THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 16, Issue of June 15, pp. 11711-11720, 1993
Printed in U.S.A.

Comparison of the Substrate-binding Pockets of the Rous Sarcoma Virus and Human Immunodeficiency Virus Type 1 Proteases*

(Received for publication, November 12, 1992, and in revised form, February 4, 1993)

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A steady state kinetic analysis of the avian myeloblastosis virus/Rous sarcoma virus (AMV/RSV) and human immunodeficiency virus Type 1 (HIV-1) retroviral proteases (PRs) was carried out using a series of 40 peptide substrates that are derivatives of the AMV/RSV nucleocapsid-PR cleavage site. These peptides contain single amino acid substitutions in each of the seven positions of the minimum length substrate required by the PR for specific and efficient cleavage. These peptide substrates are distinguished by the individual enzyme subsites of the AMV/RSV and HIV-1 PRs. The molecular basis for similarities and differences of the individual subsites for both proteases is discussed using steady state kinetic data and modeling based on crystal structures.

The retroviral protease (PR)[1] is required for production of mature, infectious virus particles (1-3). As a result, this enzyme is a suitable target for the design of antiviral agents. However, rational drug design has proven to be a formidable task since the molecular forces that drive formation of the PR-substrate complex are not fully understood.

Studies on the activity of PR on peptide substrates have shown that the enzyme requires at least 7 amino acid residues for specific and efficient processing. This includes 4 amino acid residues on the amino-terminal side and 3 amino acid residues on the carboxyl-terminal side of the scissile bond of a substrate. These are referred to as P4–P1 and P1′–P3′, respectively, using the nomenclature of Schechter and Berger (4). Inspection of these 7 residues from a variety of sequences known to be specifically and efficiently processed by PR does not reveal a simple consensus sequence.

Structural analysis of inhibitor complexes of HIV-1 PR (5–11) indicates that the substrate most likely binds to PR in an extended β conformation, maintained by a network of hydrogen bonds between the main chain amide and carbonyl groups of the substrate and PR (12, 13). In addition, subsites (S4–S3′) of the substrate-binding pocket have been identified from structural data. The inhibitors used are generally hydrophobic and are capable of interacting with the P3–P3′ residues of the substrate. Shown schematically in Fig. 1 is an AMV/RSV peptide representing the cleavage site found between the AMV/RSV nucleocapsid (NC) and PR proteins (14) and referred to as the NC-PR peptide substrate. This peptide substrate is depicted as being bound to the substrate-binding pocket. This diagram also emphasizes the relative size and position of the S4–S3′ subsites that interact with each substrate position and lists amino acids that differ between the RSV and HIV-1 PR subsites. Interestingly, essentially identical subsites have been described for the various HIV-1 PR complexes despite large differences in the primary structure of the substrate-based inhibitors (5–11). This suggests that the subsites are constrained by the structure of PR and to a large extent are independent of substrate sequence. This hypothesis is supported by data obtained from the analysis of AMV/RSV PRs with mutations in the substrate-binding pocket that demonstrate that PR subsites are capable of acting independently in the selection of substrates (12). Note that the RSV and AMV PRs, which differ in sequence by two amino acids, are biochemically indistinguishable and have been used interchangeably in these studies. In order to avoid confusion, we will use AMV/RSV throughout to refer to both PRs. However, the viral PR was purified from AMV, whereas the RSV PR was bacterially produced.

Previously, we described the activities of AMV/RSV and HIV-1 PRs on an AMV/RSV NC-PR peptide substrate that was cleaved specifically and efficiently by both enzymes. In a subsequent report, we characterized the activities of both AMV/RSV and HIV-1 PRs on analogs of the NC-PR peptide substrate that contained single amino acid substitutions beginning in the P4 position of the substrate and extending through the P3′ position (12). This set of systematically modified peptides was capable of biochemically distinguishing the subsites of these PRs. In this report, we describe results from kinetic analyses of AMV/RSV and HIV-1 PRs on the 40 modified NC-PR peptide substrates and discuss these data

* This work was supported in part by United States Public Health Service Grant CA52047 (to J. L.) and Contract N01 C0 74101 with ABL (to A. W.) from the National Cancer Institute, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of Predoctoral Fellowship GM13628 from the National Institutes of Health.

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[1] The abbreviations used are: PR, retroviral protease; RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; HIV, human immunodeficiency virus; NC, nucleocapsid.

in terms of the molecular basis of PR substrate specificity and catalytic efficiency. Furthermore, this study has identified amino acids in each of the enzyme subsites that may be important determinants for specific recognition of substrates.

