Effect of mutations on the dimer stability and the pH optimum of the human foamy virus protease

Tamás Sperka, Péter Boross, Helga Eizert, József Tözsér and Péter Bagossi

Department of Biochemistry and Molecular Biology, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, H-4010 Debrecen, Egyetem tér 1, PO Box 6, Hungary

1To whom correspondence should be addressed.
E-mail: peter@indi.biochem.dote.hu

To explore the role of residues being close to the catalytic aspartates in the higher pH optimum and in the lower dimer stability of human foamy virus (HFV) protease (PR) in comparison with human immunodeficiency virus type 1 (HIV-1) protease, single (Q8R, H22L, S25T, T28D) and double (Q8R-T28D, H22L-T28D) mutants were created based on sequence alignments and on the molecular model of HFV PR. The wild-type and mutant enzymes were expressed in fusion with maltose binding protein in Escherichia coli and the fusion proteins were purified by affinity chromatography. Specificity constant of most mutants was lower, but the value of Q8R-T28D double mutant enzyme was higher than that of the wild-type HFV PR. Furthermore, urea denaturation at two pH values and pH optimum values showed an increased stability and pH optimum for most mutants. These results suggest that the mutated residues may not be responsible for the higher pH optimum of HFV PR, but they may contribute to the lower dimer stability as compared with that of HIV-1 PR.

Keywords: aspartyl protease/dimer stability/ enzyme kinetics/ human foamy virus protease/pH optimum

Introduction

The aspartyl protease (PR) of retroviruses plays a crucial role in the maturation of virus (Oroszlán and Luftig, 1990; Tözsér and Oroszlán, 2003) and may also have a role in the early phase of life cycle (Rumlova et al., 2003; Tözsér and Oroszlán, 2003). The protease of human immunodeficiency virus type 1 (HIV-1) is a good target for chemotherapy of AIDS, and protease inhibitors are now important components of the combination anti-retroviral therapy (Eron, 2000; Tözsér, 2003). Much less is known about the PR of another human retrovirus, the human foamy virus (HFV), which has several unusual features (Flügel and Pfrepper, 2003; Rethwilm, 2003), but it is a great promise for gene therapy (Mergia and Heinkelein, 2003). The HFV PR is essential for viral infectivity, since mutation of the active site Asp residues resulted in noninfectious virions (Konvalinka et al., 1995b), as previously found for HIV-1 PR (Kohl et al., 1988). HFV PR was expressed in a vaccinia virus system but it was not purified (Luukkonen et al., 1995). Later it was cloned and found to be active as part of a thioredoxin fusion protein, but its activity was lost after elimination of the heterologous protein part fused with the enzyme coding sequence (Pfrepper et al., 1997). HFV PR was also cloned with a C-terminal His-tag and this enzyme was used to determine the cleavage sites in the Gag and Pol polyproteins (Pfrepper et al., 1998, 1999) and to characterize the specificity of the enzyme (Pfrepper et al., 2001). We have cloned the HFV PR in fusion with maltose binding protein (MBP) and characterized the fusion protein (Fenyőfalvi et al., 1999). Recently the purification protocol was improved (Boross et al., 2006), and the modifications allowed us to prepare and characterize purified, processed and active HFV PR. Comparison of the processed and fusion forms of the wild-type and mutant (S25T) PRs suggested that the fusion forms can be used instead of the processed enzymes for comparative studies (Boross et al., 2006). The obtained catalytic constants for HFV PR were much lower than those we previously determined for various mammalian retroviral proteases coded on pol genes (Fenyőfalvi et al., 1999), but similar to those obtained previously with gag-encoded avian retrovirus PR (Tözsér et al., 1996). The pH optimum of HFV PR [6.6 (Fenyőfalvi et al., 1999)] was much higher than those values published for HIV-1 PR [4.1 (Hyland et al., 1991), 4.5–5.1 (Szeltner and Polgár, 1996b), 5.5 (Darke et al., 1989), depending on the used substrate, ionic strength and other experimental conditions]. Furthermore, the dimer stability of HFV PR was much lower than that of HIV-1 PR; half-maximal enzyme activity was reached at 0.75 M (Fenyőfalvi et al., 1999) as compared with 1.85 M (Wondrak et al., 1996) denaturant concentration, respectively. To explore the molecular basis of these unusual features, we have introduced several mutations near the catalytic aspartates.

