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# Comparison of the HIV-1 and HIV-2 proteinases using oligopeptide substrates representing cleavage sites in Gag and Gag-Pol polyproteins

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The substrate specificity of the human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) proteinases was compared using oligopeptides corresponding to cleavage sites in the Gag and Gag-Pol polyproteins of both viruses. All peptides mimicking cleavage sites at the junction of major functional protein domains were correctly cleaved by both enzymes. However, some other peptides thought to represent secondary cleavage sites remained intact. The kinetic parameters ($K_m$ and $k_{cat}$) obtained for the different substrates showed several hundred-fold variation but were similar for the same substrate.

HIV-1 proteinase; HIV-2 proteinase; Oligopeptide substrate; Enzyme kinetics; Substrate specificity

## 1. INTRODUCTION

The retroviral proteinase (PR) is responsible for specific cleavages of the Gag and Gag-Pol polyproteins. Because of its crucial role in virus maturation and infectivity, the PR of both HIV-1 and HIV-2 is a potential target for chemotherapy of virus infection and associated diseases [1].

The characterization of retroviral cleavage sites requires the isolation of the viral protein products, the determination of their N- and C-terminal sequences, and comparison of these sequences to the nucleotide sequence, as has been done in our laboratory for a number of retroviruses [2] including HIV-1 [3] and simian immunodeficiency virus ($SIV_{Mne}$) [4]. Similar studies were not done to determine the cleavage sites in the HIV-2 Gag and Gag-Pol polyproteins, they were predicted on the basis of homology with $SIV_{Mne}$ and HIV-1 [4–6]. Twelve naturally occurring cleavage sites have been determined in HIV-1 Gag and Gag-Pol polyproteins [3,5–9]. A series of selected oligopeptides representing cleavage sites were tested and found to be cleaved by HIV-1 PR [10]. Similar extensive studies have not been performed for HIV-2 cleavage sites.

Our aim was to investigate the substrate specificity of HIV-1 and HIV-2 PRs by the determination of the kinetic parameters for the cleavage of oligopeptides representing all determined and/or predicted cleavage sites of the HIV-1 and HIV-2 Gag and Gag-Pol precursor polyproteins. Kinetic characterization of the PR is important for understanding its biology and is useful for inhibitor design.

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## 2. MATERIALS AND METHODS

Recombinant HIV-1 PR [11] and chemically synthesized HIV-2 PR [6,12] were used. Active enzyme content was determined by active site titration using a potent transition-state analog inhibitor [13]. Oligopeptides having amide C terminus were synthesized and characterized by amino acid analysis and sequencing as described [6]. Peptides containing the $\Psi[CH_2NH]$ pseudopeptide bond were synthesized according to Sasaki et al. [14].

Proteinase assays were performed in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 5 mM dithiothreitol, 1 mM EDTA, 0.2% Nonidet P-40 and 2 M NaCl. The reaction mixture was incubated at 37°C for 1 h and stopped by the addition of guanidine-HCl (6 M final concentration). The cleavage products were detected by reversed-phase HPLC [6], the product peaks were integrated, and the kinetic parameters were determined at less than 20% substrate turnover by fitting the data to the Michaelis-Menten equation using the Gauss-Newton iteration method. The computer program (Enz 5.0) was written and kindly provided by M. Fivash and J. Racheff of the Data Management Services, Inc., NCI-FCRDC, Frederick. The asymptotic standard errors were 5–20%. For two peptides the proper $K_m$ value could not be determined, for these peptides the $k_{cat}/K_m$ values were determined using competition assays [15]. Inhibitor constants were determined according to Dixon [16]. The estimated error of all determinations was below 20%.