EXPERIMENTAL PROCEDURES

Purification of Retroviral Proteases—AMV PR, purified from virus as described (15), was obtained from Molecular Genetic Resources, Tampa, FL. HIV-1 PR expressed in and purified from bacteria was a generous gift of Dr. J. Giam, Case Western Reserve University.

Peptides—The peptides used in this study were synthesized and purified by Advanced Chemtech (Louisville, KY). Peptides were solubilized in 1 mM 2-mercaptoethanol, and their concentrations were determined by quantitative amino acid composition analysis. Specificity of cleavage was established by direct amino-terminal analysis of product peptides (16).

Assay of PR Activity—The reaction mixture contained 100 mM sodium phosphate, pH 5.9, 2.4 M sodium chloride (1 M sodium chloride was used for HIV-1 and HIV-2 PR reactions), 10–400 μM peptide as indicated, and 0.5–5 μg/ml PR. Reaction volumes were 25 μl. Reactions were initiated by the addition of PR, incubated for various periods of time, and stopped by the addition of 175 μl of 0.5 M sodium borate, pH 8.5. Twenty μl of 0.05% (w/v) fluorescamine was then added. HIV-1 PR was never incubated more than 8 min due to its instability, presumably a result of autodegradation. The relative fluorescence, after reaction with fluorescamine, was determined on a Perkin-Elmer LS-5B spectrofluorometer using an excitation wavelength of 386 nm and an emission wavelength of 477 nm. Excitation and emission slit widths were 10 nm. Relative fluorescence intensity was converted to nanomoles of product using a standard curve described by the following equation: nmol of product = relative fluorescence intensity/313. The standard curve was obtained using a hexapeptide with a free amino terminus (14). The concentration of this peptide was determined by amino acid composition analysis. The peptides used in this study were designed with prolines at their amino termini so that the relative fluorescence intensity represents only the newly formed amino termini produced as a result of proteolytic cleavage. Arginine residues were added to the carboxyl terminus to improve solubility of the peptides. The addition of these amino acids did not influence their kinetic parameters.

Steady State Kinetic Analysis—Kinetic constants were determined using the assay described above. Concentrations of peptide ranged from 0.25–4 times the K_M value. No more than 20% of the substrate was allowed to be consumed during the course of any given experiment. Initial velocity data used to calculate kinetic constants were obtained from at least three experiments performed in duplicate. Kinetic constants were determined by a nonlinear fit of the data to the Michaelis-Menten equation using the NFIT program (14). Correlation coefficients of the fit were greater than 0.98, and the standard deviation of the constants reported was ≤20%.

Molecular Modeling—The crystal structures of the native RSV PR (17) and HIV-1 PR, as well as the complexes of the latter with inhibitors (5–9) were superimposed using C_α atoms and examined on an Evans and Sutherland ESV10 computer graphics system using the program FRODO (18). The residues forming the subsites were determined directly for the HIV-1 PR complexed with inhibitors, and the corresponding residues in RSV PR were obtained from the structural and sequence alignments (12). The NC-PR substrate was modeled as described previously (12), and different amino acid side chains were substituted at positions P4 to P3′ in order to provide a structural explanation for the kinetic data.

RESULTS AND DISCUSSION

Rationale for Choice of Substitutions in Peptide Substrates—We have synthesized 40 different peptide substrates, based on the AMV/RSV NC-PR cleavage site (PPAVS-LAMTM), which contain single amino acid substitutions in each of seven positions, P4–P3′. The amino acids present in these seven positions of the substrate have been shown by biochemical and crystallographic analyses to be necessary for specific and efficient processing by the retroviral PR. The amino acids substituted into the NC-PR peptide were chosen to explore predictions of size and polarity of each of the seven known subsites of the PR substrate-binding pocket. For instance, Gly, which has no side chain, was substituted at each position to test the importance of substrate side chain interactions with each PR subsite. A number of general features of the AMV/RSV subsites were taken into account in designing the NC-PR analogues. For example, subsite S4 is near the protein surface so that it may accommodate a variety of different types of substrate side chains. Subsite S3 is formed partially by the charged residues, Arg-10 and Arg-105. Subsite S2 is small and hydrophobic, whereas subsite S1 is large and hydrophobic. Subsite S1′ is medium-sized and hydrophobic, and the natural substrates do not show a residue larger than Leu at P1′. Subsite S2′, like S2, is small and hydrophobic. Subsite S3′ is near the surface of the enzyme and is partially exposed to solvent.

