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Kinetic and Modeling Studies of S_3–S_3' Subsites of HIV Proteinases†

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ABSTRACT: Kinetic analysis and modeling studies of HIV-1 and HIV-2 proteinases were carried out using the oligopeptide substrate

$$\begin{matrix} P_5 & P_4 & P_3 & P_2 & P_1 & P_1' & P_2' & P_3' & P_4' \\ \text{H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH}_2 \end{matrix}$$

and its analogs containing single amino acid substitutions in P_3–P_3' positions. The two proteinases acted similarly on the substrates except those having certain hydrophobic amino acids at P_2, P_1, P_2', and P_3' positions (Ala, Leu, Met, Phe). Various amino acids seemed to be acceptable at P_3 and P_3' positions, while the P_2 and P_2' positions seemed to be more restrictive. Polar uncharged residues resulted in relatively good binding at P_3 and P_2 positions, while at P_2' and P_3' positions they gave very high K_m values, indicating substantial differences in the respective S and S′ subsites of the enzyme. Lys prevented substrate hydrolysis at any of the P_2–P_2' positions. The large differences for subsite preference at P_2 and P_2' positions seem to be at least partially due to the different internal interactions of P_2 residue with P_1', and P_2' residue with P_1. As expected on the basis of amino acid frequency in the naturally occurring cleavage sites, hydrophobic residues at P_1 position resulted in cleavable peptides, while polar and β-branched amino acids prevented hydrolysis. On the other hand, changing the P_1' Pro to other amino acids prevented substrate hydrolysis, even if the substituted amino acid had produced a good substrate in other oligopeptides representing naturally occurring cleavage sites. The results suggest that the subsite specificity of the HIV proteinases may strongly depend on the sequence context of the substrate.

All replication competent retroviruses, including human immunodeficiency viruses, code for an aspartic proteinase [for review, see Hellen et al. (1989); Oroszlan & Luftig, 1990; Blundell et al., 1990; Debouck & Metcalf, 1990]. The major role of the retroviral proteinase (PR)[1] is the processing of the Gag and Gag–Pol polyprotein precursors. This processing is essential for the conversion of the immature noninfectious viral particles to mature infectious viruses (Crawford & Goff, 1985; Katoh et al., 1985; Kohl et al., 1988). Recent data suggest that the PR may also function in the early phase of viral infection by fragmenting the nucleocapsid protein (Roberts & Oroszlan, 1989; Roberts et al., 1991; Baboonian et al., 1991). The HIV-1 PR was also shown to be able to cleave cellular proteins like vimentin (Shoeman et al., 1990) and transcription factor NF-κB (Riviere et al., 1991). Furthermore, the cytoplasmic activation of the HIV PR of lytically

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[1] Abbreviations: PR, retroviral proteinase; HIV, human immunodeficiency virus; SDS, sodium dodecyl sulfate, RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB, fast atom bombardment.

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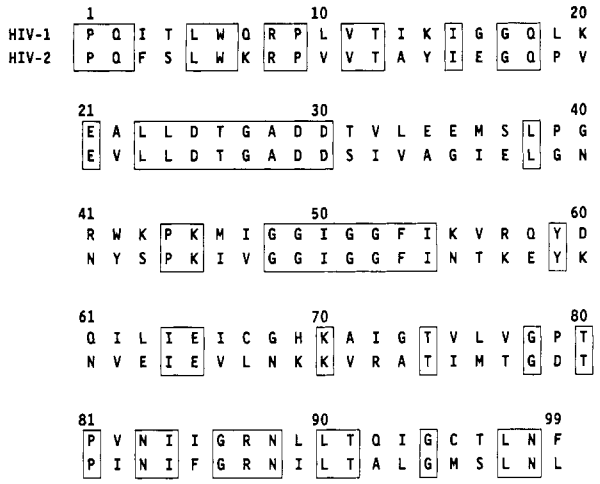

FIGURE 1: Amino acid sequence alignment of HIV-1_{IIIB} and HIV-2_{ROD} PRs [modified from Copeland and Oroszlan (1988)]. Identical amino acids are boxed. The code for amino acids is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

infected cells might contribute to cytotoxicity (Kaplan & Swanstrom, 1991). Due to its crucial role in the viral life cycle, the PR of human immunodeficiency viruses is a potential target for chemotherapy (Kräusslich et al., 1989). PR inhibitors as antiviral agents should ideally be effective against both type-1 (HIV-1) and type-2 (HIV-2) viruses since both are pathogenic in humans (Gallo & Montagnier, 1988).

The primary and three-dimensional structures of HIV-1 and HIV-2 proteinases are highly conserved (Copeland & Oroszlan, 1988; Gustchina & Weber, 1991). Their amino acid sequences are aligned in Figure 1. The substrate specificity of both proteinases has also been intensively studied, since it may help inhibitor design [for reviews, see Kay and Dunn (1990), Oroszlan and Tözsér (1990), and Tomasselli et al. (1991, and references therein)]. In a previous study we found that 17 out of 21 substrates representing naturally occurring cleavage sites in HIV-1 and HIV-2 were hydrolyzed by both proteinases, with usually similar kinetic parameters (Tözsér et al., 1991a). An investigation of the S_4 subsite [notation according to Schechter and Berger (1967)] also revealed that the two enzymes are rather similar (Tözsér et al., 1991b). Here we report kinetic and modeling investigations on the S_3–S_3' binding pockets of HIV-1 and HIV-2 proteinases.

