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Comparative Studies on the Substrate Specificity of Avian Myeloblastosis Virus Proteinase and Lentiviral Proteinases*

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The retroviral proteinase (PR) seems to play crucial roles in the viral life cycle, therefore it is an attractive target for chemotherapy. Previously we studied the specificity of human immunodeficiency virus (HIV) type 1 and type 2 as well as equine infectious anemia virus PRs using oligopeptide substrates. Here a similar approach is used to characterize the specificity of avian myeloblastosis virus (AMV) PR and to compare it with those of the previously characterized lentiviral PRs. All peptides representing naturally occurring Gag and Gag-Pol cleavage sites were substrates of the AMV PR. Only half of these peptides were substrates of HIV-1 PR. The K_m values for AMV PR were in a micromolar range previously found for the lentiviral PRs; however, the k_{cat} values were in a 10–30-fold lower range. A series of peptides containing single amino acid substitutions in a sequence representing a naturally occurring HIV cleavage site was used to characterize the seven substrate binding subsites of the AMV PR. The largest differences were found at the P4 and P2 positions of the substrate. Detailed analysis of the results by molecular modeling and comparison with previously reported data revealed the common characteristics of the specificity of the retroviral PRs as well as its strong dependence on the sequence context of the substrate.

Due to its crucial role in the early phase (1–3) as well as in the late phase of viral life cycle (for review see Ref. 4), retroviral proteinase (PR)[1] is a promising target for drug intervention; potent inhibitors developed to block its action are now in advanced clinical trials (5). However, as in the case of reverse transcriptase inhibitors, resistance rapidly develops, both *in vitro* and *in vivo* (6). Studies to determine the common features of the specificity of different retroviral PRs may help to design broad spectrum inhibitors and reduce the possibility of viable mutants. Therefore, we have extensively studied the specificity of HIV-1,[2] HIV-2 (7–9), EIAV (10, 11), and bovine leukemia virus (12) PRs using oligopeptide substrates representing the naturally occurring cleavage sites, as well as a peptide series containing single amino acid substitutions in the P4-P3′[3] positions of the cleavage site sequence between the matrix (MA) and capsid (CA) proteins of HIV-1. This MA/CA cleavage site sequence has Tyr↓Pro at the site of the cleavage (the down arrow indicates the cleavable peptide bond). Recent analysis of retroviral cleavage site sequences (13) and kinetic studies on HIV PRs (9, 14) suggest that two major types of cleavage sites exist for retroviral proteinases: one having Tyr↓Pro (type I) and the other having mainly hydrophobic residues but not Pro (type II) at the site of cleavage.

The avian type C viruses code the PR on the *gag* gene; therefore, unlike the other retroviruses, PR is made in equivalent amounts to the structural proteins and was relatively easy to purify. Many of the early studies on the role and specificity of retroviral PR used AMV/RSV[4] as a model system (for review see Ref. 15). RSV PR was the first retroviral enzyme for which the crystal structure was determined (16). Subsequently, many crystal structures have been determined of HIV-1 and HIV-2 PRs in the absence and the presence of inhibitors (17). AMV/RSV PR is still in focus of intensive specificity and mutagenesis studies (18–22). Some RSV cleavage site peptides have been previously studied as substrates of the retroviral enzymes (20, 23). Here we report the comparison of the AMV and HIV-1 proteinases based on a complete set of substrates representing naturally occurring cleavage sites. Recently Cameron *et al.* (21) used a series of substrates containing single amino acid substitutions in the type II NC/PR cleavage site sequence of RSV to compare the specificity of RSV and HIV-1 PRs. Here, we compare the specificity of AMV and HIV-1 PRs using a series of peptides containing single amino acid substitutions in the type I MA/CA sequence of HIV-1. Detailed analysis of the results by molecular modeling and comparison with previously published data on retroviral proteinases has revealed the common characteristics of the specificity of retroviral PRs as well as its strong dependence on the sequence context of the substrate.

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[1] The nomenclature of viral proteins is according to Leis *et al.* (42): MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, proteinase; RT, reverse transcriptase; IN, integrase.

[2] The abbreviations used are: HIV-1 and HIV-2, human immunodeficiency virus, type 1 and type 2; AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus; EIAV, equine infectious anemia virus; MuLV, murine leukemia virus.

[3] The notation is according to Schechter and Berger (43).

[4] The AMV and RSV proteinases differ only in two residues, which are not expected to be involved in the enzyme-substrate interaction.

MATERIALS AND METHODS

Retroviral Proteinases

AMV PR purified from virus as described (24), was obtained from Molecular Genetic Resources (Tampa, FL). Recombinant purified HIV-1 PR (25) used for the peptides representing naturally occurring cleavage sites in RSV was a kind gift of Dr. Y. S. E. Cheng (Experimental Station, The DuPont Merck Pharmaceutical Company, Wilmington, DE). Active site titration of the enzymes was performed using Pro-Pro-Cys-Val-PheSta-Ala-Met-Thr-Met for AMV PR (23) and a phosphinic acid-type substrate based inhibitor (compound 3 in Ref. 26) for HIV-1 PR.

Oligopeptides

Oligopeptides were synthesized by standard *tert*-butoxycarbonyl or 9-fluorenylmethyloxycarbonyl chemistry on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or a semiautomatic Vega peptide synthesizer (Vega-Fox Biochemicals). Some peptides were synthesized by fragment condensation as described previously (27). Amino acid composition of the peptides was determined with either a Durrum D-500 or a Waters Pico-Tag amino acid analyzer. Stock solutions and dilutions were made in distilled water (or in 10 mM dithiothreitol for peptides containing Cys residues), and the peptide concentrations were determined by amino acid analysis.

