C-terminal residues of mature human T-lymphotropic virus type 1 protease are critical for dimerization and catalytic activity

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INTRODUCTION

HTLV-1 [HTLV (human T-cell lymphotrophic virus) type 1] is associated with a number of human diseases. HTLV-1 protease is essential for virus replication, and similarly to HIV-1 protease, it is a potential target for chemotherapy. The primary sequence of HTLV-1 protease is substantially longer compared with that of HIV-1 protease, and the role of the ten C-terminal residues is controversial. We have expressed C-terminally-truncated forms of HTLV-1 protease with and without N-terminal His tags. Removal of five of the C-terminal residues caused a 4–40-fold decrease in specificity constants, whereas the removal of an additional five C-terminal residues rendered the protease completely inactive. The addition of the N-terminal His tag dramatically decreased the activity of HTLV-1 protease forms. Pull-down experiments carried out with His-tagged forms, gel-filtration experiments and dimerization assays provided the first unequivocal experimental results for the role of the C-terminal residues in dimerization of the enzyme. There is a hydrophobic tunnel on the surface of HTLV-1 protease close to the C-terminal ends that is absent in the HIV-1 protease. This hydrophobic tunnel can accommodate the extra C-terminal residues of HTLV-1 protease, which was predicted to stabilize the dimer of the full-length enzyme and provides an alternative target site for protease inhibition.

Key words: dimerization, gel filtration, human T-cell lymphotrophic virus type 1 (HTLV-1), molecular modelling, pull-down assay, retroviral protease.

1 Abbreviations used: HTLV, human T-cell lymphotrophic virus; PR, protease; HTLV-1 125 PR, wild-type stabilized HTLV-1 PR; HTLV-1 120 PR, HTLV-1 125 PR shortened by five residues; HTLV-1 116 PR, HTLV-1 125 PR shortened by nine residues; HTLV-1 115 PR, HTLV-1 125 PR shortened by 10 residues; TFA, trifluoroacetic acid.

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Figure 1  Sequence alignment of the HIV-1 (HX82) and HTLV-1 (CS) PRs

The identical residues are indicated by boxes. The sequence of the peptide reported to inhibit enzyme activity is underlined. The sequence of the longest His-tagged recombinant HTLV-1 PR form is also provided, where the stabilizing mutations (L40I, C90A and C109A) are shown in italics. wt, wild-type.

dimerization experiments, N-terminal His-tagged PRs (His–HTLV-1 125 PR, His–HTLV-1 116 PR and His–HTLV-1 115 PR) were also constructed, expressed, purified to homogeneity and studied for activity and dimerization ability. Using pull-down, gel-filtration and kinetic techniques, the present paper is the first to experimentally prove the importance of the C-terminal residues of HTLV-1 PR in enzyme dimer stability.

MATERIALS AND METHODS

Construction of the shortened HTLV-1 PR forms

The clone encoding HTLV-1 PR 125 [11] was used as a DNA template for mutagenesis. This enzyme contained alanine residues in place of cysteine residues, and a mutation (L40I) which protects the enzyme from autodegradation. Previous studies demonstrated that the activity of the stabilized enzyme is indistinguishable from that of wild-type HTLV-1 PR [11]. HTLV-1 120, 116 and 115 PR forms were generated by stop-codon insertions using the QuikChange® site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions, with the appropriate oligonucleotide primers obtained from SigmaGenosys. Incorporation of mutations was verified by DNA sequencing performed using the ABI Prism dye terminator cycle sequencing kit (PerkinElmer) and an Applied Biosystems Model 373A sequencer.

Construction of the His-tagged 10-residue shortened HTLV-1 PR

The DNA sequence of HTLV-1 125 PR and 10-residue shortened HTLV-1 PR was amplified from the clone coding for HTLV-1 125 PR and HTLV-1 115 PR respectively by PCR using the following primer set: forward, 5′-CCTCCAGTTATACCGTAGTCCCGCC-3′ and reverse, 5′-CGCGATCTCCAAGATTACGG-3′. The PCR fragments were inserted into linearized pCR-Blunt vector (Invitrogen). The plasmids were digested with Stul and BamHI restriction enzymes (New England Biolabs) to generate the final PR fragments. The N-terminal His-tagged constructs were created by replacing the capsid coding sequence (originally cloned into the Stul and BamHI restriction sites) of a recombinant HIV-1 capsid protein clone which had been created using pB6, a derivative of pET 11a that expresses N-terminal His-tagged proteins (a gift from Dr Carol Carter, Department of Molecular Genetics and Microbiology, State University of New York, Stony Brook, NY, U.S.A.). The pB6-derived plasmids were grown in Escherichia coli DH5α cells and purified with the QIAprep spin plasmid kit (Qiagen). For the construction of His–HTLV-1 116 PR, a stop codon was inserted into the His-HTLV-1 125 PR sequence using the QuikChange® site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions, using the oligonucleotide primers used previously for generating HTLV-1 116 PR. Constructs were verified by DNA sequencing performed using the ABI Prism dye terminator cycle sequencing kit (PerkinElmer) and an Applied Biosystems Model 373A sequencer.

