Analysis of substrate cleavage by recombinant protease of human T cell leukaemia virus type 1 reveals preferences and specificity of binding

Susan Daenke, 1* Hans J. Schramm 3 and Charles R. M. Bangham 1,2

1 Molecular Immunology Group, Institute of Molecular Medicine, and 2 Department of Virology, Public Health Laboratory, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K. and 3 Max-Planck-Institut für Biochemie, 82152 Martinsried, Germany

Human T cell leukaemia virus type 1 (HTLV-1) protease (PR14) was expressed in bacteria and purified by gel filtration. A continuous spectrophotometric assay was used to measure the kinetic parameters of substrate hydrolysis by PR14. Several peptide substrates containing HTLV-1 sequences known to be cleaved by PR14 were used. Cleavage analysis showed that the affinity with which PR14 binds these substrates is higher than that previously reported for HTLV-1 Gag peptides. Also, the affinities of peptides containing the sites involved in autocleavage of protease from its precursor are higher than for the peptides containing sites required for structural protein maturation. This suggests that the autocatalysis of protease from its own precursor has priority over other cleavage reactions and supports similar observations of an ordered hierarchy of processing events by retroviral proteases. As the N- and C-terminal regions of retroviral aspartic proteases are known to contribute to stability of the dimer by forming antiparallel $\beta$-strands, short peptides corresponding to these terminal sequences of HTLV-1 protease were tested for their ability to inhibit cleavage of substrates by PR14. Inhibition was seen with a C-terminal peptide corresponding exactly to the C-terminal 11 amino acids of the processed PR14, whereas a peptide containing a sequence situated further from the C terminus was less effective. An inhibitor of the protease of human immunodeficiency virus type 1, Ro 31-8959, was found to be a poor inhibitor of PR14.

Introduction

Human T cell leukaemia virus type 1 (HTLV-1) is a retrovirus associated with adult T cell leukaemia and a neurological disease, tropical spastic paraparesis. As in other retroviruses, several HTLV-1 proteins undergo proteolytic processing by the viral protease during virion assembly and maturation. Thus the Gag polyprotein is processed to generate the structural proteins p19, p24 and p15, and the Pol polyprotein is cleaved to produce the reverse transcriptase, RNase H and integrase enzymes. Processing is achieved by the action of an intrinsic viral protease that is encoded in a separate reading frame overlapping the gag/pol coding sequences (Nam et al., 1988) and is itself autoprocessed from a precursor protein (Hayakawa et al., 1991) following assembly of the virion capsid. This probably occurs in trans after precursor molecules are brought together during capsid formation by Gag polyproteins.

Sequence similarity between HTLV-1 protease (or PR14) and the proteases of other retroviruses indicates that several essential functional elements are conserved (Hayakawa et al., 1992). A hypothetical molecular model of the HTLV-1 protease dimer also shows conservation of certain structural features seen in proteases of human immunodeficiency virus type 1 (HIV-1) and Rous sarcoma virus (RSV) (Hayakawa et al., 1992), of which the crystal structures have been elucidated. Recombinant HTLV-1 protease expressed in Escherichia coli has been shown to cleave synthetic peptide substrates containing the cleavage sites of HTLV-1 Gag proteins, p19/p24 and p24/p15 (Hayakawa et al., 1991; Saiga et al., 1993; Kobayashi et al., 1991) although the $K_m$ of 500 $\mu$M reported for the p19/p24 peptide by HPLC analysis suggested that this was a relatively inefficient process.

In this paper, we describe the cleavage characteristics of recombinant HTLV-1 protease on synthetic peptide substrates containing sites used for processing HTLV-1 Gag subunits p19, p24 and p15, pol-encoded components reverse transcriptase and integrase, and the autoprocessing of the mature PR14 from its precursor molecule. Incorporation of the chromogenic reporter group p-nitro-L-phenylalanine (Nph) into the peptide substrates allowed kinetic measurements to be determined using a continuous spectrophotometric assay, which allows for greater sensitivity over assays using HPLC analysis of cleavage products. Our results show that the efficiency with which HTLV-1 protease cleaves...
its substrate is higher than that previously reported by other groups and that the binding affinities are similar to or higher than those observed in other retroviruses. Secondly, our results show that PR14 can be inhibited by a peptide corresponding to the C terminus of the active HTLV-1 protease.