Enzyme Assay

The assays for the kinetic measurements were performed in 0.25 M phosphate buffer, pH 5.6, containing 5% glycerol (7.5% glycerol for HIV-1 PR), 5 mM dithiothreitol, 1 mM EDTA, and 2 M NaCl as described previously (7–9). The reaction mixture was incubated at 37 °C for 1 h and was stopped by the addition of 9 volumes of 1% trifluoroacetic acid and then injected onto a Nova-Pak C_{18} reversed-phase chromatography column (3.9 × 150 mm, Waters Associates, Inc.) using an automatic injector. Substrates and the cleavage products were separated using acetonitrile gradient (0–100%) in water in the presence of 0.05% trifluoroacetic acid. The cleavage of peptides was monitored at 206 nm, and the peak areas were integrated. Amino acid analysis and/or N-terminal sequencing of the collected peaks was used to confirm the cleavage sites in the substrates. N-terminal sequencing was performed using a KNAUER Model 910 protein sequencer. The substrate concentrations used for the kinetic measurements were in the range of 0.01–3 mM, depending on the approximate K_m values. Kinetic parameters were determined at less than 20% substrate turnover by fitting the data to the Michaelis-Menten equation using the Gauss-Newton method using Fig.P program (Fig.P Software Corp., Durham, NC). The standard errors of the kinetic parameters were below 20%. Substrate hydrolysis followed Michaelis-Menten kinetics in the concentration range of substrates used. For the determination of relative activities, the reaction mixture containing 0.4 mM peptide was incubated at 37 °C for 1 or 24 h as described previously (11, 12). Amino acid analysis of the collected peaks was used to confirm the site of cleavage with HIV PR (9, 12). For the AMV PR, cleavage products were identified by the retention time, which was found to be identical to that obtained with HIV PR. Relative activities were calculated from the molar amounts of peptides cleaved per unit time by dividing the activity on a given peptide by the activity on the unmodified substrate SP-211 (Val-Ser-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln), at less than 20% substrate turnover, as described in Bláha *et al.* (12). Measurements were performed in duplicate, and the average values were calculated. The error was less than 20%. The relative activities for the HIV-1 PR have been reported previously (11).

Methods for Modeling Calculations

Initial Model—The crystal structures of the native RSV PR with a modeled flap and peptide substrate (20) and HIV-1 PR complexed with a modeled substrate Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln (9) were superimposed using Cα atoms and examined on a Silicon Graphics 310 computer graphics system runnung the program CHAIN (28) or a Silicon Graphics Indigo computer graphics system using the program Sybyl (Tripos Inc., St. Louis, MO). The residues forming the subsites for Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln were determined previously for HIV-1 and HIV-2 PR (8, 9), and the corresponding residues in AMV/RSV PR were obtained from the structural alignment. Models of the series of peptide variations of Pro-Ala-Val-Ser-Leu-Ala-Met-Thr that represent the NC/PR cleavage site of RSV are described separately.[5] The proteinase substrate complexes were modeled with a proton positioned midway between the closest side chain oxygens of the two adjacent catalytic aspartates, Asp-25 and Asp-25′, and the carbonyl oxygen of the scissile bond. The position of this proton has been shown to be energetically stable by molecular dynamics simulations (29). All the crystallographic water molecules were included because several appeared to be structurally important.

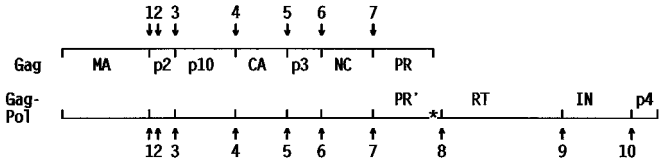

FIG. 1. **Cleavage sites in RSV Gag and Gag-Pol polyproteins.** Cleavage sites were determined by sequencing purified virion proteins (reviewed in Ref. 15). The frameshift site is marked by an *asterisk*.

Energy Minimization Calculation—The minimization and molecular dynamics were run using the program AMMP (30). No screening dielectric term or bulk solvent correction was included. A constant dielectric of one was used. No cut-off was applied for nonbonded and electrostatic terms, as described in Harrison and Weber (29). A modified version of the UFF potential set (31) was used as described separately.[5] These modifications do not significantly change the performance of the potential set on small molecules but result in constantly smaller root mean square deviations between minimized and observed protein structures. The atomic charges from the AMBER all-atom set were used for the protein and water (32). Parameters for water and for the proton were as described (29). The atomic positions for the protein and water molecules were initially tethered to those in the crystal structure of AMV PR in order to calculate and minimize the hydrogen atom positions. The side chain atoms of the substrate peptide were removed down to the Cβ atom for the substituted amino acids, and the new atomic positions were created by a variation on distance geometry (33). The new atoms were minimized with respect to bond, angle, torsion, and hybrid potentials. The proteinase structure with nonhydrogen atoms from the crystal structure and minimized hydrogen atoms was combined with each of the different peptides with single amino acid substitutions. Then each of the side chain torsion angles for substituted residues in the peptide substrate was rotated through 360 ° in steps of 15 ° to search for alternate conformations. This torsion search finds the angle(s) that have a minimum in the nonbonded energy. The substrate atoms were minimized using 300 steps of conjugate gradients. Finally, the complete model of AMV PR with each different substrate was optimized by a longer minimization using 100 steps of conjugate gradients followed by eight cycles of alternating conjugate gradients (30 steps) and short runs of molecular dynamics (20 fs steps at 300 K).

RESULTS AND DISCUSSION

Hydrolysis of Oligopeptides Representing Naturally Occurring Cleavage Sites

Ten oligopeptides representing naturally occurring cleavage sites in the RSV Gag and Gag-Pol polyproteins (Fig. 1) were tested as substrates for AMV and HIV-1 PRs (Table I). All peptides were hydrolyzed at the expected site by the AMV enzyme, except peptide 10, which was hydrolyzed at the Gly ↓ Ile bond instead of the Ala ↓ Gly bond, the site in RSV IN reported by Grandgenett *et al.* (34). For this peptide, the same site of cleavage was also observed at low ionic strength, in the absence of additional salt (data not shown). The RSV IN protein is phosphorylated at the Ser residue near the cleavage site in the C terminus (P6 position of peptide 10 in Table I; see Ref. 35). It should be noted that phosphorylation was found to alter the C-terminal processing of IN. It would be interesting to determine whether phosphorylation of the serine would cause a shift in the cleavage site. Kinetic parameters were determined at high (2 M) salt concentration. High ionic strength was found to be optimal for AMV PR (36), similar to other retroviral PRs (10, 37, 38). The range of kinetic parameters was compared with that we previously determined for the lentiviral PRs using peptides representing cleavage sites in their Gag and Gag-Pol polyproteins (Table II). The K_m was found to be also in the micromolar range, although one peptide showed an exceptionally high value (peptide 9 in Table I). However, the k_{cat} values

[5] I. T. Weber and R. W. Harrison, submitted for publication.