Enzyme purification and assays with oligopeptide substrates

HTLV-1 125 PR was purified from inclusion bodies as described previously [11,18]. The HTLV-1 PR C-terminally-truncated forms were purified using the same procedure. The His-tagged HTLV-1 PR forms were purified using two chromatography steps. The inclusion bodies were dissolved in 50 mM Tris/HCl (pH 8.0), 7.5 M guanidinium chloride and 5 mM EDTA, then loaded on to a HisTrap column (Amersham Biosciences), which was equilibrated with 20 mM sodium phosphate (pH 7.4) containing 500 mM NaCl, 3 M guanidinium chloride, 20 mM imidazole, 2% (v/v) glycerol and 0.2% Triton X-100, and the PRs were eluted by using an increasing imidazole gradient (20–500 mM) under denaturing conditions. The eluted enzymes were purified further.
by reversed-phase HPLC as described previously [11]. The correct molecular mass of the purified HTLV-1 PRs was verified by using a 4000 QTRAP mass spectrometer (Applied Biosystems). Folding of the enzymes was performed by dialysis of the HPLC fractions in a large excess of 25 mM formic acid (pH 2.8) and subsequently in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl. For the activity measurements and to check purity, folded PRs were concentrated using Amicon ultrafiltration devices (Amicon Ultra-15, 10 kDa cut-off; Millipore). Protein concentration was determined using the Bradford assay (Bio-Rad) in a 96-well plate format following the manufacturer’s instructions. Purity of the samples was determined by SDS/PAGE (16% gels). Protein (10–15 μg) was loaded in each lane. Proteins were visualized using Coomassie Brilliant Blue staining after separation. The PR assays were initiated by mixing 5 μl (8–8500 nM) of the purified enzyme with 10 μl of 2× incubation buffer A [0.5 M potassium phosphate buffer (pH 5.6) containing 10% (v/v) glycerol, 2 mM EDTA, 10 mM dithiothreitol and 4 M NaCl] or 2× incubation buffer B [100 mM sodium acetate (pH 5.0) and 0.2 M NaCl] and 5 μl of 0.01–3 mM substrate. The synthesis and characterization of the oligopeptide substrates have been described previously [8,11,18]. The substrate concentration range for kinetic measurements was 0.01–3 mM substrate. The synthesis and characterization of the substrate were initiated by mixing 5 μl (8–8500 nM) of the purified enzyme with 10 μl of 2× incubation buffer A [0.5 M potassium phosphate buffer (pH 5.6) containing 10% (v/v) glycerol, 2 mM EDTA, 10 mM dithiothreitol and 4 M NaCl] or 2× incubation buffer B [100 mM sodium acetate (pH 5.0) and 0.2 M NaCl] and 5 μl of 0.01–3 mM substrate. The synthesis and characterization of the oligopeptide substrates have been described previously [8,11,18].

Determination of apparent Kd values of the HTLV PRs
Specific activity values were measured in duplicate as a function of the dimeric enzyme concentration in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl and using 1 mM KTKVF \textsubscript{c}VVQPK oligopeptide substrate. Samples were incubated for 1 h at 37°C and processed for HPLC analysis as described above, as used previously to determine the Kd as described for HIV-1 PR [20]. Assuming that the active PR dimer (D) is in rapid equilibrium with its inactive monomer (M), the following equations can be used to describe the activity of the enzyme on the oligopeptide substrate (S) [20]:

\[
2M \xrightleftharpoons{K_d} D + S \xrightleftharpoons{K_{cat}} D + S \xrightarrow{S} D + \text{products} \tag{1}
\]

\[
2M + S \xrightarrow{S} D + 2S \xrightarrow{S} D + \text{products} \tag{2}
\]

The apparent Kd values can be obtained by plotting the relative specific activities against the concentration of the HTLV-1 PR forms and fitting a curve using the ‘Hyperbola single rectangular 2 parameters equation’ setting of SigmaPlot 8.02 software.