Materials and Methods

HIV Proteinases. HIV-2 PR was chemically synthesized using solid-phase peptide synthesis (Copeland & Oroszlan, 1988). The protein was purified in the following way: the crude polypeptide was dissolved in 6 M guanidine hydrochloride and 100 mM Tris-HCl, pH 8.0, and gel-filtered on a Sephadex G-50 or Superdex G-75 HR 16/60 column (Pharmacia-LKB Biotechnology) equilibrated with the same buffer. Fractions showing the highest specific activity after refolding by quick dilution (Copeland & Oroszlan, 1988) were acidified to pH 2 by the addition of TFA and purified using RP-HPLC on a 19 × 150 mm μ-Bondapack C_{18} column (Waters Associates, Inc.) with a linear gradient (0–60%) of acetonitrile in the presence of 0.05% TFA. Fractions of the RP-HPLC run were lyophilized, dissolved in 2 M guanidine hydrochloride and 100 mM Tris-HCl, pH 8.0, to a final concentration of 200–300 μg/mL, and refolded as follows: 1 vol was diluted with 2 vol of refolding buffer (20 mM Pipes, pH 7.0, containing 100 mM NaCl, 10% glycerol, 1 mM EDTA). The fractions were dialyzed against the refolding buffer for 4 h at 4 °C. The dialyzed fractions having the highest specific activity were gel-filtered on a Superose-12 HR 10/30 column and concentrated using an Amicon-3 concentrator. The purified and refolded proteinase migrated as a single 11-kDa band on SDS–polyacrylamide gel electrophoresis. The proper sequence of the synthesized proteinase was ensured by amino acid analysis of the full-length proteinase, and of its fragments removed during the synthesis, as well as by Edman degradation performed with a model 470A gas-phase sequencer (Applied Biosystems, Inc.).

HIV-1 PR was expressed as a fusion protein and purified as it was described (Louis et al., 1991). Active-site titration of the enzymes was performed using a peptide analog inhibitor (compound 3 in Grobleny et al., 1990).

Oligopeptide Synthesis and Characterization. Oligopeptides were synthesized by solid-phase peptide synthesis on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or a semiautomatic Vega peptide synthesizer (Vega-Fox Biochemicals) using *tert*-butoxycarbonyl chemistry and purified by RP-HPLC. Some of the peptides were synthesized by fragment condensation as described elsewhere (Bláha et al., 1991). Amino acid composition of the peptides was determined by amino acid analysis on either a Durrum D-500 or a Waters Pico-Tag analyzer. Stock solutions and dilutions were made in distilled water (for peptides containing Cys, 0.5 mM dithiothreitol was added), and the proper peptide concentration was determined by amino acid analysis. The nonhydrolyzed peptides having P_1' substitutions were also characterized by chymotryptic digestion as it was described (Bláha et al., 1991), and all nonhydrolyzable peptides were analyzed by FAB mass spectrometry.

Protease Assay. The assays were performed in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 1 mM EDTA, 0.2% Nonidet P-40, and 2 M NaCl. The reaction mixture was incubated at 37 °C for 1 h or for 24 h if indicated, and the reaction was stopped by the addition of guanidine hydrochloride (6 M final concentration). The solution was acidified by the addition of TFA, and an aliquot was injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 × 150 mm) using a WISP 710A automatic injector (both from Waters Associates, Inc.). Substrates and the cleavage products were separated using an increasing water–acetonitrile gradient (0–100%) in the presence of 0.05% TFA. The cleavage of peptides was monitored at 206 nm, and the peak areas were integrated using a Waters 745B data module. Amino acid analysis of collected peaks was used to confirm the cleavage sites in the substrates. Relative activities were determined at 0.4 mM substrate concentration by comparing the activity of each analog to that obtained with the unmodified substrate H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (SP-211) as previously described for the P_2' modified peptides (Bláha et al., 1991). Kinetic parameters were determined by using HIV-2 PR 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 Data Management Services Inc., NCI-FCRDC, Frederick, MD. The asymptotic standard errors were 5–20%. Kinetic parameters were also determined for HIV-1 PR, if the relative activities were substantially different from that of HIV-2 PR. However, with HIV-1 PR, the assay solution always contained 5 mM dithiothreitol.