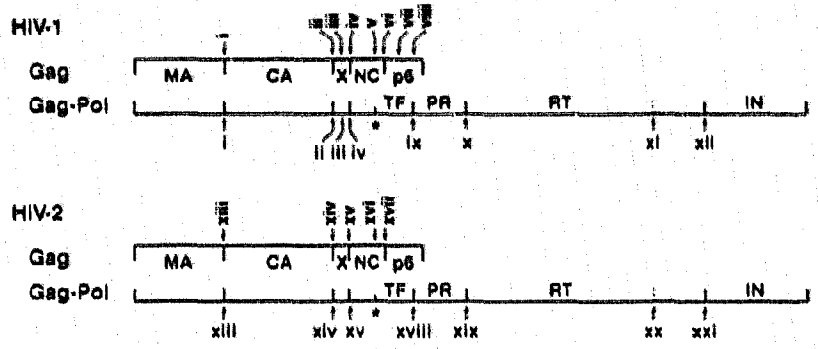


Fig. 1. Location of the cleavage sites determined in HIV-1 [3,5–9] and predicted in HIV-2 [4–6] Gag and Gag-Pol precursor polyproteins. Abbreviations: MA, matrix protein; CA, capsid protein; X, peptide between MA and CA; NC, nucleocapsid protein; TF, transframe protein; PR, retroviral proteinase; RT, reverse transcriptase; IN, integrase (nomenclature from [27]). The site of frameshift as determined for HIV-1 [28] and predicted for HIV-2 based on homology is marked with an asterisk. Numbering of the cleavage sites corresponds to the peptides synthesized as shown in Tables I and II.

## 3. RESULTS AND DISCUSSION

Oligopeptides representing determined cleavage sites in HIV-1 Gag and Gag-Pol polyproteins and predicted cleavage sites in HIV-2 Gag and Gag-Pol polyproteins (Fig. 1) were tested as substrates for both HIV-1 and HIV-2 PRs (Tables I and II). All of the oligopeptides representing cleavage sites at the junction of major functional protein domains (see Fig. 1) of HIV-1 (Table I) as well as of HIV-2 (Table II) were cleaved at the predicted sites in both homologous and heterologous systems. Peptides representing cleavage sites in p6 of HIV-1 (peptide viii) and in RT of both viruses (peptides xi and xx) were also found to be hydrolyzed by both enzymes. The specificity of the cleavage of oligopeptide substrates representing the major cleavage sites in HIV-2 polyproteins by the purified enzymes further suggests that they were predicted correctly. However, peptides mimicking another cleavage site in p6 and a cleavage site in X of HIV-1 as well as in the NC domain of both viruses (peptides vii, iii, v and xvi respectively, see Fig. 1, and Tables I and II) were not hydrolyzed by either of the enzymes even after 24-h incubation at 37°C. These noncleaved peptides contain a small, non-hydrophobic residue at either the $P_1$ or $P_1'$ position (notation according to Schechter and Berger, [17]). If these cleavages in the Gag polyproteins are performed by the viral enzyme, additional sequential and/or conformational factors may be necessary for this processing. The presence of hydrophobic side chains at $P_1$ and $P_1'$ positions are known to be preferred for efficient proteolytic activity of HIV PRs.

Table I

Kinetic parameters obtained for HIV-1 and HIV-2 PR using oligopeptides representing the cleavage sites in HIV-1 Gag and Gag-Pol polyproteins