Methods

Expression and purification of recombinant HTLV-1 protease in Escherichia coli. DNA encoding HTLV-1 protease was amplified from the genomic DNA of the HTLV-1-transformed cell line MT2 (Miysork et al., 1981) by PCR. The region amplified was from nucleotides (nt) 2074 to 2774 [precursor protease-encoding sequence; nucleotide coordinates according to Seiki et al. (1983)], using oligonucleotides that inserted an initiation codon and two extra residues 5' to the first codon for protease translation and a termination codon at the 3' end of the amplified sequence. The amino acid sequence of the translated precursor protein therefore began as Met-Gly-Ser-His1-Pro5-Thr6, and terminated at Leu208-Thr209-Leu210-Stop (see Fig. 1a). The amplified fragment was cloned into the bacterial expression plasmid pGEM7 (Studier et al., 1990) using BamHI and EcoRI restriction sites which were engineered into the primer oligonucleotides. The sequence was confirmed by direct dideoxynucleotide sequencing of the double-stranded plasmid with T7 polymerase (USB).

The pGMT7-PR plasmid was used to express recombinant PR (protease) construct and expression of the recombinant protein was induced as the addition of 0.4 mM- IPTG to growing bacterial cultures. Three hours after induction of expression, pelleted bacteria were lysed into 50 mM-MES, 1 mM-EDTA, 100 mM-NaCl, 2 % (v/v) glycerol pH 5 (MENG5). The lysate was sonicated for 30 s on ice to reduce viscosity and concentrated to 100 mg/ml total protein on Centriprep-10 filters (Bio-Rad). Protease was semi-purified by gel filtration through a Sephadex G200 column (0.9 m x 18 mm). Proteins were eluted in MENG5 solutions (10 to 20 mM) of substrate concentrations was characterized at 30 to 35 °C in a temperature-controlled cuvette, in 100 mM-sodium acetate buffer pH 5 containing 4 mM-EDTA, 5 mM-mercaptoethanol and 1 mM-NaCl (Richards et al., 1990). Cleavage was assayed in a 120 µl sample by direct spectrophotometric measurement of absorbance change at 300 nm (Richards et al., 1990). Kinetic parameters were determined from duplicate samples. A minimum of five substrate concentrations were used for calculation of Km values for each cleavage reaction.

Inhibition assays were done as described above except that inhibitors were preincubated with protease for 2 min at 37 °C prior to addition to the substrate. Reactions were followed for 3 min, at which time a linear reaction velocity was observed. Stocks of pepstatin A and Ro 31-8959 (Roche Products) were dissolved in DMSO and diluted into the protease preparations such that the concentration of DMSO was in all cases less than 5 %. An equivalent concentration of DMSO alone did not inhibit PR14 activity.

Results

Expression and purification of HTLV-1 protease (PR14)

The pGEM7–PR plasmid was used to express recombinant HTLV-1 protease precursor in E. coli strain BL21(DE3)pLysS. This strain contains a plasmid encoding the lysozyme of bacteriophage T7 that inhibits transcription by T7 RNA polymerase in the absence of induction (Studier et al., 1990). Transformants could not be established in the equivalent E. coli strain lacking this plasmid [BL21(DE3)], presumably owing to toxicity of the expressed PR14 protein. Similar observations have been made for expression plasmids encoding HIV-1 protease (Baum et al., 1990). However levels of expression of PR14 in BL21(DE3)pLysS were low, in the order of 50 to 100 µg/l bacterial culture.

The expression construct encoding the precursor form of HTLV-1 protease produced a product of 14K detectable by Western blotting in the crude bacterial lysate. Integrity of the protease was followed in this way during semipurification (Fig. 1b). The unprocessed precursor protein of expected size 26K could not be detected in the induced lysates, although this may reflect the absence of reactivity of the detecting antibody with the unprocessed precursor protein. All of the expressed PR14 processed from the precursor was in the soluble fraction of the lysate.