The studies described here utilized peptides as substrates in place of the multidomain polyproteins that are substrates *in vivo*. This strategy appears to be valid since peptides based upon the naturally occurring cleavage sites are specifically and efficiently cleaved by the PR of the virus from which the cleavage sites were derived (12, 14, 19–21). Furthermore, the relative efficiencies of processing of various sites were similar for both polyprotein and peptide substrates (22, 23).[2]

Steady State Kinetic Analysis of the NC-PR Peptide Analogues with the AMV/RSV and HIV-1 PRs—The steady state kinetic parameters for each of the NC-PR peptide analogues with AMV/RSV or HIV-1 PRs are presented in Tables I and II. The K_M and k_{cat} data are presented relative to those observed with the wild type AMV/RSV NC-PR substrate. For AMV/RSV PR, the K_M is 41.6 μM, k_{cat} is 21.6 min^{-1}, and the calculated k_{cat}/K_M value is 0.51 min^{-1} μM^{-1}. For HIV-1 PR, the K_M is 15.8 μM, the k_{cat} is 43.8 min^{-1}, and the calculated k_{cat}/K_M value is 2.77 min^{-1}. The AMV/RSV NC-PR substrate is one of the most efficiently processed cleavage sites for AMV/RSV PR (20). The HIV-1 PR binds this substrate with a greater affinity than the avian enzyme and cleaves it with an overall catalytic efficiency (k_{cat}/K_M) comparable to that of its homologous substrates (20). The AMV/RSV NC-PR cleavage site contains relatively small amino acid side chains at each position, except for leucine at P1′ and methionine at P3′. The existence of small side chains in this substrate permits the substitution of a wide variety of amino acids in each position of the cleavage site without the complication of forming destabilizing interactions between residues adjacent to one another in the substrate-binding pocket, as noted by Tozser *et al.* (24). Thus, the NC-PR cleavage site is well suited for the studies described below that probe the interaction of individual PR subsites of the AMV/RSV and HIV-1 substrate-binding pocket with substrate. We were able to perform a steady state kinetic analysis with the HIV-1 PR using all of the AMV/RSV NC-PR peptide analogues, with the exception of Trp substituted in P2′ and Gly or Asp substituted in P3′. For AMV/RSV PR, the rate of cleavage was too low for reliable kinetic analysis with Leu or Phe substituted in P4, Pro in P3, Gly and His in P2, Gln or Gly in P1′, His or Trp in P2′, and Leu, Asn, Asp, and Gly in P3′.

To provide perspective on the very complex substrate specificity of retroviral PRs, the sequence of the AMV/RSV NC-PR substrate, together with the amino acids found in the P4–P3′ positions of all natural AMV/RSV and HIV-1 *gag-pol* polyprotein-processing sites are listed in Table III. Note that these sequences are not necessarily the best possible substrates due to the need for regulation of cleavage of the polyproteins. In many cases, substitutions of more favorably fitting amino acids results in increased activity. A comparison of the amino acids predicted to form the seven HIV-1 and

[2] C. Cameron and J. Leis, unpublished observations.

TABLE I

Comparison of HIV-1 PR kinetic data for AMV/RSV NC-PR substrates with substitutions in the P4–P3′ positions

Varying concentrations of the NC-PR (PPAVS LAMTMRR) or NC-PR substrates with amino acid substitutions in the P4–P3′ positions as indicated were incubated with purified HIV-1 PR (6–24 ng), and the extent of cleavage was determined using the fluorescamine assay as described under "Experimental Procedures." The wild type amino acid in the NC-PR peptide is indicated in the parentheses in the first column. ND, cleavage products were not detected. This would indicate that the relative catalytic efficiency (k_{cat}/K_M) for these reactions was <0.001.