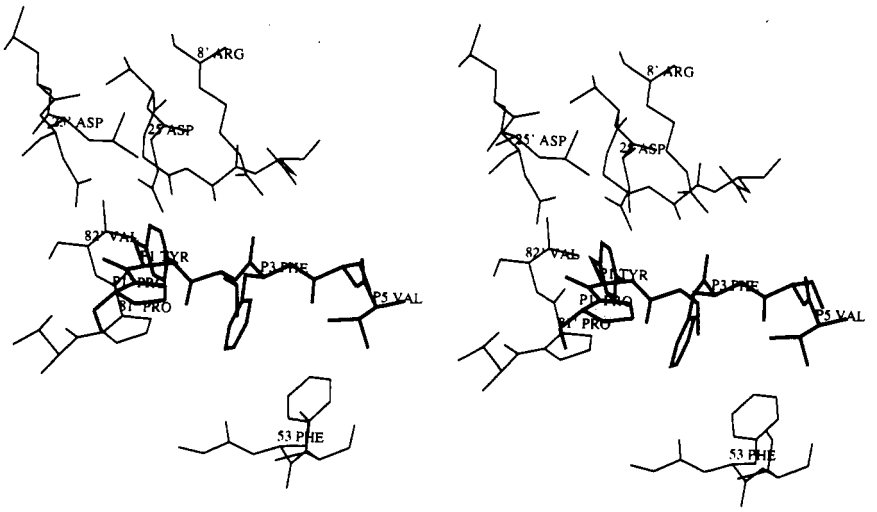

FIGURE 2: Stereoview of the model of the substrate (in thick lines) bound in the active site of HIV-1 PR (in thin lines). Residues at the positions P_5–P_1' with a sequence of Val-Ser-Phe-Asn-Tyr-Pro- are shown. Phe at P_3 makes hydrophobic contacts with the residues Pro_{81}' and Phe_{53}. Val at P_5 also contributes to these interactions. The model of HIV-2 PR is identical except residue 82 where it has Ile instead of Val.

Molecular Modeling. Atomic coordinates from the crystal structure of HIV-1 PR with different inhibitors (Miller et al., 1989; Swain et al., 1990; Jaskolski et al., 1991) were examined on an Evans Sutherland PS390 molecular graphics system using the program FRODO (Jones, 1978). The HIV-2 PR coordinates were obtained by molecular modeling (Gustchina & Weber, 1991). The amino acid side chains and their rotational flexibility at P_3–P_3' positions were examined as described for P_4 position (Tözsér et al., 1991b).

Results and Discussion

The nonapeptide, H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (SP-211, the asterisk indicates the scissile bond), representing the naturally occurring cleavage site between the matrix and capsid proteins of HIV-1 was found to be a good substrate for both HIV-1 and HIV-2 proteinases (Copeland & Oroszlan, 1988; Copeland et al., 1990). Its analogs containing single amino acid substitutions at the P_4 subsite were assayed previously as substrates of HIV proteinases (Tözsér et al., 1991b). In this study, analogs containing amino acid substitutions in P_3–P_3' positions were assayed at 0.4 mM substrate concentration with both HIV-1 and HIV-2 proteinases, and relative activities compared to that obtained with SP-211 were determined (not shown), as published for the P_2' modified peptides (Bláha et al., 1991). Kinetic parameters were determined by using HIV-2 PR. If the relative activity obtained with HIV-1 PR was significantly different from that obtained with HIV-2 PR, the kinetic parameters were also determined for this enzyme. All amino acid changes were modeled using the atomic coordinates of the model of HIV-1 PR–substrate complexes. This model was built by using the atomic coordinates of HIV-1 PR–inhibitor complexes (Miller et al., 1989; Swain et al., 1990; Jaskolski et al., 1991) and a predicted HIV-2 PR structure (Gustchina & Weber, 1991). The 17 amino acid residues that form each subsite in HIV-1 and HIV-2 PRs are listed in Table I. Most of these residues (76.5%) are identical in the two PRs. Based on the molecular model of HIV-2 PR, only four residues involved in substrate binding show differences between HIV-1 and HIV-2 PRs: Val_{32} to Ile and Leu_{76} to Met (part of subsites S_2 and S_2'), Ile_{47} to Val (part of S_4 and S_2), and Val_{82} to Ile (S_1') (Gustchina & Weber, 1991). The HIV proteinases are homodimers with C_2 symmetry (Navia et al., 1989; Wlodawer et al., 1989; Lapatto et al., 1989). Although the precise symmetry is broken in the enzyme–inhibitor complexes (Miller et al., 1989; Swain et al., 1990; Fitzgerald et al., 1990; Jaskolski et al., 1991), the respective S and S′ subsites are expected to be similar (S_3 to S_3' etc.), therefore a detailed analysis of the enzyme specificity will be carried out by comparing the respective subsites.

Table I: Residues Forming the Subsites of HIV-1 and HIV-2 PRs[a]

subsite	HIV residues
S3	Arg 8′, Asp 29, Gly 48, Phe 53, Pro 81′, Val/Ile 82′
S2	Ala 28, Asp 29, Asp 30, Val/Ile 32, Ile/Val 47, Gly 48, Gly 49, Ile 50′, Leu/Met 76, Ile 84
S1	Arg 8′, Leu 23′, Asp 25′, Asp 25, Gly 27, Gly 49, Ile 50′, Thr 80′, Pro 81′, Val/Ile 82′, Ile 84′
S1′	Arg 8, Leu 23, Asp 25, Asp 25′, Gly 27′, Gly 49′, Ile 50, Thr 80, Pro 81, Val/Ile 82, Ile 84
S2′	Ala 28′, Asp 29′, Asp 30′, Val/Ile 32′, Ile/Val 47′, Gly 48′, Gly 49′, Ile 50, Leu/Met 76′, Ile 84′
S3′	Arg 8, Asp 29′, Gly 48′, Phe 53′, Pro 81, Val/Ile 82

[a] Amino acid residues in the second subunit of the dimer are indicated by a prime. The residues that differ in the two PRs are indicated as HIV-1 residue/HIV-2 residue.