TABLE I
Hydrolysis of oligopeptides representing naturally occurring cleavage sites by AMV and HIV-1 proteinases

Place of cleavage		Peptide sequence	AMV PR			HIV-1 PR		
			K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
			mM	s^{-1}	$mM^{-1}s^{-1}$	mM	s^{-1}	$mM^{-1}s^{-1}$
In RSV								
1	MA/p2A	GTSCY ↓ HCGTA	0.96	0.062	0.06	0.125	0.31	2.48
2	p2A/p2B	GCNCA ↓ TASAP		hydrolyzed[a]			hydrolyzed[a]	
3	p2B/p10	PPYVG ↓ SGLYP	0.01	0.005	0.50		not hydrolyzed[b]	
4	p10/CA	PVVAM ↓ PVVIK	<0.03	0.04	4.9[c]		not hydrolyzed[b]	
5	CA/p3	IAAAM ↓ SSAIQ	0.51	0.005	0.01		not hydrolyzed[b]	
6	p3/NC	IQPLIM ↓ AVVNR	0.02	0.19	11.9	0.02	>0.8	318[c]
7	NC/PR	PPAVS ↓ LAMTM	0.052	0.05	2.5	0.088	0.01	0.13
8	PR′/RT	RATVL ↓ TVALH	0.04	0.04	1.00	<0.04	>0.04	1.9[d]
9	RT/IN	TFQAY ↓ PLREA	1.88	0.001	0.015	0.555	0.10	0.18
10	IN/p4	SPLFAG ↓ ISDW[e]	0.27	0.001	0.005		not hydrolyzed[b]	
In HIV-1								
11	MA/CA	VSQNY ↓ PIVQ[f]	0.38	0.006	0.015	0.15	6.8	45.3

[a] Cleaved at the expected site, but kinetics was not done. Dithiothreitol peaks covered the product peaks.
[b] Detection limit: 0.001 nmol product/s/nmol enzyme.
[c] Determined with a competition assay with peptide 11.
[d] Determined with a competition assay with peptide 9.
[e] This peptide is cleaved at Gly ↓ Ile bond, whereas the natural cleavage site was found to be between Ala and Gly. Residue corresponding to the N-terminal Ser has been found to be phosphorylated in the IN protein (35).
[f] Values for the HIV-1 PR have been published previously (9).

TABLE II
Range of kinetic constants determined for retroviral proteinases using oligopeptides representing naturally occurring cleavage sites in their Gag and Gag-Pol polyproteins

Virus	Range of K_m	Range of K_{cat}	Range of k_{cat}/K_m
	mM	s^{-1}	$mM^{-1}s^{-1}$
HIV-1[a]	0.006–0.47	0.01–6.8	0.02–202.0
HIV-2[a]	0.01–0.55	0.3–2.6	1.00–60.0
EIAV[b]	0.01–0.27	0.01–6.1	0.01–122.0
AMV[c]	0.01–1.88	0.001–0.19	0.005–11.9

[a] Taken from Tözsér *et al.* (7).
[b] Tözsér *et al.* (10) and our unpublished results.
[c] Taken from Table I.

were in a 10–30-fold lower range than those of other PRs (Table II). This is in good agreement with the relative amounts of the PR in the virions. Although the PR of the HIVs and EIAV is coded for by the *pol* gene and is synthesized in an approximately 10–20-fold lower amount than the Gag proteins, the PR of AMV is encoded in the *gag* gene (Fig. 1), and therefore it is synthesized equimolarly to the structural Gag proteins.

The peptides were also assayed as substrates of HIV-1 PR. Only half of them were substrates of this enzyme with similar kinetics to that obtained with the AMV PR except for peptide 6, which was the best substrate of both enzymes, with kinetic parameters comparable with the best values obtained for peptides representing the HIV naturally occurring cleavage sites (Table II).

By comparing the amino acids at the P1-P1′ region of the cleavage sites, it is obvious that the three lentiviral PRs prefer hydrophobic residues (7, 10), whereas AMV PR seems to tolerate glycine or polar residues. Nevertheless, the two peptides with the best k_{cat}/K_m values (peptides 4 and 6 in Table I) contain hydrophobic residues at both P1 and P1′ positions.

Mapping of the Substrate Binding Site of AMV with a Series of Peptides Containing Tyrosine and Proline at the Cleavage Site

Peptides containing single amino acid substitutions in the sequence of the cleavage site found between MA and CA of HIV-1 were assayed as substrates of AMV PR. Kinetic parameters for the unmodified peptide (peptide 11 in Table I) suggest that although it is a poor substrate of AMV PR, it is comparable with some of the peptides representing naturally occurring RSV cleavage sites. We have previously determined relative activites compared with the unmodified peptide of the HIV-1, HIV-2, and EIAV PRs for the substituted peptides to compare the specificity of the enzymes (9, 11, 12). Relative activities of AMV PR are listed in Table III. The k_{cat}/K_m values determined for a set of these substrates (data not shown) correlated well with the relative activites ($r = 0.987$; $n = 15$). Residues forming the substrate binding sites of HIV-1 have been determined from the crystal structures of HIV-1 PR inhibitor complexes. Based on homology (Fig. 2) and modeling of the substrate into the RSV PR, residues predicted to form the respective subsites compared with the HIV-1 PR are listed in Table IV together with the respective HIV-1 residues and are shown in Fig. 3. By analogy to the peptidic inhibitors (17), the substrate is predicted to bind in an extended β conformation, therefore alternative subsites such as S3 and S1 are adjacent to one another (Fig. 3*A*). The results from the type I series of peptides based on the HIV-1 MA/CA cleavage site can be compared with those using type II peptides based on the RSV NC/PR cleavage site (21) in order to identify differences due to the sequence context. Schematic representations of the HIV-1 MA/CA and RSV NC/PR substrates together with the preferred substitutions are shown in Fig. 4.

S4 Subsite—The S4 subsite of AMV PR is near the surface of the proteinase, as observed for other retroviral proteinases. In this position the side chain of P4 residue may be partially exposed to solvent. In accordance with this, polar substitutions (like Lys, Arg, and Asn) of the original Ser in P4 resulted in fairly good substrates of AMV PR (Table III). However, compared with HIV proteinases, substitution with bulky hydrophobic residues gave much higher activity in our series of peptides (see Table III). The best value was obtained with the Ile substitution. AMV PR behaves similarly to EIAV PR in preferring hydrophobic residues at P4 (11). However, with the EIAV enzyme, the Leu substitution gave the best result, and the increases in relative activities were much less pronounced. Compared with HIV proteinases, EIAV PR contains extra residues leading to the tip of the flap that could be involved in S4-P4 interaction, and the S4 pocket is predicted to be more hydrophobic than that of HIV PRs (11). Extra residues leading to the flap are also predicted for AMV PR; modeling of RSV PR predicted that Pro-62 and Gln-63, derived from the flaps, may interact with the side chain of P4 (20). These additional amino

TABLE III

Relative activities obtained with MAV and HIV-1 proteinases for substrates having single amino acid substitutions from position P4 to P3′

The numbers in parenthesis were taken from Tözse'r *et al.* (10). The relative activities were calculated by determining the molar amount of peptide cleaved and dividing the activity on a given peptide by the activity obtained with the unmodified substrate Val-Ser-Gln-Asn-Tyr ↓ Pro-Ile-Val-Gln-NH_2 at 0.4 mM concentration.

