Active site titration with U-75875 showed that the extent of refolding for both HIV-1 wild type and Q7K/L33I/L63I was 85 ± 5%.

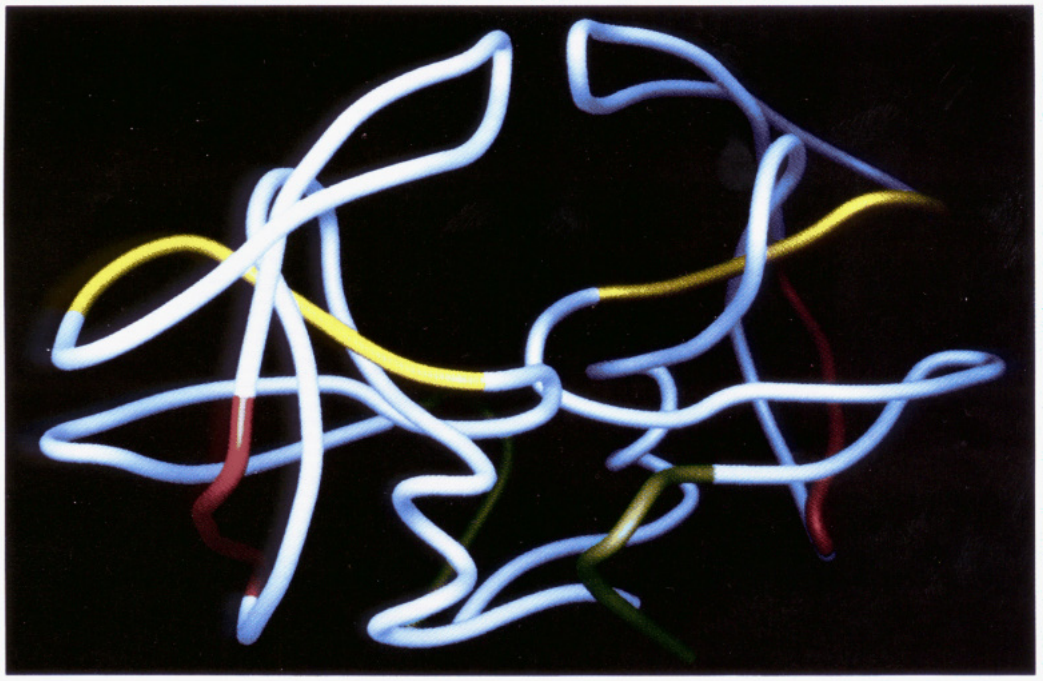

FIGURE 5: Smoothed backbone representation of an X-ray crystal structure [Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) entry 3HVP (Wlodawer *et al.*, 1989)] of uncomplexed HIV-1 protease dimer. The three autolysis sites are highlighted in green ($Leu_5$-$Trp_6$), yellow ($Leu_{33}$-$Glu_{34}$), and red ($Leu_{63}$-$Ile_{64}$). Each colored region encompasses eight residues from the $P_4$ to $P_4'$ positions around the cleavage point, none of which are accessible to binding by a second protease dimer, as long as they are in the structural environments shown here (discussed in more detail in the text). Separation of the monomers would, however, expose the green region and allow it to sample an extended conformation required for binding to a second protease dimer.

level of a protein substrate, in this case, the HIV-1 protease itself.

Our mutagenesis studies support the view that the cumulative nature of HIV-1 protease specificity can be abrogated to a considerable degree by these single-site substitutions. However, multipoint binding of substrate remains a guiding principle for understanding specificity. Indeed, cumulative specificity assures that if the rest of the sequence is optimal for hydrolysis, and if accessibility is not a problem, the protease may simply shift the peptide segment one way or the other in its binding pocket and cleave an adjacent bond. In the present study, we found that the Q7K/L33I/L63I mutant gives a minor cleavage at the $Leu_{89}$–$Leu_{90}$ bond, one corresponding closely to a site of autolysis in the HIV-2 protease at $Leu_{90}$-$Thr_{91}$ (Rosé *et al.*, 1993). Sequences surrounding these sites in the two proteases are

HIV-1 protease:

...Gly-Arg-Asn-$Leu_{89}$-↓-$Leu_{90}$-Thr-Gln-Ile...

HIV-2 protease:

...Gly-Arg-Asn-Ile-$Leu_{90}$-↓-$Thr_{91}$-Ala-Leu...

Although these sequences are quite similar, the HIV-2 protease fails to cleave the Ile–Leu bond, as would be expected from the present study and the work of Tozser *et al.* (1992), because of the Ile in $P_1$. Instead, it appears to shift a residue downstream to the $Leu_{90}$–$Thr_{91}$ bond. This example supports the idea that the specificity of these retroviral enzymes is cumulative (Poorman *et al.*, 1991). If the region, in general, binds well to the enzyme and satisfies its subsite binding requirements, it is possible to accommodate a single undesirable amino acid, such as a $P_1$ Ile, by shifting it to another subsite to which it can bind. Hydrolysis then occurs at an adjacent bond, as long as productive binding is achieved; in this case, Thr is a perfectly acceptable residue for $P_1'$ (Tomasselli *et al.*, 1993).