S_3 Subsite. The results obtained with peptides in which the P_3 Gln of SP-211 was substituted with other amino acids are presented in Table II (series A) for HIV-2 PR, and as an example, the putative binding of the peptide containing Phe at P_3 is shown in Figure 2. The S_3 binding pocket of HIV-2 PR is close to the surface. The structural model suggested that a variety of amino acids may fit into this pocket, as into the HIV-1 PR (Weber et al., 1989). In accordance with this prediction, all substituted peptides were sufficiently hydrolyzed by the HIV-2 PR, although the individual parameters varied substantially (Table II, series A). The best K_m values were obtained with large hydrophobic residues, suggesting that the S_3 pocket is rather large and the binding is mainly hydrophobic, similar to the other subsites. Phe_{53} and Pro_{81} are predicted to provide hydrophobic interactions (Figure 2). Although the Lys-containing peptide had one of the best k_{cat} values, its K_m value was high (Table II). This might be due to the electrostatic repulsion of Arg_8' or simply to solvent effects. The NH group of P_3 is not involved in a main-chain hydrogen bond interaction, although it may coordinate to the COO^- side chain of Asp_{29}. On the other hand, the CO group of P_3 may form a hydrogen bond with NH of Asp_{29}. The side chain of Asp_{29} points in the same direction as P_3, which suggests that repulsion with Asp side chain may result in a high K_m of that substrate (Table II). The good catalytic constants for the Gln-containing peptide may be due to the hydrogen

Table II: Kinetic Parameters Determined with HIV-2 PR for Substrates Having Substitutions in the P_3 Position of H-Val-Ser-Xaa-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (SP-211; series A) and H-Xaa-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (SP-2013; series B)

	Series A			Series B		
P_3 amino acid	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Gln[a]	0.18	6.2	34.4[b]	1.0	1.1	1.1[b]
Leu	<0.05[c]	2.1	90[d]	0.5	0.2	0.4[e]
Phe	<0.05[c]	2.1	111[d]	0.3	0.2	0.7
Val	0.14	4.0	28.6	1.2	0.7	0.6
Ala	0.34	7.7	22.6	1.8	3.3	1.8
Asp	1.1	2.5	2.3	ND[f]	0.8	1.2[d]
Asn	0.11	2.1	21.2	1.0	1.9	1.9
Lys	0.9	7.2	8.0	ND	ND	0.5[d]
Gly	0.09	3.0	33.3	0.8	6.1	7.6

[a] Corresponds to the original substrates (SP-211 and SP-2013, respectively) representing the cleavage site between the matrix and capsid proteins in HIV-1. [b] These values were reported previously (Tözsér et al., 1991b). [c] Increasing the substrate concentration well above the apparent K_m value, an inhibitory effect was observed. [d] Determined with competition assay, using the Gly-containing hexapeptide substrate. [e] This value was determined also with competition assay and found to be the same. [f] ND, not determined.

Table III. Kinetic Parameters Determined with HIV Proteinases for Substrates Having Substitutions in P_3' Position of H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Xaa-Gln-NH_2[a]

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	
P_3' amino acid	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR
Leu	0.23	0.19	12.3	22.8	53.5	120.0
Phe	0.45	0.35	13.8	21.4	30.6	61.1
Ala	1.8		6.6		3.7	
Asp	7.4		3.6		0.5	
Glu	3.6		4.7		1.3	
Gln	2.3		13.9		6.0	
Arg	2.2		11.4		5.2	
Lys	3.7		7.1		1.9	
Ser	2.6		9.0		3.5	
Thr	2.5		8.6		3.4	
Gly	5.0		6.9		1.4	

[a] Xaa is Val in the original substrate (SP-211) representing the cleavage site between the matrix and capsid proteins in HIV-1. Kinetic parameters for SP-211 are listed in Table I, series A.

bond formation of the Gln side chain with Arg_8'. The substrate binding pocket of HIV-2 PR seems to be very similar to that of HIV-1 PR. The relative activities were very similar with HIV-1 PR or HIV-2 PR (not shown), therefore the kinetic parameters with HIV-1 PR were not determined. Interestingly, with the peptides containing Leu or Phe at P_3 position, an apparent substrate inhibition was observed, increasing the substrate concentration well above K_m (not shown). This suggests the possibility of increased nonproductive binding, for example, the P_3 Phe or Leu might occupy the S_1 subsite. The preference for large hydrophobic residues at the P_1 position has been noted earlier. Another possibility is that intramolecular hydrophobic interactions of P_3 with P_1 and P_5 residues may not be advantageous for the catalytic activity. Gly in this position resulted in good binding and also good kinetic constants, indicating that side-chain interactions at this position may not be crucial for proper enzyme–substrate interaction.