Active PR14, semipurified from the bacterial lysate by gel filtration, eluted coincidently with proteins of Mr 25K to 30K, indicating that the protease was in dimeric form. The final preparation used in cleavage assays represented a yield of approximately 20 µg protease/l bacterial culture and was estimated to be greater than 95 % pure

Proteolytic assays

(i) Substrates. Decapeptide substrates were synthesized by solid phase using a Zinsser synthesizer and Fmoc chemistry. The reporter group Nph was incorporated at the P1' position in peptides corresponding to the cleavage sites of HTLV-1 p19/p24 (APQVLN-phVMHPL), p24/p15 (KTQVLNpHVQPK), Gag/PR (PASILNpH-VIPL), PR/RT (reverse transcriptase) (PpVILNpHQLPL), RT/IN (integrate) (TDALLNpHTPVLQ) and the HIV-1 ca/x peptide substrate (KARVLNpHEAM) (ca, capsid protein; X, peptide between matrix and capsid proteins in HIV Gag). As the specificity of substrate cleavage is largely determined by the residues flanking, but not immediately adjacent to, the scissile bond (positions P4, P2, P2' and P3'; Griffiths et al., 1992), substitution of the P1' amino acid by Nph in peptide substrates maintains the specificity of the reaction. Stock solutions (10 to 20 mM) of peptide substrates were prepared in 100 % DMSO and stored at -80 °C.

(ii) Cleavage of synthetic peptide substrates. The hydrolysis of synthetic peptide substrates was characterized at 30 to 35 °C in a temperature-controlled cuvette, in 100 mM-sodium acetate buffer pH 5 containing 4 mM-EDTA, 5 mM-mercaptoethanol and 1 mM-NaCl (Richards et al., 1990). Cleavage was assayed in a 120 µl sample by direct spectrophotometric measurement of absorbance change at 300 nm (Richards et al., 1990). Kinetic parameters were determined from duplicate samples. A minimum of five substrate concentrations were used for calculation of K_m values for each cleavage reaction.

Inhibition assays were done as described above except that inhibitors were preincubated with protease for 2 min at 37 °C prior to addition to the substrate. Reactions were followed for 3 min, at which time a linear reaction velocity was observed. Stocks of pepstatin A and Ro 31-8959 (Roche Products) were dissolved in DMSO and diluted into the protease preparations such that the concentration of DMSO was in all cases less than 5 %. An equivalent concentration of DMSO alone did not inhibit PR14 activity.
Fig. 1. (a) Full-length HTLV-1 protease precursor sequence expressed in E. coli is shown (top line) aligned with the processed sequence of HIV-1 protease (bottom line). Sequence identity between the two protease sequences is indicated by a bullet (●). The processed form of HTLV-1 PR14 is shown in bold type. (b) pGMT7-PR-transformed bacteria were lysed after IPTG induction and lysates were analysed after separation by 18% SDS PAGE. Semipurification of PR14 from bacterial lysates was achieved by successive separation by gel filtration on Sephadex G75 and G200. PR14-containing fractions from the gel filtration separation were identified by Western blotting using sheep anti-HIV-1 P9 antibody plus donkey anti-sheep IgG-HRPO conjugate. The blots were developed with 4-chloronaphthol as the substrate. Mₗ markers are shown on the left of the figure; left lane, G75 pooled fractions; right lane, G200 pooled and concentrated fractions.

Table 1. Kinetic parameters of HTLV-1 and HIV-1 proteases using synthetic peptide substrates

<table>
<thead>
<tr>
<th>Cleavage site</th>
<th>Protease</th>
<th>Peptide sequence*</th>
<th>Kₘ (µM)†</th>
<th>kₗ (s⁻¹)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p19/p24</td>
<td>PR14</td>
<td>APQVLNpHVMHPL</td>
<td>15.3±3.2</td>
<td>148±24</td>
</tr>
<tr>
<td>p24/p15</td>
<td>PR14</td>
<td>KTKVLNpHVPK</td>
<td>30.9±3.8</td>
<td>330±57</td>
</tr>
<tr>
<td>Gag/PR</td>
<td>PR14</td>
<td>PASILNpHVIPL</td>
<td>5.2±1.3</td>
<td>17.5±24</td>
</tr>
<tr>
<td>PR/RT</td>
<td>PR14</td>
<td>PPVILNpHQAPl</td>
<td>3.8±0.6</td>
<td>21.8±3.6</td>
</tr>
<tr>
<td>RT/INT</td>
<td>PR14</td>
<td>TDALLNpHTPVLQ</td>
<td>176±41</td>
<td>259±46</td>
</tr>
<tr>
<td>HIV-1</td>
<td>PR9</td>
<td>KARVLNpHEAM</td>
<td>25.0±5.6</td>
<td>9.7±3.3</td>
</tr>
</tbody>
</table>