Materials and methods

Site-directed mutagenesis of the HFV PR

Cloning of the wild-type and S25T mutant HFV PR in fusion with MBP were described previously (Fenyőfalvi et al., 1999; Boross et al., 2006). For single mutants, the wild-type residues were exchanged using the Quick-Change mutagenesis protocol (Stratagene, La Jolla, CA, USA) with the following oligonucleotide pairs obtained from Sigma-Genosys (The Woodlands, TX, USA). Mutated positions are indicated by underlined letters. Q8R: 5’-CTTCAGCTGTATACGGCGTTTCGCCGC-G-3’ and 5’-CGCCGAAGCGGGCTTACAGCTAGAA-3’; H22L: 5’-GGGACTAATATGGTGTTGCCTGGAGTTCG-3’ and 5’-GGGAGGCAAC-3’; S25T: 5’-ACAGGGGCAAC-3’ and 5’-GGGATACACGCCAACCATAC-3’; T28D: 5’-CTTCAGCTGTATACGGCGTTTCGCCGC-3’ and 5’-GGGGACAT-3’; Q8R-T28D: 5’-GGGACTAATATGGTGTTGCCTGGAGTTCG-3’ and 5’-GGGGACAT-3’. For Q8R-T28D double mutant, the second mutation was introduced into the S25T mutant HFV PR using the above-mentioned Q8R oligonucleotide pairs.
For H22L-T28D double mutant, the second mutation was introduced into the T28D mutant HFV PR using the following oligonucleotides: 5'-GGGACTAAATTGTAGCCCTTCGG-GATTCAAGGGGAGAC-3' and 5'-GTCGCCCCGTGAAATCCAGAGGCTAACAATTGATCC-3'. Mutations were verified by DNA sequencing performed using the ABI Prism dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Model 373A sequencer (Applied Biosystems, Foster City, CA, USA).

Purification of the wild-type and mutant HFV PRs
A total of 500 ml freshly prepared Escherichia coli culture bearing the plasmid construct coding for the wild-type or a mutant enzyme was grown at 37°C up to an absorbance at 600 nm of 0.7–1.0, in Luria–Bertani medium containing 100 µg/ml ampicillin. Then induction with IPTG (1.0 mM) was performed for 5 h and cells were harvested by centrifugation at 2000 g for 10 min at 4°C. After removal of the supernatant, 25 ml lysis buffer (50 mM Tris, pH 7.2, 1 mM EDTA and 100 mM NaCl) was added. Cells were disrupted by freezing–thawing followed by sonication on ice. Samples were centrifuged at 9000 g for 15 min at 4°C. The supernatant was loaded on a column containing amylose resin (25 ml) applied to AKTApurifier automated liquid chromatography system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and extensively washed with the lysis buffer. The fusion protein was eluted with lysis buffer containing 20 mM maltose. The fusion protein (Table I). The specificity constant of S25T mutant was wild-type-like, but the values of T28D, Q8R and H22L single mutants were 2.6, 2.7 and 10.8 times lower than that of the wild-type protein, respectively. The specificity constant of H22L-T28D double mutant was also 4.2 times lower; however, the unusual parameters. The activity of mutant MBP-HFV protease (Morozov et al., 1997) was used for immunoblotting, followed by manual corrections based on the structural alignment. Homologous model of HFV PR was built by Modeller (Salis and Blundell, 1993) from the crystal structure of HIV-1 PR complexed with an inhibitor [PDB code: 7HVP (Swain et al., 1990)]. Structures were examined on Silicon Graphics workstations using Sybyl program package (Tripos, St Louis, MO, USA).