Substitutions	Ser P4	Gln P3	Asn P2	Tyr P1	Pro P1′	Ile P2′	Val P3′
Ala	2.2 (0.3)	0.42 (0.34)	40.7 (0.53)	0.01 (<0.01)		1.8 (0.17)[a]	0.43 (0.34)
Leu	11.3 (0.02)	2.0 (0.40)	4.3 (0.06)	0.21 (0.21)	0 (0)	0.44 (0.44)[a]	1.0 (3.2)
Val	9.1 (0.05)	0.50 (0.71)	21.5 (0.17)	0.02 (0)	0 (0)	2.2 (0.93)[a]	
Ile	19.3 (0.03)		12.4 (0.1)	0 (0)	0 (0)		
Phe	10.2 (0.03)	2.4 (0.22)	0.25 (0.03)	1.6 (1.7)	0 (0)	0.07 (0.07)[a]	12.0 (2.4)
Trp				0.02 (0.01)	0 (0)		
Gly	0.16 (0.76)	0.03 (0.18)	1.6 (0.12)	0 (0)		0.05 (<0.01)[a]	0.17 (0.24)
Ser				0 (0)	0 (0)		0.46 (0.36)
Thr			0.82 (0.02)				1.00 (0.40)
Met	5.8 (0.04)			0.17 (0.23)			
Asp	0.78 (0.17)	0.01 (<0.01)	0.47 (0.13)	0 (0)		0.01 (<0.01)[a]	0.13 (0.05)
Asn	1.4 (0.17)	0.05 (0.50)				0.01 (0.01)[a]	
Glu							0.40 (0.1)
Gln						0.01 (0.03)	0.89 (0.67)
Lys	2.9 (0.06)	0.07 (0.12)	0 (0)	0 (0)		0 (0)[a]	0.27 (0.1)
Arg	0.8 (0.15)						2.70 (0.57)
Cys			35.6 (0.92)	<0.01 (<0.01)			

[a] Taken from Bláha *et al.* (27).

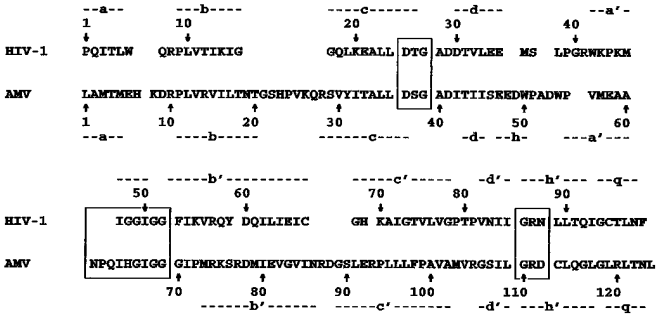

FIG. 2. **Sequence of HIV-1 and AMV proteinases.** The sequence of AMV PR differs only at two residues from RSV PR, the latter contains Thr instead of Ala-52 and Leu instead of Val-82. These residues are not expected to change the substrate specificity.

acid residues in AMV/RSV PR are predicted to provide a more enclosed S4 subsite, capable of accommodating hydrophobic residues. When residues 61–63 were deleted from AMV/RSV PR, the avian enzyme utilized substrates with polar residues (Asn or His) in P4, similarly to HIV-1 PR (22). Based on mapping RSV PR subsites with a series of peptides based on the NC/PR cleavage site, the original P4 Pro and its His substituted analog gave the best result (Fig. 4), whereas substitution of Leu or Phe resulted in nonhydrolyzable peptides (21). This is predicted to be due to the presence of β-branched Val in P2 position of the NC/PR peptide, which may restrict the internal space of S4 for P4 residue, whereas in the MA/CA peptide the P2 Asn may allow larger hydrophobic residues to be accommodated (Fig. 5). Interestingly, MuLV PR also behaved similarly to EIAV PR and AMV PR but differently from HIV PRs when mapped by the same Tyr ↓ Pro peptide series (38). However, MuLV PR does not seem to have extra residues leading to the flap but was predicted to have a more hydrophobic pocket due to extra residues from another part of the molecule.

Subsites S3 and S3′—With the P3 substitutions, less dramatic results were obtained than with the P4 changes, as was also found with EIAV and MuLV proteinases (11, 38). The ability of the S3 subsite to accommodate a variety of residues is also seen with substitutions in the P3 position of the NC/PR cleavage site peptide (21). Substitution of Gln to Leu and especially to Phe in the Tyr ↓ Pro peptide provided substantially better substrates (Table III and Fig. 4). Interestingly, the change of P3′ Val to Phe gave a dramatic increase in the relative activity, whereas Leu did not provide any increase over the original peptide. The S3 subsites of HIV-1 and AMV/RSV PR consist of relatively open and deep pockets that are near the surface (21). This could allow side chains of amino acids in the P3 position freedom of movement to either interact with hydrophobic residues near the S1 subsite or polar residues at the surface of the enzyme (21). In our series, hydrophobic side chains are greatly preferred over polar residues. In the NC/PR peptide, the P1 residue is a small Ser, whereas in our series the large P1 Tyr may restrict the conformations available for the P3 residue. However, there may be a favorable hydrophobic interaction of Phe P3 with the P1 Tyr side chain. Interestingly, Val and Leu substitutions gave very poor substrates for EIAV PR (11) but fairly good ones for AMV PR.

The most marked changes in the S3 pocket of AMV PR are Arg-105′ instead of Pro-81′ and Gly-106′ instead of Val-82′. In both of these positions EIAV PR contains the same residue as the HIV-1 PR. The preference for Phe in P3 by AMV/RSV PR relative to HIV-1 PR can be explained by the presence of Gly-106′ in AMV/RSV PR, as compared with Val-82′ in the analogous position of HIV-1 PR (21) and Val-87′ of EIAV PR. Val-82′ is at the top of the HIV-1 S3 subsite pointing into the pocket (Fig. 3*B*) so that it could sterically interfere with the binding of a large amino acid residue such as Phe. This steric hindrance is not present with Gly in the AMV/RSV subsite.