| Peptide | Location of cleavage | Sequence | HIV-1 PR | | | HIV-2 PR | | |
|---|---|---|---|---|---|---|---|---|
| | | | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) |
| i. | MA/CA | VSQNY○PIVQ[a] | 0.15 | 6.8 | 45.3 | 0.18 | 6.2 | 34.4 |
| ii. | CA/X | KARVL○AEAMS | 0.01 | 0.09 | 90.0 | 0.03 | 0.9 | 30.0 |
| iii. | in X | VLAEAM●SQVTN[b] | | not hydrolyzed | | | not hydrolyzed | |
| iv. | X/NC | TATIM○MQRGN | 0.05 | 3.7 | 74.0 | 0.08 | 3.5 | 43.8 |
| v. | in NC | TERQAN●FLGKI | | not hydrolyzed | | | not hydrolyzed | |
| vi. | NC/p6 | RPQNF○LQSRP[c] | 0.53 | 0.3 | 0.6 | 0.30 | 0.2 | 0.7 |
| vii. | in p6 | ESFRSG●VETTT | | not hydrolyzed | | | not hydrolyzed | |
| viii. | in p6 | DKELY○PLTSL | 0.47 | 0.01 | 0.02 | 0.42 | 0.09 | 0.21 |
| ix. | TF/PR | VSFNF○PQITL | <0.01 | 0.06 | 6.9[d] | <0.01 | 0.06 | 6.2[d] |
| x. | PR/RT | CTLNF○PISP | 0.07 | 1.5 | 24.1 | 0.06 | 0.4 | 6.7 |
| xi. | in RT | AETF○YVDGAA | 0.04 | 0.4 | 10.0 | 0.17 | 1.0 | 5.8 |
| xii. | RT/IN | IRKIL○FLDG | 0.006 | 1.2 | 202.0 | 0.005 | 1.2 | 240.0 |

[a] Cleavage sites proved by amino acid analysis are marked with open circles in the substrate sequences

[b] These peptides were subjected to a 24-h incubation with the enzymes. Cleavage sites found by sequence analysis but not hydrolyzed in the oligopeptide are marked with solid circles

[c] Sequence from protein sequencing [3]. Deducted from the nucleotide sequence [29], there is Gly at the $P_3$ position. RPGNF○LQSRP was also investigated, and the kinetic parameters for its hydrolysis were found to be very similar to that of peptide vi

[d] Measured with competition assay [15] with the MA/CA cleavage site (peptide i)

Table II

Kinetic parameters obtained for HIV-1 and HIV-2 PR using oligopeptides representing the predicted cleavage sites in Gag and Gag-Pol precursor polyproteins of HIV-2

| Peptide | Location of cleavage | Sequence | HIV-1 PR $K_m$ (mM) | HIV-1 PR $k_{cat}$ ($s^{-1}$) | HIV-1 PR $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) | HIV-2 PR $K_m$ (mM) | HIV-2 PR $k_{cat}$ ($s^{-1}$) | HIV-2 PR $k_{cat}/K_m$ ($mM^{-1}\cdot s^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| xiii. | MA/CA | EKGGNY PVQHV[a] | 0.60 | 2.4 | 4.0 | 0.55 | 2.0 | 3.7 |
| xiv. | CA/X | KARLM AEALK | 0.13 | 0.3 | 2.3 | 0.08 | 1.2 | 15.0 |
| xv. | X/NC | IPFAA AQQRK | 0.28 | 0.3 | 1.1 | 0.07 | 0.8 | 11.4 |
| xvi. | in NC | DRQAG•FLGLG[b] | | not hydrolyzed | | | not hydrolyzed | |
| xvii. | NC/p6 | KPRNF PVAQV | 1.25 | 0.8 | 0.6 | 0.70 | 0.7 | 1.0 |
| xviii. | TF/PR | RGLAA PQFSL | 0.13 | 1.9 | 14.6 | 0.07 | 2.6 | 37.1 |
| xix. | PR/RT | MSLNL PVAKV | 0.02 | 2.2 | 110.0 | 0.02 | 1.2 | 60.0 |
| xx. | in RT | GAETF YTDGS | | not determined[c] | | | not determined[c] | |
| xxi. | RT/IN | IRQVL FLEKI | <0.01 | 0.3 | 58.0[d] | <0.01 | 0.3 | 33.0[d] |

[a] Cleavage sites are marked as in Table I
[b] This peptide was subjected to a 24-h incubation with the enzymes
[c] A small amount of the substrate was hydrolyzed at the indicated place after a 16-h incubation with the enzymes
[d] Measured with competition assay [15] with the TF/PR cleavage site (peptide xviii)