Proteolytic assay
Oligopeptide substrate SRAVN*TVTQS (where asterisk shows the site of cleavage) was synthesized as described previously (Fenyőfalvi et al., 1999). Kinetic parameters were determined in 50 mM MES, 100 mM Tris, 50 mM acetate, 1 M NaCl, pH 6.3 buffer (META). The pH of the buffer system was sensitive to the temperature, therefore it was adjusted at 37°C. The reaction mixtures contained 1.4–22 µM purified enzyme and 0.2–1.0 mM substrate and they were incubated at 37°C for 1 h. The reactions were stopped by the addition of 180 µl 1% trifluoroacetic acid, and an aliquot was analyzed by reversed-phase HPLC as described previously (Fenyőfalvi et al., 1999). Cleavage products of PR-catalyzed hydrolysis were previously identified by amino acid analysis for wild-type HFV protease (Fenyőfalvi et al., 1999) and mutant enzymes produced the same cleavage fragments as indicated by identical retention times. Kinetic parameters were determined by fitting the data obtained at <20% substrate hydrolysis to the Michaelis–Menten equation by using the Fig. P program (Fig. P Software Corp., Durham, NC, USA).

The pH optimum of the enzymes was determined in META buffer but having pH in the range of 3–9. Symmetrical bell-shaped pH optimum curves were fitted by nonlinear regression module of SigmaPlot program (Systat Software, Inc., Point Richmond, CA, USA) using the following equation (Polgár et al., 1994):

$$\text{relative activity} = \frac{a_{\text{max}}}{[1 + 10^{pK_1-pH} + 10^{pK_2-pH}]}$$

where pK_1 and pK_2 are the negative logarithms of the acidic ionization constants of the catalytically competent ionizing groups.

The urea denaturation curves were determined in META buffer having pH 6.0 or 7.2 and in the presence of 0–3 M urea. Sigmoidal urea denaturation curves were fitted by the nonlinear regression module of SigmaPlot using the following equation (Jandu et al., 1990; Szeltner and Polgár 1996a):

$$f_u = 1 - \exp((m[D] - \Delta G_u)/RT)$$

$$1 + \exp((m[D] - \Delta G_u)/RT),$$

where $f_u$ is fraction of unfolded protein, $[D]$ is the urea concentration, $\Delta G_u$ is the conformational stability of the protein at zero concentration of urea, $m$ is a measure of the dependence of $\Delta G$ on the urea concentration, R is the gas constant and $T$ is the absolute temperature. The urea concentration leading to 50% loss in enzymatic activity can be calculated from the following equation: $D_{1/2} = \Delta G_u/m$.

Sequence alignment and molecular modeling
The sequences of the retroviral proteases were aligned to the sequences of structurally aligned HIV-1, HIV-2, SIV (simian immunodeficiency virus), EIAV (equine infectious anemia virus), FIV (feline immunodeficiency virus) and RSV (Rous sarcoma virus) proteases. Structural alignment was made by Whatif (Vriend, 1990), and the initial multiple sequence alignment was made by ClustalW (Thompson et al., 1994), followed by manual corrections based on the structural alignment. Homologous model of HFV PR was built by Modeller (Salis and Blundell, 1993) from the crystal structure of HIV-1 PR complexed with an inhibitor [PDB code: 7HVP (Swain et al., 1990)]. Structures were examined on Silicon Graphics workstations using Sybyl program package (Tripos, St Louis, MO, USA).