The AMV/RSV PR S3 subsite also contains 2 basic residues, His-65 and Arg-105, not found in the HIV-1 S3 subsite. These residues confer on portions of the avian subsite a greater degree of hydrophilicity (21). However, the charged groups of these amino acids are not positioned to provide strong ionic interactions with substrate as evident from the very poor catalytic efficiency of Asp substituted peptide. The aliphatic side chain of Arg-105 in AMV/RSV PR may have an important hydrophobic role in defining the S3 subsite. In agreement with these results, Konvalinka *et al.* (39) found that HIV-1 PR will accept a variety of residues in the P3 position, whereas Strop *et al.* (23) reported that the AMV enzyme has a preference for large polar or nonpolar residues at this position.

Regarding the S3′ pocket, the best value was obtained with Phe substitution, similar to the results obtained with the S3 substituted peptides. Substitution with Arg also gave a substrate better than the original one. The preference for Phe over the unsubstituted Val was also found for HIV-1 and HIV-2 PRs (9) and EIAV PR (11). An interesting difference from these

TABLE IV
Residues forming the subsites of AMV and HIV-1 PRs

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 PR residue/AMV PR residue and are shown in bold. The residues in the extra loop of the AMV PR flap are in parentheses.

Subsite	HIV-1/AMV residues
S4	Asp 29/41, **Asp 30/Ile 42, (Pro 62, Gln 63),** Ile 47/64, **Val 56/Met 73, Leu 76/Ala 100**
S3	Arg 8′/10′, Leu 23′/35′, Asp 29/41, **Gly 48/His 65,** Gly 49/66, **Phe 53/Gly 70, Pro 81′/Arg 105′, Val 82′/Gly 106′**
S2	Ala 28/40, Asp 29/41, **Asp/Ile 42, Val 32/Ile 44,** Ile 47/64, **Gly 48/His 65,** Gly 49/66, Ile 50′/67′, **Val 56/Met 73, Leu 76/Ala 100,** Ile 84/108
S1	Arg 8′/10′, Leu 23′/35′, Asp 25′/37′, Asp 25/37, Gly 27/39, Gly 49/66, Ile 50/67, **Thr 80′/Val 104′, Pro 81′/Arg 105′, Val 82′/Gly 106′,** Ile 84′/108′
S1′	Arg 8/10, Leu 23/35, Asp 25/37, Asp 25′/37′, Gly 27′/39′, Gly 49′/66′, Ile 50′/67′, **Thr 80/Val 104, Pro 81/Arg 105, Val 82/Gly 106,** Ile 84/108
S2′	Ala 28′/40′, Asp 29′/41′, **Asp 30′/Ile 42′, Val 32′/Ile 44′,** Ile 47′/64′, **Gly 48′/His 65′,** Gly 49′/66′, Ile 50/67, **Val 56/Met 73, Leu 76′/Ala 100′,** Ile 84′/108′
S3′	Arg 8/10, Leu 23/35, Asp 29′/41′, **Gly 48′/His 65′,** Gly 49′/66′, **Phe 53′/Gly 70′, Pro 81/Arg 105, Val 82/Gly 106**