Since with certain sequences the presence of P_4 residue seems to be required for sufficient substrate hydrolysis (Moore et al., 1988; Darke et al., 1988; Tözsér et al., 1991b) while with others it is not (Phylip et al., 1990; Tomasselli et al., 1990), the effect of amino acid changes on the kinetic parameters were also investigated in the absence of P_4 and P_5 residues (Table II, series B). Removal of the Val-Ser- group from the N terminus of the substrate resulted generally in an increase in K_m values. If the P_3 residue is relatively large, the k_{cat} value substantially decreased by the removal of Val-Ser-, while if the P_3 residue was relatively small (like Ala, Asp, Asn), the decrease in k_{cat} value was less pronounced. In the case of Gly, the shorter peptide even had a higher k_{cat} value.

S_3' Subsite. Results with peptides having amino acid substitutions in P_3' position of SP-211 are shown in Table III. In general, the tendency of the K_m and k_{cat} values is similar to that found with P_3 substitutions: hydrophobic residues (Leu, Phe, Val) produced the best binding (low K_m) and proteolytic constants (k_{cat}/K_m). As mentioned earlier, due to the symmetry of the PR dimer, S_3–P_3 interactions should be similar to S_3'–P_3', but not identical, due to the asymmetry produced by substrate binding (Gustchina & Weber, 1990; Miller et al., 1989; Swain et al., 1990; Jaskolski et al., 1991). Nevertheless, basically the same amino acids of the enzyme are involved in S_3–P_3 interactions as in S_3'–P_3' interactions, although the individual distances are different.

Similar to that found in the case of P_3 substitutions, charged residues resulted in drastic increase of the K_m values. An interesting difference from the P_3 results is the very high K_m values for the polar residues (2–8 mM). On the other hand, the k_{cat} values do not seem to be dramatically changed. The results concerning the K_m values indicate that hydrophobic contact at this position is crucial for proper binding of the substrate in this peptide sequence context. While Pro_{81}'–P_3 distance seems to be the same as Pro_{81}–P_3' (measured as C_α–C_α distance), Phe_{53}' is further (about 0.8 Å) from P_3' than Phe_{53} is from P_3. Interaction of the substrate with Phe_{53} and Phe_{53}' is predicted to help the proper closure of the flap on the substrate and keep the hydrophobic environment of the substrate binding cleft. However, the postulated role of Phe_{53} and Phe_{53}' in the substrate binding should be investigated by mutation of this residue. In addition to the differences in distances, interaction with the P_5 Val may substitute for the P_3 interaction (Figure 2), resulting in generally lower K_m values of the P_3-modified peptides compared to the P_3' substitutions since no P_5' is present.

Compared to the P_3 position, substantial differences were found in the relative hydrolysis rates for the Leu- and Phe-

Table IV: Kinetic Parameters Determined with HIV-2 PR for Substrates Having Substitutions in P_2 Position of H-Val-Ser-Gln-Xaa-Tyr*Pro-Ile-Val-Gln-NH_2[a]

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	
P_2 amino acid	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR
Leu	0.17	0.12	3.4	0.4	20.0	3.3
Phe	0.36	0.18	1.5	0.2	4.2	1.1
Cys	0.20	0.10	7.2	7.4	36.0	74.0
Val	0.43		2.5		5.8	
Ile	0.17		1.2		7.1	
Ala	0.08	0.23	6.1	3.9	76.3	17.1
Asp	0.27		2.2		8.1	
Thr	1.3	0.41	0.6	0.7	0.5	1.7
Lys			[b]			
Gly	0.78		2.3		3.0	

[a] Xaa is Asn in the original substrate (SP-211), representing the cleavage site between the matrix and capsid proteins in HIV-1. [b] This peptide was not hydrolyzed by HIV-1 and HIV-2 PRs during a 24-h incubation period (k_{cat} is estimated to be smaller than 0.01 s^{-1}).

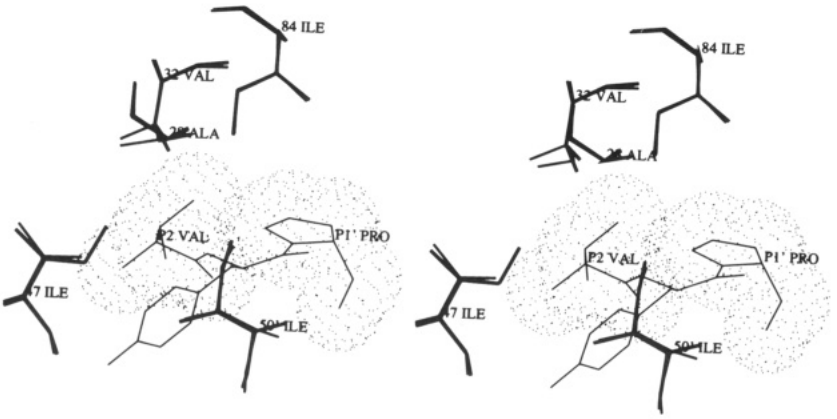

FIGURE 3: Close contacts between the residues Val at P_2 and Pro at P_1' in the model of the substrate (in thin lines) at the active site of HIV-1 PR (in thick lines) and HIV-2 PR (in medium thick lines). Van der Waals surfaces are indicated by dots for P2 and P1′ side chains.

containing substrates by HIV-1 and HIV-2 PRs (not shown); therefore, for those peptides the kinetic parameters were also determined with HIV-1 PR (Table III). While the K_m values were similar, both peptides had higher k_{cat} value with HIV-1 PR. HIV-1 PR may have higher hydrolysis rates with Leu and Phe compared to HIV-2 PR due to the presence of Val_{82} rather than Ile_{82} in the distal part of S_3'. The P_1 Tyr may interfere with the P_3–residue 82′ interaction, while the combination of P_3' Phe or Leu with P_1' Pro makes their interaction with residue 82 more favorable.