As mentioned above, the kinetics of cleavage of model peptides are generally in accord with autolysis observed at the corresponding sites in the protease. The transition to a protein substrate, however, introduces much higher levels of complexity for any kinetic analysis. First, from what we understand of its crystallographic structure, the protease dimer would not be predicted to be a substrate. From the depiction given in Figure 5, it is clear that if this crystal structure (PDB entry 3HVP; Wlodawer *et al.*, 1989) is at all representative of that in solution, another protease dimer would not have access to any of the three sites of autolysis. Both the $Leu_{33}$-$Glu_{34}$ (Figure 5, yellow region) and $Leu_{63}$-$Ile_{64}$ (Figure 5, red region) autolysis sites are in $\beta$-strands that are engaged in extensive interactions with neighboring $\beta$-strands on both sides. In each case, it would require disruption of seven hydrogen bonds before a $P_4$-to-$P_4'$ section could be removed from its sheetlike environment to allow accessibility for binding a second protease dimer. The $Leu_5$-$Trp_6$ site (Figure 5, green region) is in the N-terminal region, part of which is in a four-stranded intramolecular $\beta$-sheet; the remainder is engaged in a stacking interaction with an $\alpha$-helix, also in the other monomer. It should be noted that the structural environments of the three cleavage sites are the same regardless of whether the protease is bound to an inhibitor (*e.g.*, PDB entry 4HVP; Miller *et al.*, 1989) or uncomplexed, as in the structure shown in Figure 5 (PDB entry 3HVP; Wlodawer *et al.*, 1989). It seems clear, therefore, that unfolding or loosening of the structure is necessary to provide access to the peptide bonds in question.

The protease dimer is in equilibrium with monomeric species, the conformations of which may vary considerably. Of the three sites of autolysis, that at $Leu_5$-$Trp_6$ would appear to be the one most likely to undergo hydrolysis in a monomer since it alone relies totally on *inter*molecular interactions to maintain its conformation seen in the dimeric crystal structure (Figure 5). In a monomer, these interactions are broken, and the

$Leu_5$-$Trp_6$ region could well adopt an extended conformation required for hydrolysis. Furthermore, cleavage at this primary site (Rosé *et al.*, 1993) should block re-formation of active dimer, since the excised N-terminal regions participate in 8 of the 12 hydrogen bonds in the $\beta$-sheet that stabilizes the dimer (Wlodawer *et al.*, 1989; Weber, 1990). The other two regions comprising autolysis sites $Leu_{33}$-$Glu_{34}$ and $Leu_{63}$-$Ile_{64}$ are engaged only in *intra*molecular interactions and should be more stable within the dissociated monomer. Monomers cleaved at $Leu_5$ would favor production of further disordered species for subsequent attack at residues 33 and 63.

Our results support a simple model of autolysis in which the active HIV-1 protease dimer cleaves dissociated monomers at $Leu_5$, taking them out of an equilibrium pool, and then further degrades these and other disordered monomer species at $Leu_{33}$ and $Leu_{63}$. Any condition which favors dissociation of the dimer would be expected to promote autolysis, and this is especially true during the process of refolding of recombinant or synthetic protease preparations. The generation, during refolding, of active enzyme which can proceed to hydrolyze not yet folded or disordered polypeptides can account for discrepancies in the literature with regard to what the specific activity of "purified" protease should be. Moreover, we have actually seen an *increase* in protease activity over time during incubations of some of our stabilized mutants at pH 4 where disordered species can continue to generate active dimer over a period of days without being destroyed (Figure 4). Generally, in incubations at pH 4 as opposed to pH 5.5, there is a greater degree of autolysis. This is consistent with a shift in the equilibrium toward disordered species at low pH; in protected mutants, autolysis still takes place but at slow rates reflective of those seen with model peptides (Table 1).

The model may also explain the effect of DTT in promoting stabilization of the wild-type and mutant HIV-1 proteases. Prevention of oxidation of cysteine residues 67 and 95 might be expected to preserve enzyme activity and thus lead to more rapid autolysis. However, the fact that DTT *stabilizes* the enzymes against autolysis suggests that its preservation of the reduced form of the thiols helps maintain the integrity of the protease dimer and, thus, minimizes generation of disordered species that serve as substrates for the active forms present in equilibrium. Support for this inference comes from the fact that DTT has no effect on autolysis of the HIV-2 protease, an enzyme lacking thiols. Breakdown of this enzyme is comparable to that seen with the HIV-1 protease in the presence of DTT. Rosé *et al.* (1993) also proposed an effect of DTT on dimer stabilization based upon kinetic evidence.

Along more practical lines, we have engineered a mutant of the HIV-1 protease, Q7K/L33I/L63I, that has the same kinetic properties as those of the wild-type enzyme but is highly resistant to autolysis. Incubation of this mutant for 11 days at room temperature, pH 5.5, was occasioned by no loss of enzymatic activity and minimal loss of protein (Figures 3 and 4), whereas under these same conditions the wild-type protease retained 60% of its activity and only 30% of the protein. Again, the fact that protein loss does not parallel loss of enzymatic activity in the wild-type protease is explained by the rapid breakdown of unfolded species in the early phase of incubation (Figure 4).

Autolysis is a problem that continues to plague structure–function studies of the protease. The resulting instability of the enzyme upon storage and during studies in solution at room temperature where kinetic properties are constantly changing has frustrated functional characterization. Autolysis can also pose difficulties for crystallographic analysis if breakdown products hamper growth of good crystals. Mutant Q7K/L33I/L63I should be useful in circumventing some of these analytical obstacles.

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