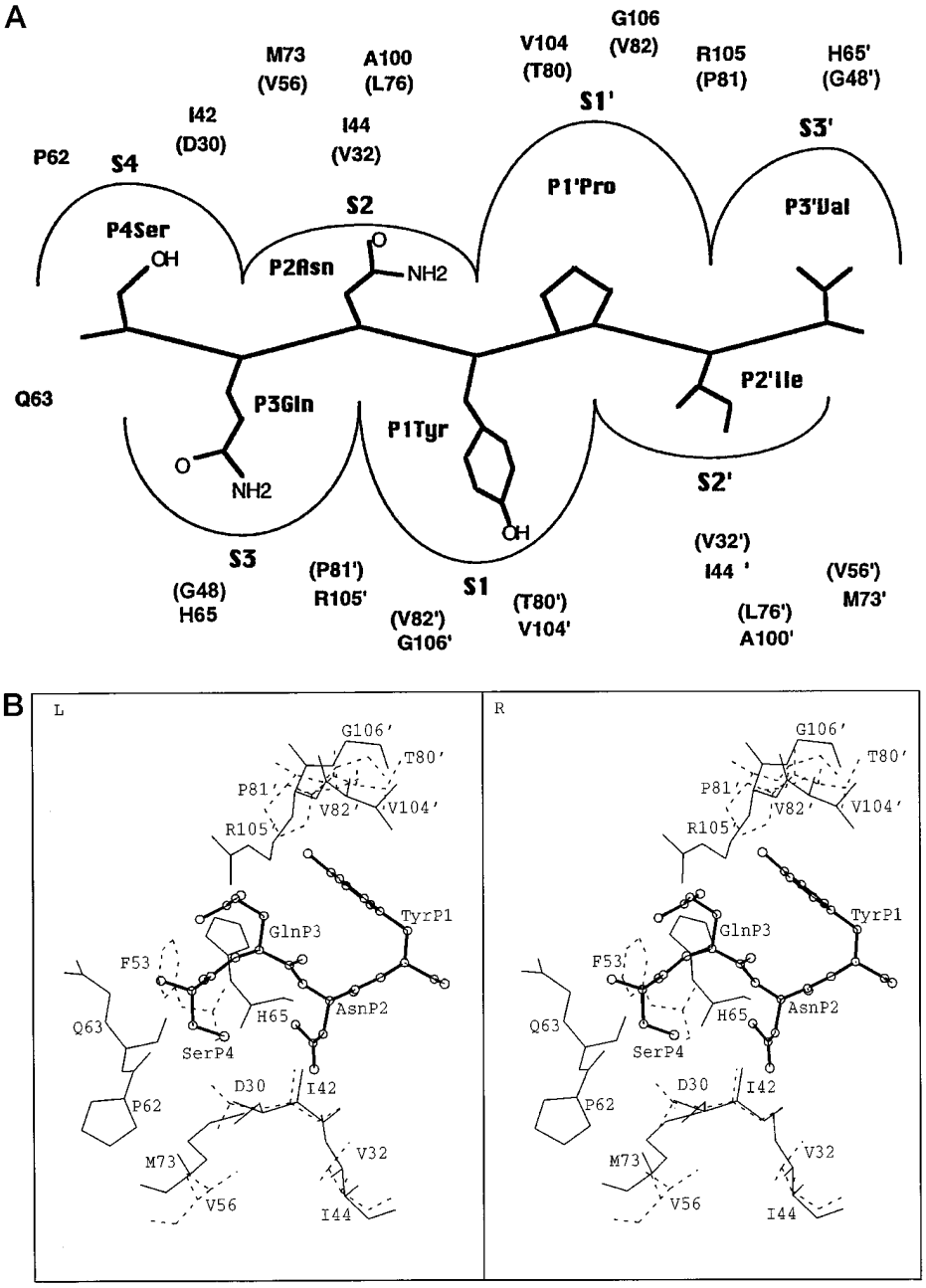

FIG. 3. *A*, schematic representation of the HIV-1 MA/CA substrate in the S4-S3′ subsites of PR. The relative size of each subsite is indicated approximately by the area enclosed by the *curved line* around each substrate side chain. Proteinase residues forming the subsites are shown for those that differ between the AMV and HIV-1 PRs. The HIV-1 PR residues are in *parentheses*. Many of the residues contribute to more than one subsite, as indicated by the position of the label. *B*, stereoview of residues P4-P1 of the HIV-1 MA/CA cleavage site with the substrate-binding residues that differ in AMV and HIV-1 PRs. The same residues are shown as indicated in the scheme of *A* and Table IV. The AMV PR residues (*thin continuous lines*) are from the crystal structure of RSV protease (16). The HIV-1 PR residues (*dashed lines*) and the substrate residues (*thick lines* in a *ball-and-stick representation*) are from a model based on the crystal structures of HIV protease with peptide-like inhibitors. The modeling procedure is described under "Materials and Methods."

enzymes is that they also prefer Leu in this position while the AMV PR does not.

Subsites S2 and S2′—By changing the P2 Asn of SP-211 to small or medium sized hydrophobic residues, a substantial increase in relative activities was obtained, whereas for HIV-1 PR (and also for HIV-2 PR), the original Asn gave the best results. Strop *et al.* (23), using AMV PR, also found a preference for small hydrophobic residues in S2. EIAV PR (11) and MuLV

PR (38) also preferred medium sized hydrophobic residues at this position, although the increases in relative activities or k_{cat}/K_m values were much less pronounced (maximum 10-fold increase) than observed for AMV PR. There is a difference in the preferred size of the hydrophobic side chains. For AMV and EIAV PR the smaller Ala, Cys, and Val gave the best results (Table III., Ref. 11), whereas MuLV PR preferred the bulkier aliphatic Leu and Ile (38). However, it seems to be a common result that Phe at P2 forms a poor substrate; modeling suggests that the Phe side chain is too bulky for this subsite. Therefore, in the type I sequence context, the preference for Asn by the HIV PRs is rather exceptional, because the other studied retroviral proteinases showed much higher preference for small or medium sized hydrophobic residues.

The S2 subsites of all PRs are sterically more restricted compared with the S4 and S3 subsites and are predicted to accommodate hydrophobic residues. The P2 side chain is surrounded by five Ile side chains in AMV PR (residues 42, 44, 64, 108, and 67′), and three of them are conserved in HIV-1 PR, but the residue corresponding Ile-44 is Val-32, and that corresponding to Ile-42 is Asp-30. The ability of HIV-1 PR to accommodate more polar residues may be related to the presence of Asp-30 (21). In analogous positions, the Thr in EIAV (11) and His in MuLV (38) also may be responsible for accommodation of more polar P2 residues. The two residues that seem to be crucial in determining the preference for Val over Leu at P2 in our substrate series are Ile-44 and Ile-64. The corresponding side chains of retroviral proteinases are shown in Table V. Based on this comparison, in both positions Ile favors Val at P2 in the substrate, whereas Val favors Leu. However, in this respect the residue equivalent to Ile-64 seems to be more crucial in the comparison of HIV-1 and HIV-2 values. A Val preference over Leu was obtained for wild type AMV/RSV PR, but a Leu over Val preference for HIV-1 PR using a substrate series based on the NC/PR cleavage site and predicted to be due to the role of Ile-44 compared with Val-32 in HIV-1 PR (21). A mutant RSV PR containing Val in place of this Ile resulted in higher relative activity than the wild type enzyme for the Leu substituted peptide, but the preference for Val still remained (22). The removal of a methyl group in the side chain of the substrate can be compensated for by the addition of a methyl group to the side chain of the amino acid in the enzyme subsite, as suggested by Cameron *et al.* (21); optimization of van der Waals' interactions in the individual enzyme subsites is a pri-

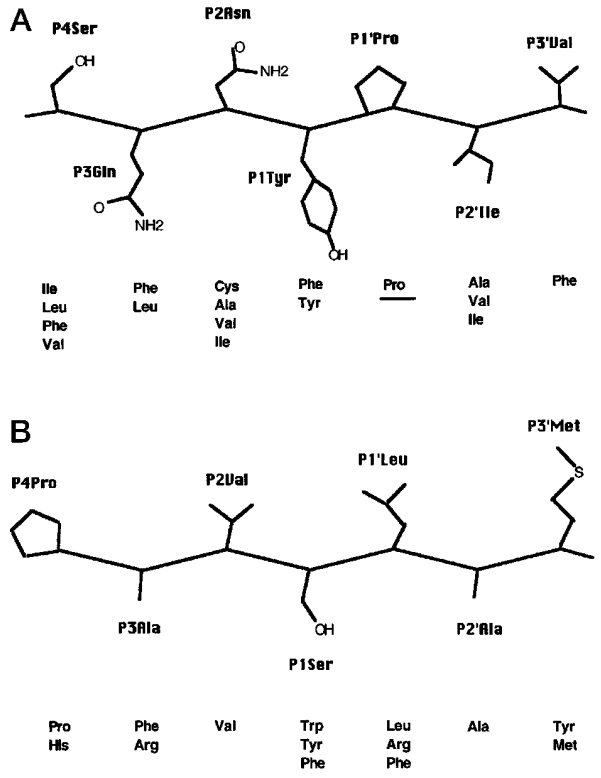

FIG. 4. **Schematic representation of the HIV-1 MA/CA (*A*) and RSV NC/PR (*B*) substrates.** Preferred substitutions for the HIV-1 peptide by the AMV PR (based on Table III) and those for the RSV substrate by AMV PR (based on Ref. 21) are listed under the residues.