S_2 Subsite. Results with peptides having substitutions at P_2 position are shown in Table IV. The S_2 subsite for HIV-2 PR seems to be also hydrophobic and smaller than the S_3 and S_3'. The NH of P_2 amino acid coordinates to Gly_{48} of the flap, while its CO coordinates to the $water_{301}$ at the tip of the flap as observed in all crystal structures of HIV-1 PR with inhibitors. The P_2 side chain is surrounded by Ile_{50}' and Val_{47} (Ile in HIV-1 PR) of the flaps, Ala_{28}, Ile_{32} (Val in HIV-1 PR), and Asp_{30}. The side chain of P_2 may coordinate to NH groups of Asp_{29} and Asp_{30}. The best k_{cat} values were obtained with small residues [Asn (see Table II), Cys, Ala]. While Leu fits relatively well into this pocket, the bulkier aromatic Phe resulted in worse kinetic parameters; it is apparently too large for this subsite. Also, β-branched residues such as Val do not fit very well, probably due to the steric collision with P_1' Pro of the substrate (Figure 3). Interestingly, using another series of substrates having *p*-nitro-Phe at P_1' position, the β-branched residues gave the best kinetic parameters for HIV-2 PR (and also for HIV-1, Phylip et al., 1990). These results may provide a rational basis for the classification of cleavage sites of immunodeficiency viruses (Henderson et al., 1988): the P_2 tends to be Asn in class I cleavage sites having Tyr/Phe*Pro at the scissile bond, while P_2 is generally hydrophobic in class II and class III cleavage sites in which P_1' is not proline. While Gly was not acceptable in a different sequence context (Phylip et al., 1990), it was satisfactory in our series. Substitution of Asn to Asp in this position resulted in weaker binding and lower catalytic activity, probably due to unfavorable interaction with Asp_{30}. Substitution with Lys prevented substrate hydrolysis. It has been suggested that differences in the S_2 pocket of HIV-1 and HIV-2 may result in substantial differences in inhibitor binding: the difference in residue 32 (Val in HIV-1 PR and Ile in HIV-2 PR resulting in larger subsite for HIV-1 PR) was postulated to be responsible for differential inhibition of HIV-1 and HIV-2 PRs by acetyl-pepstatin and H-261 (Gustchina & Weber, 1991). The relative hydrolysis rates for the P_2-substituted peptides showed differences for the Leu-, Phe-, Cys-, Ala-, and Thr-containing peptides; therefore, the kinetic parameters were also determined for these peptides using HIV-1 PR (Table IV). The Leu-, Phe-, and Ala-containing peptides were better substrates for the HIV-2 PR. The effect on K_m values seems to depend on the size of residue 32: Cys, Leu, and Phe bind better to HIV-1 PR with Val_{32}, while the smaller Ala binds better to HIV-2 PR due to the presence of larger Ile_{32}. The larger hydrophobic Leu and Phe show better k_{cat} values with HIV-2 PR, which may reflect the presence of Val_{47} rather than Ile as in HIV-1.

S_2' Subsite. The results obtained with the P_2'-modified peptides are shown in Table V. As for S_2 position, the binding at this subsite is mainly hydrophobic in nature. In common with the P_2-modified peptides, Phe and Asp produced inefficient substrates, while the peptide containing Lys was not hydrolyzed. In contrast to the findings for P_2 residue, the best catalytic constant was obtained with the β-branched Val. Another substantial difference is that while the peptide containing Gly at P_2 was reasonably hydrolyzable, at P_2' Gly

Table V: Kinetic Parameters Determined with HIV Proteinases for Substrates Having H-Val-Ser-Gln-Asn-Tyr*Pro-Xaa-Val-Gln-NH_2 Structure[a]

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	
P_2' amino acid	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR
Leu	0.27	0.25	7.2	4.7	26.6	18.8
Phe	0.31		0.9		3.0	
Val	0.56		9.3		16.7	
Ala	1.09		4.8		4.7	
Asp	4.1		2.6		0.6	
Asn	5.2		1.5		0.3	
Gln	0.87		4.4		5.1	
Lys			*b*			
Gly	5.0		1.4		0.3	

[a] Xaa is Ile in the original substrate (SP-211), representing the cleavage site between the matrix and capsid proteins in HIV-1. [b] Not hydrolyzed during 24-h incubation by HIV-1 and HIV-2 PRs (k_{cat} estimated to be smaller than 0.01 s^{-1}).

Table VI: Kinetic Parameters Determined for Substrates Having Substitutions in P_1 Position of H-Val-Ser-Gln-Asn-Xaa*Pro-Ile-Val-Gln-NH_2[a]

	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	
P_1 amino acid	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR	HIV-2 PR	HIV-1 PR
Leu[b]	0.34		2.8		2.8	
Phe	0.22	0.13	14.8	17.7	67.3	136.2
Cys	ND[c]		0.2		ND	
Ala	ND		<0.5		ND	
Met	0.40	0.76	5.1	1.4	12.8	1.8
Trp	<0.05		0.2		ND	

[a] Xaa is Tyr in the original substrate (SP-211), representing the cleavage site between the matrix and capsid proteins in HIV-1. Substitutions which resulted in nonhydrolyzable peptides (k_{cat} smaller than 0.01): Val, Ile, Pro, Ser, Asp, Lys, and Gly. [c] ND, not determined.