Results and discussion
HFV and its protease have several unusual features (Flügel and Pfeffer, 2003; Rethwilm, 2003). Our previous characterization of the HFV PR suggested that in comparison with HIV-1 PR the enzyme has lower catalytic efficiency, lower dimer stability and higher pH optimum (Fenyőfalvi et al., 1999; Boross et al., 2006). Therefore, various residues in the vicinity of the catalytic aspartates were selected for mutagenesis study (Figures 1 and 2) to explore their potential contributions to the unusual parameters. The activity of mutant MBP-HFV proteases was compared with that obtained for the wild-type fusion protein (Table I). The specificity constant of S25T mutant was wild-type-like, but the values of T28D, Q8R and H22L single mutants were 2.6, 2.7 and 10.8 times lower than that of the wild-type protein, respectively. The specificity constant of H22L-T28D double mutant was also 4.2 times lower; however,
enzymatic activity (pH values. The urea concentration leading to 50% loss in measurable activity on all mutant forms of the enzyme at both have the highest possible distance between them and to have sides of the bell-shaped curves on the activity versus pH plots. To get a more profound effect, the pH values were chosen to be 6.0 and 7.2 (Table III). These values were located at the two by measuring their urea denaturation curves at two pH values:

The small variation of the apparent $k_{\text{cat}}$ values of the mutant enzymes compared with that of the wild-type enzyme may reflect small variation of the ‘true’ catalytic constant and in parallel small variation in the concentration of the active, correctly folded enzyme. The other possibility to get unchanged $V_{\text{max}}$ value requires an increased ‘true’ catalytic constant and a decreased folding capability or vice versa, which is highly unlikely.

We have determined the pH profile of the wild-type and the mutant fusion enzymes (Table II). The lowest pH optimum was found to be about 6.2 in the case of the Q8R mutant, while the highest was about 6.8 for the T28D mutant. Dimer stabilities of the wild-type and mutant fusion enzymes have been compared by measuring their urea denaturation curves at two pH values: 6.0 and 7.2 (Table III). These values were located at the two sides of the bell-shaped curves on the activity versus pH plots. To get a more profound effect, the pH values were chosen to have the highest possible distance between them and to have measurable activity on all mutant forms of the enzyme at both pH values.

Sequence alignment of retroviral proteases (Figure 1) showed that several unusual residues can be found around the active site aspartates: the residue corresponding to His22 is Leu in all other retroviral PRs (Leu23 in HIV-1 PR) except those of foamy viruses in which aromatic residue (Phe or Tyr) also can be found. All retroviral proteases contain a hydrophobic patch around the catalytic aspartyl residues that may help to isolate the catalytic residues from the aqueous environment for maintaining the proper catalytic power and it also may contribute to the interaction energy of the dimer by providing hydrophobic contacts between the monomers. A hydrophobic cluster is formed by Leu10, Leu23, Ala27, Val90 and Ile84 residues of the ‘fireman’s grip’ in HIV-1 PR demonstrated that it is crucial for linked wild-type and T26S/T26S HIV-1 homodimers (Bagossi et al., 1995). By comparison studies, we have suggested that the proteases containing Ser at the active site triplet instead of Thr may form less stable dimers (Bagossi et al., 1996). A detailed study on the role of Ser to Thr in the avian sarcoma leukemia virus protease (S38T) substantially increased its activity (Arad et al., 1995), while the reverse mutation in HIV-1 PR (T26S) decreased the catalytic efficiency (Konvalinka et al., 1995a; Rose et al., 1995). By comparing the kinetic values obtained for the T26S mutant HIV-1 PR (Konvalinka et al., 1995a; Rose et al., 1995) and the values for linked wild-type and T26S/T26S HIV-1 homodimers (Bagossi et al., 1996), and supported by our molecular modeling studies, we have suggested that the proteases containing Ser at the active site triplet instead of Thr may form less stable dimers (Bagossi et al., 1996). A detailed study on the role of the ‘fireman’s grip’ in HIV-1 PR demonstrated that it is crucial for stabilization of the PR dimer and for overall stability of

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**Fig. 1.** Sequence comparison of N-terminal parts of retrovirus proteases. Manual alignment is given, based on sequence and structure comparison made with ClustalW and Whatif programs, respectively. The active site aspartates are shown in bold. Foamy virus proteases are shown in italics. Mutated positions are indicated by gray boxes.
**Fig. 2.** Molecular model of HFV PR. Mutated residues are shown with ball and stick representation in both monomers, but residues are labeled only in one monomer.