Substrate position	Substituted amino acid	K_M	Relative k_{cat}[a]	k_{cat}/K_M
P4 (Pro)	His	0.5	1.1	2.3
	Gly	1.7	1.2	0.7
	Asn	3.1	1.6	0.5
	Phe	15.9	0.3	0.02
	Leu	33.1	0.4	0.02
P3 (Ala)	Arg	0.6	1.0	1.6
	Pro	0.7	0.9	1.2
	Phe	0.7	0.8	1.2
	Gly	0.8	0.8	1.0
	Asp	1.0	0.9	0.9
	His	1.1	0.9	0.8
	Asn	1.2	1.0	0.8
P2 (Val)	Leu	0.7	0.8	1.2
	Gly	1.4	0.9	0.6
	Ser	1.9	0.8	0.4
	Trp	9.8	0.8	0.08
	His	12.6	0.2	0.02
P1 (Ser)	Leu	1.1	12.8	11.6
	Trp	0.3	2.7	8.1
	His	0.8	1.4	1.6
	Ala	1.0	1.5	1.5
	Arg	0.9	1.2	1.4
	Glu	1.5	1.2	0.8
	Gly	1.2	0.7	0.6
P1′ (Leu)	Phe	1.0	0.7	0.7
	Arg	1.7	0.2	0.09
	Gln	3.2	0.1	0.04
	Glu	4.7	0.04	0.01
	Gly	3.1	0.01	0.002
P2′ (Ala)	Leu	1.3	0.3	0.3
	Ser	4.6	0.4	0.09
	Gly	20.2	0.9	0.05
	His	9.2	0.1	0.02
	Trp	ND	ND	
P3′ (Met)	Leu	0.7	0.4	0.5
	Tyr	8.8	0.4	0.04
	Asn	5.5	0.03	0.007
	Gly	ND	ND	
	Asp	ND	ND	

[a] The kinetic parameters for the P4–P3′-substituted NC-PR substrates are given relative to the unmodified wild type substrate, which is defined as equal to 1 in each case. For HIV-1 PR, the K_M is 15.8 μM, the k_{cat} is 43.8 min^{-1}, and the calculated k_{cat}/K_M value is 2.77 min^{-1}.

TABLE II

Comparison of AMV/RSV PR kinetic data for AMV/RSV NC-PR substrates with substitutions in the P4–P3′ positions

Varying concentrations of the same NC-PR (PPAVS LAMTMRR) or NC-PR substrates with amino acid substitutions in the P4–P3′ positions as indicated were incubated with purified AMV/RSV PR (25–100 ng), and steady state kinetic data were analyzed. Table notations are as described in the legend to Table I.

Substrate position	Substituted amino acid	K_M	Relative k_{cat}[a]	k_{cat}/K_M
P4 (Pro)	His	1.5	1.0	0.7
	Asn	1.0	0.3	0.3
	Gly	0.6	0.06	0.09
	Leu	ND	ND	
	Phe	ND	ND	
P3 (Ala)	Arg	0.6	3.0	5.0
	Pro	ND	ND	
	Phe	0.16	1.2	7.5
	Gly	1.8	0.65	0.36
	Asp	2.3	1.76	0.76
	His	0.5	1.8	3.6
	Asn	1.0	2.0	2.0
P2 (Val)	Leu	1.6	0.1	0.06
	Gly	4.9	0.2	0.04
	Ser	57.7	0.6	0.01
	Trp	24.5	0.52	0.02
	His	ND	ND	
P1 (Ser)	Leu	0.5	4.4	8.5
	Trp	0.2	5.1	25.5
	His	0.76	1.5	2.0
	Ala	1.9	0.95	0.5
	Arg	0.7	3.0	4.3
	Glu	0.9	1.5	1.7
	Gly	0.7	0.1	0.1
	Tyr	0.59	11.3	19.1
	Phe	0.42	7.0	16.7
P1′ (Leu)	Phe	1.1	0.4	0.4
	Arg	0.9	0.7	0.8
	Gln	ND	ND	
	Glu	3.7	0.23	0.06
	Gly	ND	ND	
P2′ (Ala)	Leu	0.6	0.06	0.1
	Ser	7.2	0.1	0.01
	Gly	28.3	0.21	0.007
	His	ND	ND	
	Trp	ND	ND	
P3′ (Met)	Leu	ND	ND	
	Tyr	1.2	1.9	1.6
	Asn	ND	ND	
	Gly	ND	ND	
	Asp	ND	ND	

[a] The kinetic parameters for the P4–P3′-substituted NC-PR substrates are given relative to the unmodified wild type substrate, which is defined as equal to 1 in each case. For AMV PR, the K_M is 41.6 μM, k_{cat} is 21.6 min^{-1}, and the calculated k_{cat}/K_M value is 0.51 min^{-1} μM^{-1}.

AMV/RSV PR subsites, which highlights the differences between the two enzymes, is presented in Table IV and in Fig. 1. Note that because the peptide substrate binds in an extended β conformation, alternate subsites, such as S1 and S3, are adjacent to one another (Fig. 1).