* All peptides were synthesized with the Nph group at the P1' position relative to the scissile bond.
† Values represent the mean of two experiments, each comprising duplicate assays ± s.e.m. Kₘ values for p19/p24, p24/p15 and RT/INT differ significantly from those for Gag/PR and PR/RT; P < 0.001. The concentration of DMSO in all assays did not exceed 5% final.

by SDS-PAGE. Specific activity of the concentrated PR14 was approximately 1 µmol/min/mg of HTLV-1 p24/p15 peptide substrate.

Cleavage of peptide substrates by PR14

The pH optimum for cleavage of a Gag synthetic peptide by HTLV-1 protease has previously been reported as pH 5 (Kobayashi et al., 1991). Bovine leukaemia virus (BLV) protease, which is the most closely related of the retroviral proteases to that of HTLV-1 by sequence similarity, has been shown to require a salt concentration of 1 to 2 M for maximum activity (Menard et al., 1993). Therefore all cleavage assays were done at this pH and at a salt concentration of 1 M. HIV-1 and Moloney murine leukaemia virus (Mo-MuLV) proteases prefer higher salt concentrations of 3 M (Richards et al., 1990; Menendez-Arias et al., 1993). No difference in kinetic parameters was observed for PR14 when 1 M-ammonium sulphate was substituted for sodium chloride in the cleavage buffer (data not shown).

To analyse the kinetic parameters of PR14 and the effect of protease inhibitors, we used a sensitive chromogenic peptide assay (Richards et al., 1990) that allows the continuous quantification of peptide substrate hydrolysis. Synthetic decapeptides containing putative maturation sites found in the HTLV-1 Gag, Pol and PR precursor proteins were synthesized with incorporation of the chromogenic reporter group. As shown in Table 1,
Table 2. Inhibition of PR14 by active site inhibitors, pepstatin A and Ro 31-8959

<table>
<thead>
<tr>
<th>Protease</th>
<th>Cleavage site</th>
<th>Peptide sequence</th>
<th>Inhibitor</th>
<th>$K_i$ (M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-1</td>
<td>p24/p15</td>
<td>KTKVLNphVQPK</td>
<td>Pepstatin A</td>
<td>116.8 ± 21.5 x 10^{-6}</td>
</tr>
<tr>
<td>PR14</td>
<td>p19/p24</td>
<td>APOVLNphVMHIPL</td>
<td>Pepstatin A</td>
<td>89.1 ± 7.9 x 10^{-6}</td>
</tr>
<tr>
<td>HIV-1</td>
<td>ca/x</td>
<td>KARVLNphEAM</td>
<td>Ro 31-8959</td>
<td>3.7 ± 0.6 x 10^{-6}</td>
</tr>
<tr>
<td>PR9</td>
<td>ca/x</td>
<td>KARVLNphEAM</td>
<td>Pepstatin A</td>
<td>49.3 ± 2.7 x 10^{-6}</td>
</tr>
</tbody>
</table>

* Values represent the mean of four experiments ± S.E.M. Inhibitors were preincubated with protease at 35 °C for 2 min before addition to substrate. Tenfold and twofold titrations of inhibitors allowed the calculation of $K_i$ at a substrate concentration equivalent to $K_m$.

$K_i$ values were determined using the formulae $K_i = \frac{K_i(app)}{1 + [S]/K_m}$, where $V_0 =$ reaction velocity - inhibitor, $V_i =$ velocity + inhibitor, $S =$ substrate, $I =$ inhibitor, $K_m =$ Michaelis constant for protease-substrate reaction (Beynon & Bond, 1989).