Molecular modelling
The crystal structures of HIV-1 PR (PDB code 1K1T [21]) and HTLV-1 116 PR (PDB code 2BF7 [14]) complexed with inhibitors, and a molecular model of HTLV-1 125 PR were used for structural analysis. The nine-residue-long C-terminal tail of HTLV-1 125 PR was built manually into the crystal structure.
of the HTLV-1 116 PR using the Sybyl software package (Tripos). Unfavourable interactions were removed by energy minimization (Kollman all-atom force field [22], 10 Simplex and 100 Powell iterations, 8 Å (1 Å = 0.1 nm) cut-off). Lipophilic potentials on molecular surfaces were generated by the MOLCAD module of Sybyl. The model of HTLV-1 125 PR is available from the authors upon request.

RESULTS

The sequence alignment of HTLV-1 PR and HIV-1 PR, based on crystal structures, is shown in Figure 1 for the wild-type PRs and for the His–HTLV-1 125 PR. All of the other constructs used in the present paper contained shortened versions of this HTLV-1 PR sequence or no His tag (starting with the first Pro residue as indicated), HTLV-1 125 PR has been characterized in detail previously [8,11,18]. On the basis of the active-site titrations, this form folded completely and efficiently processed the oligopeptide substrates at high ionic strength (Table 1). At lower ionic strength, the enzyme exhibited an approx. 30–80-fold reduction in the specificity constants (Table 1). These results suggest that the C-terminal residues are important for efficient substrate hydrolysis, and the N-terminal extra sequence with the His tag is not favourable for enzyme activity. Interestingly, extension of the N-termimus of HIV-1 PR also had an unfavourable effect on the activity of the enzyme [25]. To determine the apparent dimer stability of the HTLV-1 125 and HTLV-1 120 PR forms, an activity-based assay originally used for HIV-1 PR [20] was used. The assay was performed in low-ionic-strength conditions (Buffer B). As the fully formed dimer should have a constant specific activity, a decrease in the specific activity by decreasing the enzyme concentration is considered to be the result of less efficient dimerization; hence the apparent dimer stability of both PR forms is shown in Figure 2. The Kd values were much lower compared with those obtained with HTLV-1 125 PR, resulting in extremely low specificity constants (Table 1). These results suggest that the C-terminal residues are important for efficient substrate hydrolysis, and the N-terminal extra sequence with the His tag is not favourable for enzyme activity. Interestingly, extension of the N-termimus of HIV-1 PR also had an unfavourable effect on the activity of the enzyme [25].

To determine the apparent dimer stability of the HTLV-1 125 and HTLV-1 120 PR forms, an activity-based assay originally used for HIV-1 PR [20] was used. The assay was performed in low-ionic-strength conditions (Buffer B). As the fully formed dimer should have a constant specific activity, a decrease in the specific activity by decreasing the enzyme concentration is considered to be the result of less efficient dimerization; hence the enzyme concentration corresponding to a 50% loss in enzyme activity represents the apparent dissociation constant (Kd) [20]. Substantial differences were observed in the apparent dimer stability of HTLV-1 125 PR (Kd = 491 nM) and HTLV-1 120 PR (Kd > 3000 nM), as shown in Figure 3. These results indicated that the removal of the C-terminal five residues substantially decreased

### Table 1: Kinetic parameters determined for various HTLV-1 protease forms

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Substrate</th>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K_m (mM)</td>
<td>k_cat (s⁻¹)</td>
</tr>
<tr>
<td>HTLV-1 125 PR</td>
<td>KTKVL↓VVQPK</td>
<td>0.003 ± 0.004*</td>
<td>10.0 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td>KTKVF↓VVQPK</td>
<td>0.049 ± 0.006*</td>
<td>16.4 ± 0.9*</td>
</tr>
<tr>
<td>His–HTLV-1 125 PR</td>
<td>KTKVL↓VVQPK</td>
<td>0.088 ± 0.013</td>
<td>0.0020 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td>KTKVF↓VVQPK</td>
<td>0.116 ± 0.014</td>
<td>0.021 ± 0.001</td>
</tr>
<tr>
<td>HTLV-1 120 PR</td>
<td>KTKVL↓VVQPK</td>
<td>0.024 ± 0.003</td>
<td>0.150 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>KTKVF↓VVQPK</td>
<td>0.014 ± 0.001</td>
<td>0.160 ± 0.001</td>
</tr>
<tr>
<td>HTLV-1 116 PR</td>
<td>KTKVL↓VVQPK</td>
<td>No cleavage‡</td>
<td>No cleavage‡</td>
</tr>
<tr>
<td></td>
<td>KTKVF↓VVQPK</td>
<td>Residual activity§</td>
<td>Residual activity§</td>
</tr>
</tbody>
</table>