Subsite S4—The S4 subsite of HIV-1 PR is located near the enzyme surface such that amino acids in the P4 substrate position may be partially exposed to surrounding water molecules (Fig. 2*a*). This is consistent with the observation that amino acids found in the P4 position of HIV-1 PR *gag* and *pol* polyprotein cleavage sites are mostly polar (Table III). Similarly, the S4 subsite of AMV/RSV PR is near the surface. However, modeling of this site predicts that Pro-62 and Gln-63, derived from the flaps, may interact with the side chain of the P4 amino acid (12) (Fig. 2*a*). These additional amino acid residues in AMV/RSV PR provide for a more enclosed S4 subsite, capable of accommodating hydrophobic residues. This is consistent with the observation that mostly small hydrophobic amino acids residues are found in the AMV/RSV *gag* and *pol* polyprotein cleavage sites at this position (Table III). When residues 61–63 are deleted from AMV/RSV PR, the avian enzyme utilizes substrates with polar residues (Asn or His) in P4 similarly to HIV-1 PR.[3] This conclusion that the AMV/RSV PR S4 subsite is more enclosed than the HIV-1 PR S4 subsite is further supported by the kinetic data summarized in Tables I and II, which demonstrate that HIV-1 PR is less sensitive to changes in the P4 position than

[3] C. Cameron, T. Ridky, J. Leis, H. Burstein, A. M. Skalka, I. Weber, T. Copeland, and A. Wlodawer, manuscript in preparation.

TABLE III

Amino acid residues in gag and pol AMV/RSV and HIV-1 protease cleavage sites

The amino acid sequence in bold letters represents the AMV/RSV NC-PR cleavage site. Numbers in parentheses indicate the multiple of times the amino acid is found in the natural *gag* and *pol* cleavage sites.

Virus	Amino acids required for cleavage						
	P4 **Pro**	P3 **Ala**	P2 **Val**	P1 **Ser**	P1′ **Leu**	P2′ **Ala**	P3′ **Met**
RSV	Pro (4)	Ala (2)	Ile	Met (3)	Ala	Val (3)	Val (2)
	Ala (2)	Leu (2)	Val (3)	Ser	Leu	Ala	Met
	Val	Thr	Ala (3)	Tyr (2)	Thr	Leu	Ala (2)
	Phe	Gln	Phe	Leu	Gly	Ile	Ser
	Thr	Val	Cys	Ala	Pro (2)	Ser	Arg
		Tyr		Gly	Ser (2)	Gly	Leu
		Ser			His	Cys	Gly
HIV	Arg (2)	Arg	Ile (2)	Leu (2)	Ala	Gln (3)	Ala
	Ala (2)	Lys	Val	Met	Met	Leu (2)	Asp
	Ser (2)	Thr	Ala	Tyr	Pro (3)	Ile (2)	Arg
	Thr	Leu	Asn (4)	Phe (3)	Phe (2)	Glu	Val
	Pro	Phe		Asn	Leu		Ile
		Gln (2)					Ser (2)
		Gly					Gly

TABLE IV

Comparison of the amino acid residues forming the subsites of the AMV/RSV and HIV-1 proteases

Subsite	Amino acid residues[a]	RSV/HIV protease residues[b]
S4	Nonconserved	*I42/D30*, M73/V56, R105′/P81′
	Unique	H7′, P62, Q63
	Conserved	R10′/8′, *D41/29*, I64/47
S3	Nonconserved	*H65/G48*, V104′/T80′, R105′/P81′, G106′/V82′
	Unique	Q63
	Conserved	R10′/8′, L35′/23′, D37′/25′, G39/27, *D41/29*, G66/49, *I67/50*, I108′/84′, R111/87
S2	Nonconserved	*I42/D30*, I44/V32, *H65/G48*, M73/V56, A100/L76
	Conserved	A40/28, D41/29, I64/47, G66/49, *I67′/50′*, I108/84
S1	Nonconserved	*H65/G48*, V104′/T80′, R105′/P81′, G106′/V82′, S107′/N83′
	Conserved	R10′/8′, L35′/23′, D37′/25′, D37/25, *G39/27*, G66/49, I67/50, I108′/84′
S1′	Nonconserved	*H65′/G48′*, V104/T80, R105/P81, G106/V82, S107/N83
	Conserved	R10/8, L35/23, D37/25, D37′/25′, *G39′/27′*, G66′/49′, *I67′/50′*, I108/84
S2′	Nonconserved	*I42′/D30′*, I44′/V32′, *H65′/G48′*, M73′/V56′, A100′/L76′, V104′/T80′
	Conserved	A40′/28′, *D41′/29′*, I64′/47′, G66′/49′, *I67/50*, I108′/84′
S3′	Nonconserved	S38′/T26′, *H65′/G48′*, V104/T80, R105/P81, G106/V82
	Unique	Q63′
	Conserved	R10/8, L35/23, *G39′/27′*, *D41′/29′*, I64′/47′, G66′/49′, *I67′/50′*, I108/84, R111′/87′

[a] The amino acid residues that are not conserved or unique to the AMV/RSV PR (not present in the HIV-1 PR) or are conserved between the AMV/RSV and HIV-1 PR subsites as indicated.