Table 3. Inhibition of HTLV-1 and HIV-1 protease by N- and C-terminal peptides*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin (N- or C-terminal)</th>
<th>Inhibition constant (IC_{50}† or $K_i$‡)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVIPLDPARRPV-OH</td>
<td>HTLV-1(N)</td>
<td>17.1 nM†‡</td>
</tr>
<tr>
<td>QMQGVLYL-OH</td>
<td>HTLV-1(C)</td>
<td>&gt; 400 µM†</td>
</tr>
<tr>
<td>YLPEAKRP/CVL-OH</td>
<td>HTLV-1(C)</td>
<td>84 µM‡</td>
</tr>
<tr>
<td>Ac-POQ/TLQARP-NH₂</td>
<td>HIV-1(N)</td>
<td>&gt; 2 mM†</td>
</tr>
<tr>
<td>Ac-TLNF-OH</td>
<td>HIV-1(C)</td>
<td>1.5 mM‡</td>
</tr>
<tr>
<td>Ac-TVFSNF-OH</td>
<td>HIV-1(C)</td>
<td>251.5 µM‡</td>
</tr>
<tr>
<td>Ac-LKAIHF-OH</td>
<td>MuLV(C)</td>
<td>80 µM†; 61 µM‡‡</td>
</tr>
</tbody>
</table>

* PR9 or PR14 were preincubated with peptides shown above for 2 min prior to the addition of substrate. Peptides were titrated as for the data in Table 2 to determine $K_i$ parameters.
† Schramm et al. (1992, 1993).
‡ $K_i$ values determined in this study.
§ No inhibition at 800 µM [test as in Schramm et al. (1993)].

the $K_m$ values for all the HTLV-1 peptides were between 3 and 35 µM. The $K_m$ values for the Gag/PR and PR/RT peptides, which correspond with the protease autocleavage sites from the precursor protein, were consistently lower (5 µM and 3.8 µM respectively) than those obtained for the Gag- and Pol-derived peptide substrates. This indicates that the affinity of these peptides for PR14 is higher than that of the other peptides. As the $k_{cat}$ values for all the substrates were similar, the mechanism of hydrolysis of these peptides once bound would appear to be the same. A peptide substrate containing the HIV-1 Gag ca/x cleavage site was cleaved so slowly by PR14 that the kinetic parameters could not be reliably determined. This peptide was cleaved by HIV-1 PR9 at pH 4.7 with a $K_m$ of 25 µM and $k_{cat}$ of 9.7 s⁻¹.

Inhibition of PR14 by pepstatin A and Ro 31-8959

The data in Table 2 show that pepstatin A inhibited PR14 activity poorly, with an apparent $K_i$ of 80 to 130 µM. This is significantly higher than the value obtained by Kobayashi et al. (1991) using a 15-residue peptide spanning the HTLV-1 Gag p19/p24 site. The hydroxyethylamine-based inhibitor Ro 31-8959 (Roberts et al., 1990) gave a $K_i$ value of 3.7 µM. Although still inhibitory for HTLV-1 PR14, this contrasts with its extreme potency in inhibiting HIV-1 PR9 activity ($K_i = 11$ pm; Table 2).

Inhibition of PR14 by N- and C-terminal HTLV-1 peptides

The N- and C-terminal regions of retroviral proteases are known to contribute to the stability of the dimeric enzyme by the formation of anti-parallel β-strands (Hayakawa et al., 1992; Wlodawer & Erickson, 1993). Destabilization of the dimer is one mechanism by which inhibition of protease activity might be achieved (Zhang et al., 1991; Schramm et al., 1993). Peptides corresponding to the N- and C-terminal regions of HTLV-1 and HIV-1 were analysed for inhibitory activity on PR14 and HIV-1 proteases (Table 3). $K_i$ values for QMQGVLYL (C-terminal amino acids 140 to 147 corresponding to HIV-1 amino acids 92 to 99) and PVIPLDPARRPV
(N-terminal amino acids 33 to 44) of 70 μM and 87 μM respectively, indicate relatively poor inhibition of PR14 by these peptides. In contrast, a peptide corresponding to the exact C terminus of HTLV-1 PR14 (YLPEAKPVIL; amino acids 147 to 157) inhibited PR14 with an apparent $K_i$ of 5.7 μM. This peptide also showed some inhibition of HIV-1 protease with an apparent $K_i$ of 84 μM. Peptides derived from the HIV-1 N- and C-terminal protease sequences (Schramm et al., 1993) were ineffective on PR14 (Table 3). A peptide (Ac-LKAQ1HF, amino acids 101 to 107) derived from the C terminus of MuLV, which is closely related in sequence to HTLV-1, showed specificity for PR9 (Table 3). These results indicate that N- and C-terminal peptides of HIV-1 were ineffective on PR14 with an apparent $K_i$ of 50 μM (Table 3) but was ineffective on HIV-1 protease (Schramm et al., 1993).