**Table I.** Kinetic parameters determined for the wild-type and mutant HFV proteases in fusion with MBP for substrate SRAVN*TVTQS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (nM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (nM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.41 ± 0.02</td>
<td>0.0053 ± 0.0001</td>
<td>0.013 ± 0.001</td>
</tr>
<tr>
<td>Q8R</td>
<td>1.3 ± 0.3</td>
<td>0.0061 ± 0.0008</td>
<td>0.0047 ± 0.0012</td>
</tr>
<tr>
<td>H22L</td>
<td>–</td>
<td>–</td>
<td>0.0012 ± 0.0001*</td>
</tr>
<tr>
<td>S25T</td>
<td>0.26 ± 0.04</td>
<td>0.0031 ± 0.0001</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>T28D</td>
<td>0.35 ± 0.06</td>
<td>0.0017 ± 0.0001</td>
<td>0.0049 ± 0.0009</td>
</tr>
<tr>
<td>Q8R-T28D</td>
<td>0.17 ± 0.02</td>
<td>0.0071 ± 0.0002</td>
<td>0.042 ± 0.005</td>
</tr>
<tr>
<td>H22L-T28D</td>
<td>–</td>
<td>–</td>
<td>0.0031 ± 0.0001*</td>
</tr>
</tbody>
</table>

*Due to the apparently high $K_m$ values for these enzymes the $k_{cat}/K_m$ value was calculated from measurements performed in first-order kinetic conditions.

**Table II.** $pK_1$, $pK_2$ and pH optimum values for wild-type and mutant HFV PRs determined for MBP fusion proteins using substrate SRAVN*TVTQS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$pK_1$ (±SE)</th>
<th>$pK_2$ (±SE)</th>
<th>pH optimum$^*$ (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5.61 ± 0.16</td>
<td>7.00 ± 0.15</td>
<td>6.30 ± 0.11</td>
</tr>
<tr>
<td>Q8R</td>
<td>5.71 ± 0.26</td>
<td>6.70 ± 0.24</td>
<td>6.21 ± 0.18</td>
</tr>
<tr>
<td>H22L</td>
<td>5.64 ± 0.24</td>
<td>6.83 ± 0.23</td>
<td>6.24 ± 0.17</td>
</tr>
<tr>
<td>S25T</td>
<td>5.49 ± 0.15</td>
<td>7.81 ± 0.18</td>
<td>6.65 ± 0.12</td>
</tr>
<tr>
<td>T28D</td>
<td>5.79 ± 0.14</td>
<td>7.82 ± 0.16</td>
<td>6.81 ± 0.11</td>
</tr>
<tr>
<td>Q8R-T28D</td>
<td>5.59 ± 0.15</td>
<td>7.58 ± 0.17</td>
<td>6.59 ± 0.11</td>
</tr>
<tr>
<td>H22L-T28D</td>
<td>6.01 ± 0.29</td>
<td>6.98 ± 0.29</td>
<td>6.50 ± 0.20</td>
</tr>
</tbody>
</table>

*The pH optimum of HIV-1 PR was 4.1 (Hyland et al., 1991), 4.5–5.1 (Szeltner and Polgár, 1996b), 5.5 (Darke et al., 1988), depending on the used substrate, ionic strength and other experimental conditions.