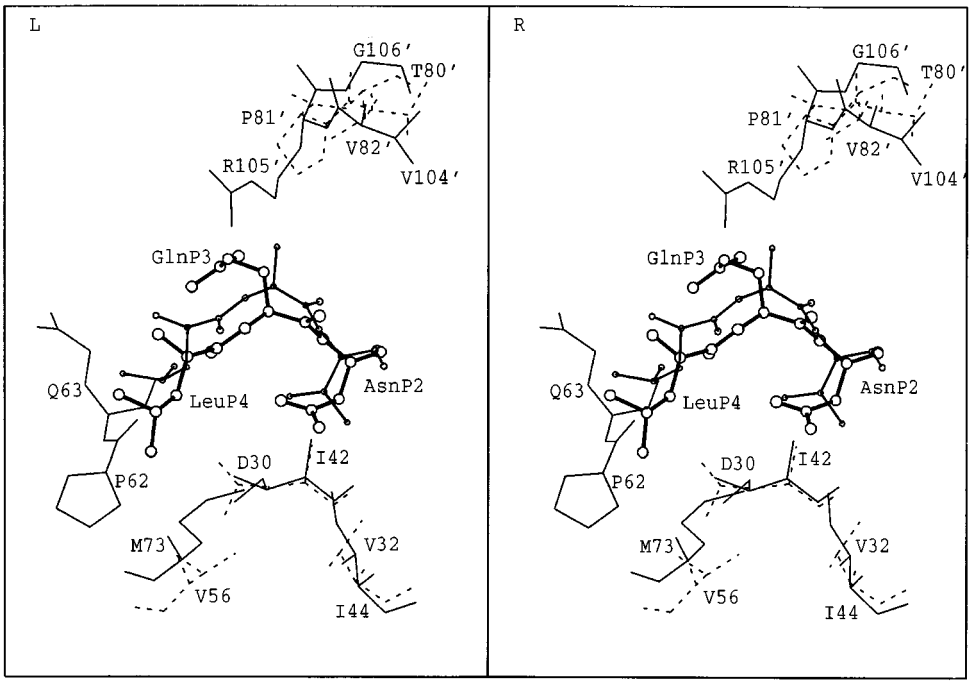

FIG. 5. **Interactions of P4 and P2 residues.** Residues forming the S4 to S2 subsites of RSV (*thin continuous lines*) and HIV-1 (*dashed lines*) proteinases are shown together with P3-P1 residues of two different substrates (in a *ball-and-stick representation*). The MA/CA substrate of HIV-1 with Leu at P4 is shown in *thicker lines*, and the NC/PR substrate of RSV with Leu at P4 is in *thinner lines*. The HIV-1 PR residues and the substrate residues are from models based on the cystal structures of HIV-1 PR with peptide-like inhibitors.

mary determinant in selection of amino acids in the different substrate positions.

The S2′ subsite, like S2, is also predicted to accommodate smaller hydrophobic residues. In accordance with this, the best result was obtained with Ala substitution. It is interesting to note that the changes in relative activities are not as marked than as for the P2 substitutions, the Ile residue of the unmodified peptide could already provide good interactions. This also seems to be common with EIAV PR and MuLV PR using the same substrate series (11, 38) and for HIV-1 and RSV PR using the NC/PR based series (21) in which Ala at P2′ gave the best result (21). For AMV PR, Strop *et al.* (23) reported an apparently symmetrical requirement for small hydrophobic residues in both P2 and P2′ and large hydrophobic residues in P1 and P1′. However, their series was based on a type II cleavage site containing two hydrophobic residues at both P1 and P1′ positions (Tyr and *p*-nitophenyl residues, respectively), whereas our series was based on a type I (Tyr ↓ Pro) cleavage site peptide. Studying HIV proteinase specificity, marked differences were found in subsite specificity of these two types of aromatic-Pro and hydrophobic-hydrophobic (not Pro) cleavage sites; for example β-branched P2 residues are the best in type II sites, but they are not optimal in type 1 sites due to steric collision with P1′ Pro (9, 14).

Subsites S1 and S1′—Relative activities obtained with the avian enzyme for the P1 substituted peptides were very similar to those obtained with HIV-1 PR (see Table III) and EIAV PR (11). None of the P1 modified peptides showed a relative activity higher than that obtained with the unsubstituted peptide except that containing Phe instead of Tyr. Also, the k_{cat}/K_m values for HIV-2 PR (10) and MuLV PR (38) suggested a very similar preference for the P1 side chains, having Phe > Tyr > Leu > Met for AMV, HIV-1, EIAV and MuLV PRs (for HIV-2, Met substitution produced a higher k_{cat}/K_m value than substitution with Leu). It is common in all cases that substitutions having Gly, Ser, Asp, or Lys at P1 site gave nonhydrolyzable substrates, whereas peptides with Ala, Val, Ile, or Trp gave either noncleavable or very poor substrates. However, in the NC/PR series Trp and Leu substitutions of the P1 Ser gave the best values. The S1 and S3 subsites are overlapping (Fig. 6). The P3 Gln of the MA/CA peptide restricts the available space for P1 residue, whereas the P3 Ala in the NC/PR peptide does not (Fig. 6). In naturally occurring cleavage sites for AMV, HIV-1, HIV-2, and EIAV and also of other retroviral proteinases, hydrophobic amino acids predominate in P1 position (15). Our results imply that the S1 binding site of all the studied retroviral proteinases is very similar for the SP-211 substituted peptides and in this sequence context the S1 sites do not contribute substantially to the observed differences in specificity of the PRs.

Based on molecular modeling, the S1 subsite is mainly hydrophobic, located deep inside the protein. Many of the residues forming the S1 site of the HIV-1 and RSV PRs are conserved. However, there are some nonconserved changes, including Val-104′ (Thr-80′), Arg-105′ (Pro-81′), and Gly-106′ (Val-82′). Arg-105′ seems to contribute to both S1 and S3 subsites, whereas Gly-106′ renders the RSV PR S1 pocket bigger than that of HIV-1 PR (Fig. 6). It is an interesting feature of our substrate series that the P1 Tyr side chain is predicted to occupy that region, which is also a part of the S3 pocket. The S1 and S3 subsites seem to be overlapping much more than S4-S2, S2-S1′, S1-S2′, and S1′-S3′ pockets. This could be a major factor in sequence dependence of the results of specificity studies based on different starting peptide sequences. Modeling also suggests that depending on the P3 residue, OH of Tyr and the bulky Trp side chain does not fit well into the AMV pocket, as was also observed for HIV proteinases (9).