**Discussion**

We describe the expression of recombinant HTLV-1 protease in bacteria and cleavage analysis of this protease on several peptide substrates. The active protease was found to be autocatalytically processed from a precursor molecule (amino acids 1 to 234) encoded by the prokaryotic vector pGMT7-PR. Attempts in our laboratory to express the mature form of HTLV-1 protease directly resulted in the protease being inactive (data not shown). These constructs were of two types: (i) a protein comprising Pro33 to Leu157 and (ii) a protein comprising Pro101 to Leu147. That neither of these expressed proteins was active suggests that correct folding of the protein in bacteria requires N- and C-terminal sequences of the precursor which had been cleaved off, although the same pathway may not be required in eukaryotic cells. Others have shown a requirement for residues Leu147 to Leu157 for proteolytic activity (Hayakawa et al., 1992). Alternatively it is possible that N-terminal processing of the expressed proteins resulted in the product being truncated to Pro18 (see Rangawala et al., 1992).

Modelling of the structure of HTLV-1 protease has suggested that the N- and C-terminal sequences, as in other retroviruses, form antiparallel β-strands (Hayakawa et al., 1992) that are essential for the stability of the dimer. It was hypothesized that N- and C-terminal peptides might also destabilize the PR14 dimer by binding to these regions and disrupting the β-strand pairing. The N- and C-terminal peptides of HIV-1 were poor inhibitors of PR14 activity. The HTLV-1 C-terminal peptide (YLPEAKRPPVIL-OH) was a more effective inhibitor than the C-terminal HIV-1 peptides, although the $K_i$ of 5.7 μM suggests that the peptides either fit poorly, or the monomeric form is not accessible for binding to free peptides. Similar experiments with HIV-1 protease indicate that the dissociation of the dimeric enzyme is slow (Darke et al., 1994) and that inhibition can be increased by preincubation of the protease with peptide at 37 °C for 2 to 3 h (Schramm et al., 1992). However long preincubation of PR14 at 37 °C resulted in the inactivation of the enzyme in the absence of N- and C-terminal peptides and hence this could not be tested (data not shown). Alternatively, poor inhibition may reflect low affinity of the peptides for monomeric PR14 subunits. Comparison of the efficacy of N- and C-terminal peptides in inhibiting PR14 and PR9 (Schramm et al., 1993; Table 3) may result from significant differences in the interface part of the two proteases. The C-terminal region of HTLV-1 protease is 10 residues longer than that of HIV-1 based on sequence homology (Fig. 1a) and may form a more complex and stable structure in HTLV-1. Hence, the dissociation equilibrium of HTLV-1 PR14 might be so slow that monomeric protease subunits are rarely available for binding by peptides, although complete dissociation may not be required for inhibition to be achieved by destabilization of the structure (Schramm et al., 1993). It is also possible that the peptide YLPEAKRPPVIL-OH, which contains the N-terminal part of the HTLV-1 PR/RT substrate sequence, binds to the active site of the protease and is therefore inhibitory by competing with other substrates.

We have shown that HTLV-1 PR14 shows specificity for HTLV-1 substrates, being unable to hydrolyse a peptide of the HIV-1 Gag sequence. Blaha et al. (1992) likewise showed specificity of BLV protease for the closely related BLV, HTLV-1 and MuLV substrates, but not for HIV-1 substrates. However the substrate specificity of retroviral proteases accommodates some flexibility in their recognition sequences, although some preference for substrates corresponds with the structural composition of retroviral enzyme subsites (Cameron et al., 1993). Cellular proteins, which may have sequences similar to viral substrates, may be protected from cleavage either because the protease becomes active only when encapsulated within the virion, or owing to the presence of an intrinsic viral protease inhibitor. In HIV-1, an inhibitory role for the P6 protein has been hypothesized (Schramm et al., 1993).