*Results are from [8].† These values were determined using first-order kinetic conditions ([S] < K_m).‡ No hydrolysis was observed either low or at high protease concentrations.§ Residual activity was observed when the substrate was incubated with 3.1 μM protease under assay conditions.
the apparent dimer stability of the enzyme. A substantial decrease in the specific activity was observed for HTLV-1 125 PR above 2500 nM (results not shown), and a similar effect was demonstrated for HTLV-1 120 PR above 7000 nM (results not shown). This effect was predicted to be the result of the high aggregation tendency of HTLV-1 PR, as observed in the gel-filtration experiments (see below) and is in good agreement with our observations of aggregation tendencies during the crystallization of HTLV-1 PR forms.

To further characterize the effect of truncation on the dimer stability, as well as the dimerization capability of the enzymes, gel-filtration experiments were performed using the same buffer conditions as used for the dimerization assay. As shown in Figure 4, although wild type HTLV-1 125 PR appeared to be mostly in the dimeric form under the gel-filtration assay conditions, the equilibrium was shifted towards the monomeric form for HTLV-1 120 PR, whereas HTLV-1 115 PR was completely in the monomeric form, suggesting that the elimination of the 10-residue-long C-terminal-flanking sequence is detrimental for dimerization of the enzyme in low ionic strength conditions.

As HTLV-1 115 PR and His-tagged HTLV-1 PRs were not active at low ionic strength (Table 1), a pull-down assay was performed to obtain more information about the effect of the C-terminal residues of HTLV-1 PR on dimerization. Although HTLV-1 125 PR formed a heterodimer with His-tagged HTLV-1 125 PR with a molar ratio of close to 1:1, HTLV-1 120 and 115 PRs showed significantly weaker associations with His–HTLV-1 125 PR (Figure 5). Interestingly, the dimerization capability of HTLV-1 115 PR was similar when binding to either His–HTLV-1 116 and His–HTLV-1 125 PRs.

To obtain a feasible explanation for these results, we analysed the nature of the C-terminal extension in HTLV-1 PR (Figure 1), as well as creating a molecular model for HTLV-1 125 PR. Various surface representations of HIV-1 and HTLV-1 116 PRs showed substantial differences between the hydrophobicities of the C-terminal regions of the two enzymes (Figures 6A and 6B). The more hydrophilic HIV-1 PR also showed a more compact...
structure in this region (Figure 6A), whereas residues of the terminal \(\beta\)-sheet and the Arg\(^{77}\)–Pro\(^{84}\) loop of HTLV-1 116 PR formed a groove (Figure 6B). On the basis of the experimental results, we predicted that this hydrophobic groove may be filled by the extra C-terminal residues which may preferentially interact with the hydrophobic ‘outer’ surface of the terminal \(\beta\)-sheet. To obtain a molecular model for the full-length HTLV-1 PR, we have incorporated the nine missing residues into the crystal structure of HTLV-1 116 PR in such a way that this interaction may occur. The structural requirements of this step and the characteristics of PEAKGPPVIL fragment (residues 116–125) were in good agreement: the PEAKGPP section was dominated by proline, glycine and charged residues with strong turn-forming propensity, whereas the residues of the terminal valine-isoleucine-leucine section possessed hydrophobic and \(\beta\)-sheet-forming characteristics. The C-terminal residues were predicted to form two new ‘strands’ for the terminal \(\beta\)-sheet (Figure 6C), however they were stabilized not by hydrogen bonds of the peptide backbone, but by hydrophobic interactions of the side chains. Interestingly, cellular aspartic PRs also have six strands in their corresponding \(\beta\)-sheet region, but the topologies are completely different (results not shown). The presence of the additional C-terminal residues in the full-length PR model resulted in a substantial decrease in size of the hydrophobic patch, although it did not disappear completely (Figure 6D).