Kinetic parameters for the substrates were determined using both HIV-1 and HIV-2 PR at high (2 M) salt concentration (Tables I and II). As for the avian enzyme [18], this high ionic strength was found to be optimal for HIV PRs (our unpublished result). The $K_m$ values were found to be in the micromolar range (5–1250 $\mu$M) showing an approximate 300-fold variation, while the $k_{cat}$ values differed at least 600-fold (0.01–6.8 $s^{-1}$) considering both the homologous and heterologous systems. The kinetic parameters for the two PRs showed remarkable similarities. The kinetic parameters for the hydrolysis of some oligopeptides were found to be similar also by others [19,20]. Furthermore, the interchange of the PR domain of the *pol* genes of HIV-1 and HIV-2 resulted in complete, though less efficient, autocatalysis of the chimeric polyproteins in an *E. coli* expression system [5]. The HIV-1 and HIV-2 PRs show a 70% homology [6]. Comparing the side chains of HIV-1 PR involved in the enzyme–substrate interaction in the $S_3$–$S_3'$ positions [21] to the respective side chains in HIV-2 PR, one finds only conservative changes (two Val–Ile exchanges and an Ile–Val exchange). The strong homology in the $S_3$–$S_3'$ region may explain the similar kinetic constants obtained for both enzymes. However, some differences were also found, regarding both the $K_m$ and the $k_{cat}$ values (Tables I and II). Recently Tomasselli et al. [20] have reported, that while a peptide with X/NC cleavage site sequence ($P_5$–$P_4'$) of HIV-2 was hydrolyzed only by HIV-2 PR, another peptide with NC/p6 sequence ($P_5$–$P_3'$) of HIV-2 was not hydrolyzed by either of the enzymes. On the contrary, the related but longer peptides ($P_5$–$P_5'$) in our series were hydrolyzed by both enzymes (see Table II). The apparent discrepancies may be due to the difference in the lengths of the peptides and/or to different assay conditions.

A classification of the cleavage sites found in HIV-1 and SIV as well as predicted for HIV-2 Gag and Gag-Pol polyproteins has been published from our laboratory, based on the scissile bonds and the surrounding sequences [4]. While some of the peptides representing Class 2 or Class 3 cleavage sites were not hydrolyzed, all of the peptides representing Class 1 cleavage sites were hydrolyzed by HIV PRs. However, a strong relationship between the classification and the kinetic data of the substrate hydrolysis was not found. For example, the two peptides, one with the highest and the other with the lowest catalytic constant in the HIV-1 homologous series (peptide i and viii respectively) belong to Class 1, characterized by the Tyr(Phe)/Pro scissile bond. Furthermore, the kinetic parameters for peptide ix and x are also substantially different, although both have an -Asn-Phe/Pro- sequence at the cleavage site. These findings suggest, that amino acids relatively far from the cleavage site may significantly contribute to the binding and activation energies, as was found by substitution of amino acids at $P_4$–$P_2$ positions [19,22].

Two substrates (ix and x in Table I) containing the highly specific Phe/Pro cleavage site were selected to synthesize their pseudopeptide analog containing a $\Psi[CH_2NH]$ bond. The analogs were found to be competitive inhibitors of both HIV PRs (Table III). Their

Table III

Inhibitory constants obtained by peptides containing reduced bond

| Peptide | $K_i$ ($\mu$M)[a] | |
|---|---|---|
| | HIV-1 PR | HIV-2 PR |
| $\Psi$-ix VSFNF($\Psi$)PQITL | 0.030 | 0.025 |
| $\Psi$-x CTLNF($\Psi$)PISP | 0.7 | 0.6 |

[a] Using peptide i as substrate, the $K_i$ value was determined according to Dixon [16], in the presence of 2 M NaCl. Both inhibitions were of the competitive type

$K_i$ values are related to the $K_m$ values of the respective substrates, and very similar for the two enzymes. Potent inhibitors of the HIV-1 PR were published recently [13,23–25]. A few of them were also tested and found to be potent on HIV-2 PR [24]. However, dramatic differences in potency of some renin inhibitors toward the HIV PRs have been reported [20]. Since both viruses are pathogenic in humans [26], a proteinase inhibitor ideally should be effective against both.

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