In our study, the poor inhibition of HTLV-1 PR14 by Ro 31-8959 reinforces the distinction between the substrate binding site specificities of HTLV-1 and HIV-1. Ro 31-8959 is one of a series of transition state mimetic compounds based on the HIV-1 Pol substrate sequence Leu166 to Ile169 in which the Phe167 to Pro168 scissile bond is replaced by a Phe[CH(OH)CH$_2$N]Pro moiety (Roberts et al., 1990). In the case of Ro 31-8959, Pro is replaced by decarboxyisoprinolone. This compound maintains the Asn166 at the P2 position and fulfils the requirements of a class I cleavage site which does not occur in BLV or HTLV-1 Gag proteins and provides a poor substrate for both of these retroviral proteases.
(Henderson et al., 1988). Jacobsen et al. (1993) have noted that a mutation from Gly to Val in the substrate-binding region of HIV-1 protease significantly reduces the effect of Ro 31-8959, probably by directly inhibiting binding of the compound to the protease.

The kinetic parameters calculated for the cleavage of HTLV-1 peptides by PR14 show that the highest affinity binding is found in peptides containing the sequences corresponding to Gag/PR and PR/RT sites. For these peptides, the $K_m$ values were three- to 10-fold lower than the values obtained for Gag-derived peptide substrates, whereas the turnover number ($k_{cat}$) remained approximately the same. Thus the autocatalytic cleavage of PR14 from its precursor could be favoured over subsequent processing events by having a higher affinity of the recognition site for the protease active site. Although the sequence of events leading to autoprocessing of viral proteases from their polyprotein precursors is not well understood, recent evidence suggests that multimerization of the precursor, directed by Gag sequences, may precede processing (Luban et al., 1993). Since in HTLV-1, PR14 is encoded in a separate reading frame from Gag or Pol sequences, the N- and C-terminal flanking regions of the protease precursor may play this role. In addition, an ordered series of cleavage events of the HIV-1 Gag precursor at different processing sites has been noted. This order preference may be influenced by the amino acid composition and surface accessibility of these sites (Pettit et al., 1991). A high affinity interaction between autoprocessing sites within the protease precursor molecule and the PR14 active site could partially compensate for the relative inaccessibility of the site during multimerization of the precursor protein within the assembled viral capsid, whereas subsequent cleavage of structural proteins by PR may not be so constrained. The recognition hierarchy of maturation sites by PR may therefore begin with the autoprocessing sites of PR itself.

The rate of hydrolysis of substrates ($k_{cat}$) by HTLV-1 PR14 is similar to that reported for other retroviral proteases (Richards et al., 1990; Griffiths et al., 1992; Tozser et al., 1993). Substrates containing aromatic/Pro cleavage sites have been shown to be cleaved up to 60-fold more slowly than hydrophobic/hydrophobic sites-containing substrates (Griffiths et al., 1992). In our study, peptides p19/p24, Gag/PR and PR/RT conform to the aromatic/Pro type whereas the p24/p15 substrate is typical of a hydrophobic/hydrophobic substrate. In fact, the p24/15 substrate was cleaved more efficiently than the rest (Table 1), although this difference was not statistically significant.

Recognition by proteases of substrates and inhibitors designed to bind the active site depends on specificity largely conferred by structural elements within the binding site. The data presented here show that PR14 is a highly stable enzyme that binds its native substrates with high affinity. Part of the stability may relate to complex structural pairing of the C termini of the PR14 monomeric subunits. The definition of the crystal structure of HTLV-1 protease will clarify some of these interactions and allow modifications to be made in drug design to accommodate the differences between retroviral proteases.

We wish to thank Roche Products Ltd. for supplying Ro 31-8959 used in this study and John Kay for helpful advice on the kinetic assays. This work was supported by the Wellcome Trust.

References


Analysis of HTLV-I protease cleavage


(Received 22 March 1994; Accepted 15 April 1994)