**DISCUSSION**

On the basis of structure-based sequence comparisons, HTLV-1 PR appears to contain a 10-residue-long C-terminal-flanking sequence, as shown in Figure 1. The C-terminal extension is a characteristic feature of the PRs belonging to the deltaretrovirus family. Although some other PRs also appear to contain C-terminally-extended sequences, none of the PRs for which the three-dimensional structure is available contain such C-terminal extensions [12], including the newly determined structure of HTLV-1 116 PR [14]. The importance of this C-terminal-flanking sequence is controversial. In the case of BLV (bovine leukaemia virus), another deltaretrovirus, the C-terminal-flanking region of
the PR appeared to be dispensable for the activity of purified recombinant PR [26], where at least five out of the 10 C-terminal-flanking amino-acid residues were required for the self-processing activity of HTLV-1 PR [15]. Inhibition by a peptide representing the C-terminal 12-amino-acid-residue-long region of the PR also suggested the importance of this region in enzyme dimerization [16]. However, a purified, N-terminal His-tagged but C-terminally shortened (by 10 residues) HTLV-1 PR has been described previously, which possessed an activity equivalent to that of the full-length His-tagged PR [17]. Surprisingly, both the wild-type and the shortened enzyme showed very low catalytic efficiency (0.004–0.006 s−1 kcat values) compared with the catalytically active HTLV-1 125 PR and HTLV-1 120 PR forms described in the present paper. HTLV-1 116 PR, which was used for crystallization, appeared to have 60% activity of that of the full-length PR, but no kinetic data were provided [14], therefore the folding/activity level of this form is uncertain, but HTLV-1 116 PR appeared to have residual catalytic activity (Table 1). We have speculated that the activity of His–HTLV-1 115 PR described by Herger et al. [17] might be the result of the presence of the N-terminal His tag, therefore, we have also expressed HTLV-1 115 PR with an N-terminal His tag, but this form appeared to be completely inactive in our assay systems. Furthermore, N-terminal His-tagged HTLV-1 116 PR did not show any activity (results not shown), whereas the activity of His–HTLV-1 125 PR was substantially lower than that of HTLV-1 125 PR (Table 1).

To further characterize the role of the C-terminal extension in HTLV-1 PR, we have determined the apparent Kcat values of HTLV-1 125 and 120 PRs using a specific activity-based method. The shorter enzyme appeared to have a dramatically lower dimer stability (Figure 3). The importance of the C-terminal residues in dimerization is also supported by the result of the gel-filtration experiments, which showed that HTLV-1 115 PR appeared to be present only in the monomeric form in low-ionic-strength conditions (Figure 4). An aggregation effect was observed for HTLV-1 125 PR when the protein concentration was increased (Figure 3), an effect also seen for HTLV-1 120 PR at a 3-fold higher protein concentration (results not shown). This phenomenon partly explains the sustained failure of the crystallization of full-length HTLV-1 PR and the fact that HTLV-1 116 PR was favourably crystallized, although only in the presence of a ligand that is expected to strongly facilitate dimerization [14]. These results suggest that the C-terminal 10 residues may also have a critical role mediating the in vitro aggregation of the PR.

The results of the pull-down assay further confirmed our findings that the C-terminal extension of HTLV-1 PR has a critical role in dimerization in the absence of a ligand that may also be a prerequisite for sufficient catalytic efficiency. There is no structural evidence for how these extended residues can contribute to dimerization. Retroviral PR dimers are held together by an intricate hydrogen-bond network at the catalytic aspartate residues called the ‘fireman’s grip’, as well as by the β-sheets formed by C- and N-terminal residues [12]. The two regions are not independent: in the case of HIV PR, the residues above the β-sheet form a hydrophobic cluster which interacts with the side chain of the threonine residues after the catalytic aspartate residues. The side chains below the β-sheet are hydrophilic and they interact with the bulk solvent. Unlike in HIV-1 PR, the residues of the β-sheet exposed to water are hydrophobic in HTLV-1 PR, which might explain the high aggregation tendency of the enzyme. Furthermore, it also provides a surface for interaction with the residues of the extreme C-terminal, which may contribute to the dimer stability of full length HTLV-1 PR. However, the exact way in which these residues contribute to the dimer stability of HTLV-1 PR requires further structural studies. We have created a molecular model for the positioning of the C-terminal residues. This model explains the importance of these residues in the dimerization process and does not contradict the observed high aggregation and the low crystal-forming propensities of full-length HTLV-1 PR. The flexibility of the C-terminal tail may allow the dimer to switch between a more compact and a more open structure, in which the tail does not interact with the body of the PR, which may interfere in the crystallization processes. It is important to note that the C-terminal tail of HTLV-1 PR was also found to be critical for the infectivity of recombinant HTLV-1 virions: although the five-residue truncation already decreased infectivity, elimination of the 10 C-terminal residues practically abolished infectivity in a wild-type PR sequence background (M. Mitchell and D. Derse, personal communication). Therefore the proposed extended dimerization interface of HTLV-1 PR may provide an additional target site for the design of inhibitors against this enzyme.

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REFERENCES


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