The conserved Arg8-Asp29$^*$ (Figure 5) and Arg8$^+$-Asp29 ion-pairs at the outer side of the substrate-binding site in HIV-1 PR significantly contribute to the stability of the dimer (Lapatto et al., 1989; Loeb et al., 1989; Miller et al., 1989; Wlodawer et al., 1989; Wlodawer and Erickson, 1993; Manchester et al., 1994). However the corresponding residues in HFV PR are Gln8 and Thr28, respectively, which cannot form an ion-pair. To study the role of these residues in stabilization of the enzyme structure, two single mutants as well as that the specificity constant of HFV PR did not alter when Ser25 was changed to Thr, hence the presence of Ser in the active site triplet does not appear to be an important determinant for the low catalytic efficiency of the enzyme (Boross et al., 2006). However, the dimer stability was increased by the Ser25 to Thr mutation (Table III), in good agreement with the role of this residue in dimerization. It is of interest to note that mutation of Ser to Thr in the active site triplet increased the pH optimum. Aspartyl proteases are usually active at acidic pH, since the catalytically competent enzyme has one protonated and one deprotonated Asp at the active site, and this can be typically achieved at an acidic pH. However, exceptions are also known, for example renin is active in the physiological pH (Inagami et al., 1984). The increased pH optimum might be a consequence of increased dimer stability. The dimerization of HIV-1 PR is strongly dependent on the pH, forming less stable dimers at higher pH (Darke et al., 1994; Szeltner and Polgár, 1996a). The reverse mutation in HIV-1 PR (T26S) resulted in a lower pH optimum, as compared with that of the wild-type enzyme (Konvalinka et al., 1995a; Strisovsky et al., 2000), and the T26S mutant retained substantially smaller activity at pH 6.8 than the wild-type enzyme, in comparison with their activity at pH 4.7 (Strisovsky et al., 2000). While the pH optimum of Ser-containing wild-type HFV PR was also lower than the pH optimum of Thr-containing S25T mutant, the urea stability of S25T mutant increased at higher pH.
Q8R-T28D double mutant were constructed. The T28D and the Q8R single mutants showed higher stability against urea than the wild-type enzyme (Table III). We expected only marginal effect in the case of single mutants, when only the half of the ion-pair was regenerated, and a more pronounced effect was expected in the case of the double mutant. However, the T28D mutant was even more stable than the double mutant Q8R-T28D enzyme, which suggests that other structural features may also play a role. The stability values of these enzymes were separated into two groups: low stability group consisted of the Q8R mutant and the wild-type enzymes, while T28D and Q8R-T28D mutants had higher $D_{1/2}$ values at both pH values.

The stability of enzymes correlated with the hydrogen-bond forming capability of these residue pairs. Only one hydrogen bond can be formed between Gln-Thr and Arg-Thr residue pairs in contrast to Gln-Asp and Arg-Asp pairs, where two hydrogen bonds can be formed. It is also possible that the HFV PR structure significantly deviates from the known retroviral protease fold, which may cause, at least partially, the unusual features of the HFV PR. Recently, the crystal structure of human T-cell leukemia virus type 1 (HTLV-1) protease was solved and showed an example for unexpected structural variations (Li et al., 2005). However, specific structural features of HFV PR remain unpredictable until an experimental structure of a protease belonging to foamy virus family will be solved.

Interestingly, T28D mutant has the highest pH optimum among the studied mutant forms of HFV PR (Table II).

Our results showed that several features of HFV PR were substantially different from those of other retroviral proteases. The pH optimum of wild-type HFV PR was higher than that of HIV-1 PR, but the wild-type sequence was not optimized for that: all mutants had the same or higher pH optimum than the wild-type HFV PR. Interestingly, the studied mutants showed the same or higher stability against urea at both pH values, suggesting that during evolution HFV PR did not evolve to maximize the dimerization energy, as compared with HIV-1.