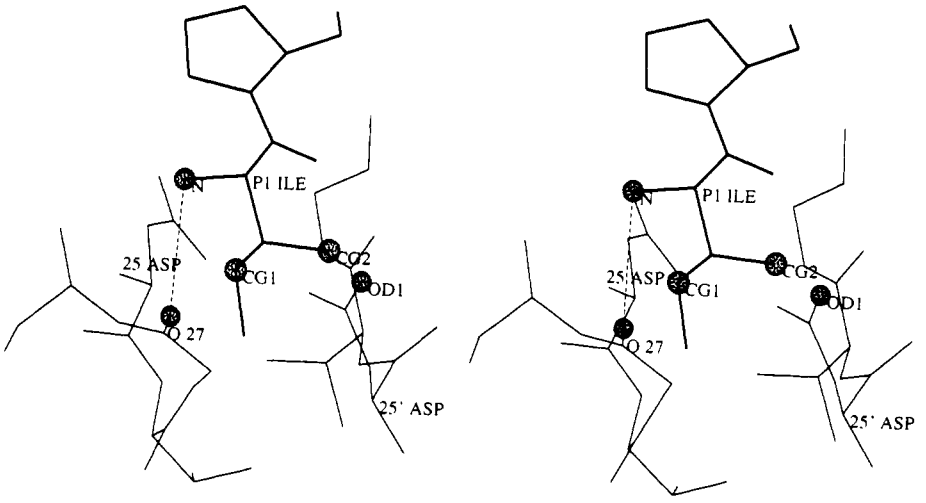

FIGURE 4: Model of HIV-PRs with the substrate-containing Ile, a β-branched residue in P_1 position based on the structure of the complex of HIV-1 PR with inhibitor JG-365 (Swain et al., 1990). Atoms involved in close contacts are indicated by dotted circles.

produced a very inefficient substrate. It should be noted that in another substrate having different amino acid sequence, Gly in P_2 position abolished activity (Konvalinka et al., 1990). Another difference is the very high K_m values for the Ala-, Asp-, and Asn-containing peptides. While Asn at P_2 resulted in a very good substrate (unmodified SP-211, see Table II), it resulted in a very inefficient one in the P_2' position, and the explanation is not obvious. Generally, our results concerning the P_2' subsite agree with that found for P_2 by Phylip et al. (1990), who used a substrate in which both P_1 and P_1' amino acids were aromatic (Tyr and *p*-nitro-Phe).

Similar to that found for the P_2 position, the peptide having P_2' Leu was less efficiently hydrolyzed by the HIV-1 PR (Table V).

S_1 Subsite. As expected from the naturally occurring cleavage sites (Oroszlan & Tözsér, 1990), oligopeptides containing hydrophobic residues at P_1 were hydrolyzed (Table VI). The model for substrate binding suggests that the NH of P_1 amino acid interacts with CO of Gly_{27}, while the carbonyl O interacts with Asp_{25} and Asp_{25}'. The side chain is in a well-defined hydrophobic pocket, comprised of Ile_{82}' (Val in HIV-1 PR), Pro_{81}, and Ile_{84}' as well as Leu_{23}. The best result was obtained with Phe. Tyr resulted in a substantial decrease in the value of the catalytic constant. Modeling studies suggested that the OH group of Tyr may be difficult to fit into the substrate binding pocket. While the flexible Met fits reasonably well into the pocket, Trp is too bulky to fit well. β-Branched residues are not hydrolyzable, as also noted by others (Phylip et al. 1990), due to an unfavorable collision with the carbonyl oxygen of Asp_{25}' and CO of Gly_{27} (Figure 4).

S_1' Subsite. The following amino acids were introduced into P_1' position: Ala, Asp, Lys (Blaha et al., 1991), Tyr, Phe, Leu, Met, Gly, Val, Ile, Ser, and Trp. Using HIV-2 proteinase (0.4 mM substrate, 20 nM enzyme in the assay solution), none of the substrates were hydrolyzed during a 24-h incubation. With HIV-1 (using 80 nM enzyme and 24-h incubation), a small amount of hydrolysis was found with all peptides, except that containing Lys; however, the predicted k_{cat} values were less

than 0.01 s^{-1}. Furthermore, all peptides were able to inhibit SP-211 cleavage, (20–80% inhibition if used in equimolar amount to the substrate), indicating that their binding constants are similar to that of SP-211. In addition, while SP-211 containing Pro at P_1' position was not hydrolyzed by chymotrypsin, all the modified peptides were hydrolyzable, to a similar extent as was found with the Ala-, Asp-, and Lys-containing peptides (Blâha et al., 1991).