It is worth noting that the peptide containing Val at P1 was hydrolyzed by AMV PR, but that containing Ile was not hydrolyzable. The peptides containing β-branched residues (Val and Ile) at P1 of the MA/CA peptide were not hydrolyzable by HIV-1

TABLE V
Relative preference for Val over Leu substitutions of P2 Asn of SP-211 as function of side chains in the proteinases

Proteinase	Residues in equivalent positions		Relative preference
RSV	Ile-44	Ile-64	6.6
EIAV	Val-32	Ile-53	3.4
HIV-1	Val-32	Ile-47	2.8
HIV-2	Ile-32	Val-47	0.5
MuLV	Val-39	Val-54	0.05[a]

[a] Taken from k_{cat}/K_m value (38). MuLV PR has many other changes in the S2 pocket that might interfere with the role of the Val-39 and Val-54 in the enzyme-substrate interaction.

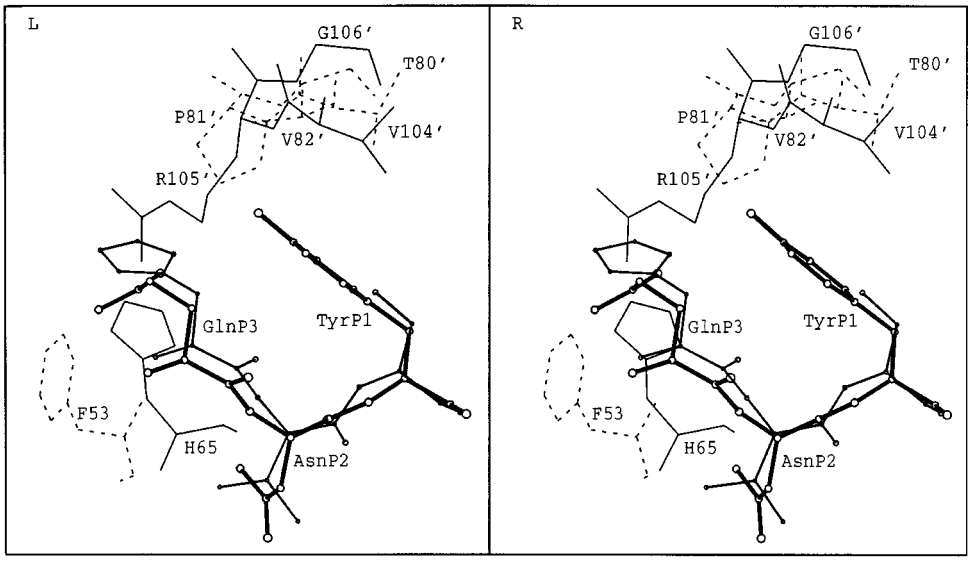

FIG. 6. **Interactions of P1 and P3 residues.** Residues forming the S1 and S3 subsites of RSV (*thin continuous lines*) and HIV-1 (*dashed lines*) proteinases are shown together with P3-P1 residues of two different substrates (in a *ball-and-stick representation*). The MA/CA substrate of HIV-1 is shown in *thicker lines*, and the NC/PR substrate of RSV with His at P3 is in *thinner lines*. The HIV-1 PR residues and the substrate residues are from models based on the cystal structures of HIV-1 PR with peptide-like inhibitors.

and HIV-2 proteinases (9) and by EIAV PR (11). Similar results were obtained for HIV-1 PR using another peptide series (40, 41). However, a small rate of hydrolysis was obtained with P1 Val and P1 Ile substituted SP-211 analogs with MuLV PR, and this was attributed to the presence of Ala-57 instead of Ile in HIV PRs. In the analogous position, AMV PR also contains Ile-57, but the change of Gly-106 in place of Val-82 of HIV-1 may also provide a somewhat larger substrate binding pocket, accepting β-branched residue.

Another common characteristic for the studied retroviral proteinases is that changing the P1′ proline in the type I MA/CA peptide to any other tested amino acids (see Table III) gave nonhydrolyzable or very poor substrates (9, 11), whereas substitutions in Type II sequences provided good substrates (21, 41). These results indicate that specificity at P1′ position of the retroviral PRs is also strongly dependent on the surrounding sequence.

Conclusion

Comparison of the specificity of the AMV PR to the HIV-1, HIV-2, and EIAV proteinases using the type I MA/CA substrate series, as well as comparing the AMV PR to HIV-1 PR specificity with the type II NC/PR substrate series suggests that these PRs have many common features. All prefer hydrophobic residues at the P1 position, although the optimal size of the residue may depend on the residues forming S1 subsite and may also be a function of the residue at P3. P1′ Pro is unique in the type I MA/CA peptide, because changing it to any other tested amino acid prevented hydrolysis by the retroviral proteinases (Table III and Refs. 9, 11, and 38). The size of the S2 and S2′ subsites is restricted, and these are predicted to be the smallest subsites in all cases, but the preference for P2 is highly variable due to the different PR residues forming the S2 subsite and also depends on the P1′ residue (9, 14), P1, and perhaps the P4 residue. In the type I sequence context, the preference for Asn by the HIV PRs is rather exceptional, because all of the other studied retroviral proteinases showed much higher preference for small or medium sized hydrophobic residues. Subsite S3 is more open than S2 and can accept a variety of residues. Specificity in S3 is a function of the P1 residue; a large P1 side chain restricts the size of the P3 residue that can be accommodated. Although S4 is close to the surface, it shows a preference for hydrophobic residues except for HIV PRs, although the size of the preferred residue is a function of P2. Our results suggest that the specificity of retroviral proteinases is very complex and strongly depends on the context of the substrate sequence. The preference at a given position may depend not only on the complementarity of residues at the same side of the β sheet (like P3 and P1, P2 and P1′) but also on those at the opposite side. However, modeling in many cases could give an explanation for the sequence context dependence and is a promising tool to "decode" the specificity of the retroviral proteinases. The strong sequence context dependence should be taken into account in the design of proteinase inhibitors, because developing resistance is one of the most serious problems in treatment of AIDS. A mutation in a substrate binding subsite of the PR indirectly could influence the specificity of the other binding sites. Conversely, changing the ligand at those other affected positions could complement the changes and regain the high potency of the enzyme-ligand interaction.

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