Table III. Stability parameters for wild-type and mutant HFV PRs determined using MBP fusion proteins

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH = 6.0</th>
<th></th>
<th></th>
<th>pH = 7.2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta G^0$ ($\pm$ SE) (kJ/mol)</td>
<td>$m$ ($\pm$ SE) (kJ/mol/M)</td>
<td>$D_{1/2}$ ($\pm$ SE) (M)</td>
<td>$\Delta G^0$ ($\pm$ SE) (kJ/mol)</td>
<td>$m$ ($\pm$ SE) (kJ/mol/M)</td>
<td>$D_{1/2}$ ($\pm$ SE) (M)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>-6.48 ± 0.66</td>
<td>-11.88 ± 1.10</td>
<td>0.55 ± 0.07</td>
<td>-5.58 ± 0.92</td>
<td>-9.75 ± 1.43</td>
<td>0.57 ± 0.13</td>
</tr>
<tr>
<td>Q8R</td>
<td>-5.82 ± 0.85</td>
<td>-9.60 ± 1.25</td>
<td>0.61 ± 0.12</td>
<td>-6.09 ± 1.05</td>
<td>-8.32 ± 1.30</td>
<td>0.73 ± 0.17</td>
</tr>
<tr>
<td>H22L</td>
<td>-5.33 ± 0.80</td>
<td>-7.76 ± 1.02</td>
<td>0.69 ± 0.14</td>
<td>-7.29 ± 0.88</td>
<td>-9.26 ± 1.04</td>
<td>0.79 ± 0.13</td>
</tr>
<tr>
<td>S25T</td>
<td>-8.31 ± 0.80</td>
<td>-10.00 ± 0.92</td>
<td>0.83 ± 0.11</td>
<td>-7.61 ± 0.51</td>
<td>-6.63 ± 0.44</td>
<td>1.15 ± 0.11</td>
</tr>
<tr>
<td>T28D</td>
<td>-6.74 ± 0.64</td>
<td>-7.86 ± 0.70</td>
<td>0.86 ± 0.11</td>
<td>-5.80 ± 0.58</td>
<td>-6.29 ± 0.59</td>
<td>0.92 ± 0.13</td>
</tr>
<tr>
<td>Q8R-T28D</td>
<td>-7.47 ± 1.14</td>
<td>-9.31 ± 1.33</td>
<td>0.80 ± 0.17</td>
<td>-7.55 ± 0.70</td>
<td>-8.79 ± 0.77</td>
<td>0.86 ± 0.11</td>
</tr>
<tr>
<td>H22L-T28D</td>
<td>-5.50 ± 0.76</td>
<td>-7.38 ± 0.91</td>
<td>0.75 ± 0.14</td>
<td>-6.55 ± 0.83</td>
<td>-8.03 ± 0.95</td>
<td>0.82 ± 0.14</td>
</tr>
</tbody>
</table>

$D_{1/2}$ value for HIV-1 PR: 1.85 M (Wondrak et al., 1996).

Fig. 3. Hydrophobic residues being around the catalytic aspartates in the crystal structure of HIV-1 PR. Catalytic aspartates are shown with ball and stick representation, and hydrophobic residues around them are shown as lines (enzyme) or tubes (inhibitor). Residues of the second monomer are labeled by primed numbers.
PR. While the wild-type HFV PR had the same sensitivity against urea at both pH values, mutant enzymes showed higher sensitivity against urea at pH 6.0 than at pH 7.2. The overall results of these mutational studies suggest that requirements of HFV PR structure may differ from that of other retroviral protease structures, in response to a different selective pressure caused by the different life cycle of foamy viruses. Unlike conventional retroviruses, the majority of mature, enveloped, infectious foamy virus particles remain in the endoplasmic reticulum and only ~5% can be found in the cell culture supernatant (Yu and Linial, 1993; Linial and Eastman, 2003). Since it was shown that the ProPol polyprotein is not efficiently cleaved between PR and RT (Flügel and Pfrepper, 2003), it is also possible that C-terminal flanking sequences, including the reverse transcriptase of Pol polyprotein, may modify the features of HFV PR, for example by providing additional dimerization interfaces, which are not provided after maturation of other viral proteases.

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