The lack of the sufficient hydrolysis of these peptides by both HIV-1 and HIV-2 PRs is surprising since some of these amino acids can be found in P_1' of naturally occurring cleavage sites, and the respective oligopeptides were good substrates (Tözsér et al., 1991a). Although Partin et al. (1990) observed polyprotein processing at or near the MA/CA cleavage site of a modified recombinant Gag–Pol polyprotein (expressing equimolar amount of PR to the Gag proteins in an in vitro translation system) in which Thr, Leu, or Ala was substituted for Pro, they used 50–100-fold excess of purified HIV-1 PR over the substrate in trans assay, and the actual cleavage site was not determined by sequence analysis. Using an *Escherichia coli* expression system, the same MA/CA polyprotein junction was not processed when Tyr*Pro was substituted with Met*Met (VSQNM*MIVQ), although Met*Met is the cleavage site at the X/NC junction and very efficiently hydrolyzed in its own sequence context (Tritch et al., 1991). Our results also indicate that the productive binding of an amino acid at P_1' position is strongly dependent on the surrounding sequence.

Conclusions

The substrate interaction with HIV proteinases is primarily hydrophobic in nature, as predicted by Weber et al. (1989). In agreement with the experimental results that seven residues of the substrate should be recognized to obtain efficient hydrolysis, analysis of the HIV proteinase structures shows at least seven distinct substrate binding pockets, one for each substrate side chain. Interactions at all seven subsites at both P and P′ sides are important for proper enzyme substrate interaction. The specificity of HIV proteinases has been intensively studied using oligopeptide substrates (Phylip et al., 1990; Konvalinka et al., 1990; Richards et al., 1990; Tözsér et al., 1991a,b) or polyproteins (Tomasselli et al., 1990; Partin et al., 1990; Tritch et al., 1991). Many of the effects on the kinetics observed in the present study by using various substitutions are predictable or explainable by molecular modeling. However, modeling did not predict the important role of P_1' proline in the investigated sequence. On the contrary, modeling studies indicated that most of the investigated residues would fit into the substrate binding pocket. In accordance with this, the peptides carrying P_1' substitutions were able to bind to the enzyme and inhibit SP-211 cleavage (data not shown). Modeling is able to describe binding capabilities rather than catalytic efficiency, partly because it is very difficult to model the conformation change leading from the enzyme–substrate complex to the transition state. On the other hand, it is also difficult to predict what portion of the binding energy will be utilized by the enzyme for the enzyme substrate interaction and for the lowering of the activation energy. Tyr/Phe*Pro cleavage sites in retroviral Gag and Gag–Pol polyproteins have been classified as class I cleavage sites, having distinct preference for the side chains farther from the scissile bond compared to the class II and class III cleavage sites (Henderson et al., 1988). Our results suggest a rather "unique" role of the Pro in a typical class I cleavage site (e.g., SP-211). It is a special characteristic of the PR to be able to hydrolyze efficiently the peptide bond involving the proline nitrogen (Oroszlan et al., 1978). Proline is the most frequently occurring single amino acid at the P_1' site, and it is invariably the N terminus of the capsid (CA) protein in all retroviruses (Oroszlan & Luftig, 1990; Pettit et al., 1991). Proline residues have a relatively high intrinsic probability of forming the cis rather than the trans isomer of the preceding peptide bond (Brandts et al., 1975), and interestingly Tyr-Pro seems to have the highest frequency of occurrence in cis in proteins among all Xaa-Pro pairs (MacArthur & Thornton, 1991). However, modeling studies suggested that the Pro would bind in trans rather than in cis in P_1' position of the SP-211 (unpublished results). It remains to be determined whether the trans isomer is hydrolyzed more efficiently than the cis and whether isomerization of the peptide bond involving proline nitrogen has a role in polyprotein processing and virus maturation. It has been shown that substitution of Tyr*Pro to Ser-Arg in HIV-1 Gag polyprotein prevents cleavage at the MA/CA junction and results in alteration of virion morphology and noninfectious virus (Gottlinger et al., 1989).

The specificity of HIV proteinases is rather complex and difficult to understand. Occurrence of various amino acids at the P_4–P_3' positions in naturally occurring cleavage sites of HIV-1 and HIV-2 Gag and Gag–Pol polyproteins (Oroszlan & Tözsér, 1990) would suggest that the retroviral proteinases are rather unspecific enzymes; however, they process their natural substrates, the Gag and Gag–Pol polyproteins, in a restrictive way. Kent et al. (1989) emphasized the importance of the primary sequence of the substrate for determining specificity. However, the importance of substrate conformation on the specificity of the HIV proteinases was noted by Billich et al. (1988). Our results suggest that, although the respective S and S′ subsites in the substrate binding site share some similarities, they are substantially different. The differences may be attributed to the asymmetry introduced by the binding of the substrate and/or the different amino acid sequence context. Due to the β-sheet conformation of the substrate, the side chains of P_4, P_2, P_1', and P_3' lie on the same side of the peptide backbone, while P_3, P_1, and P_2' side chains are directed to the opposite side. Therefore adjacent side chains may interact, for example, P_2 and P_1' or P_3 and P_1, as observed in the crystallographic structures of enzyme–inhibitor complexes. At least at certain subsites (P_2, P_1', and P_2') these interactions may strongly influence the subsite specificity. This context-dependence substantially contributes to the specificity of the enzyme.

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