[b] Amino acid residues in the second subunit of the dimer are indicated by a prime. The first amino acid number refers to AMV/RSV, whereas the second refers to HIV-1 PR. The underlined (*italicized*) residues are involved in substrate main chain hydrogen bond formation.

AMV/RSV PR. For example, the NC-PR analogue with Gly at P4 is a reasonable substrate for HIV-1 PR but not for AMV/RSV PR, where the k_{cat}/K_M is reduced to 10% of that with Pro.

Substitution of Leu or Phe for Pro in the P4 position of the NC-PR substrate resulted in a substantial loss in binding and a small decrease in the catalytic rate by HIV-1 PR (Table I). These substitutions also resulted in a substantial decrease in cleavage by AMV/RSV PR. This result suggests that large hydrophobic residues are not tolerated in the P4 position by either enzyme, which is consistent with the presence of several polar residues in the S4 subsite (Fig. 2*a*, Table IV). In contrast, when polar residues were substituted into the P4 position, all of the peptides were substrates, although the k_{cat}/K_M values varied depending upon the amino acid substituted (Tables I and II). It is worth noting that introduction of His in the P4 position of the NC-PR substrate increased its relative K_M for HIV-1 PR but decreased it for AMV/RSV PR (Tables I and II). This result can be explained by the presence in S4 of Ile-42 in AMV/RSV PR, as compared with Asp-30 in HIV-1 PR. Molecular modeling suggests that Asp-30 of HIV-1 PR can form an ionic interaction with His in the P4 substrate position, which would improve its K_M. This has been tested experimentally by substituting Asp for Ile-42 in the AMV/RSV PR. The resulting enzyme showed a 2-fold increase in efficiency of cleavage of the His-P4 substituted NC-PR substrate,[2] similar to that observed for HIV-1 PR (Table I). Tozser *et al.* (13) found comparable results in their analysis of HIV-1 and HIV-2 PRs, *i.e.* most P4 analogues tested were utilized as substrates with the exception of those with larger hydrophobic residues. These studies used a peptide with a totally different sequence from NC-PR. Also, they observed that amino acids frequently found in turns in the three-dimensional structure of proteins (Gly, Pro, Asn), when substituted in the P4 position, produced good substrates for HIV-1 PR (13).

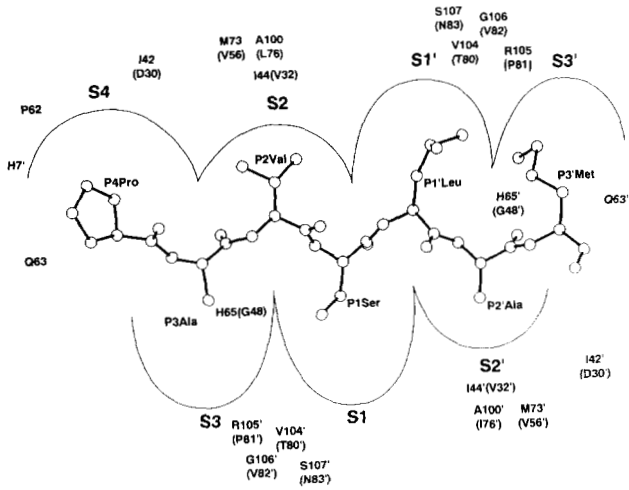


FIG. 1. **A schematic representation of the AMV/RSV NC-PR substrate, PAVSLAM, from P4–P3′ in the S4–S3′ subsites of PR.** The relative size of each subsite is indicated approximately by the area enclosed by the *curved line* around each substrate side chain. Protease residues forming the subsites are shown for those that differ between the AMV/RSV and HIV-1 PRs. The AMV/RSV PR residue is shown outside the *parentheses*, whereas the HIV-1 PR residue is shown in the *parentheses*. Most of the residues contribute to more than one adjacent subsite, and this is indicated by the position of the label.

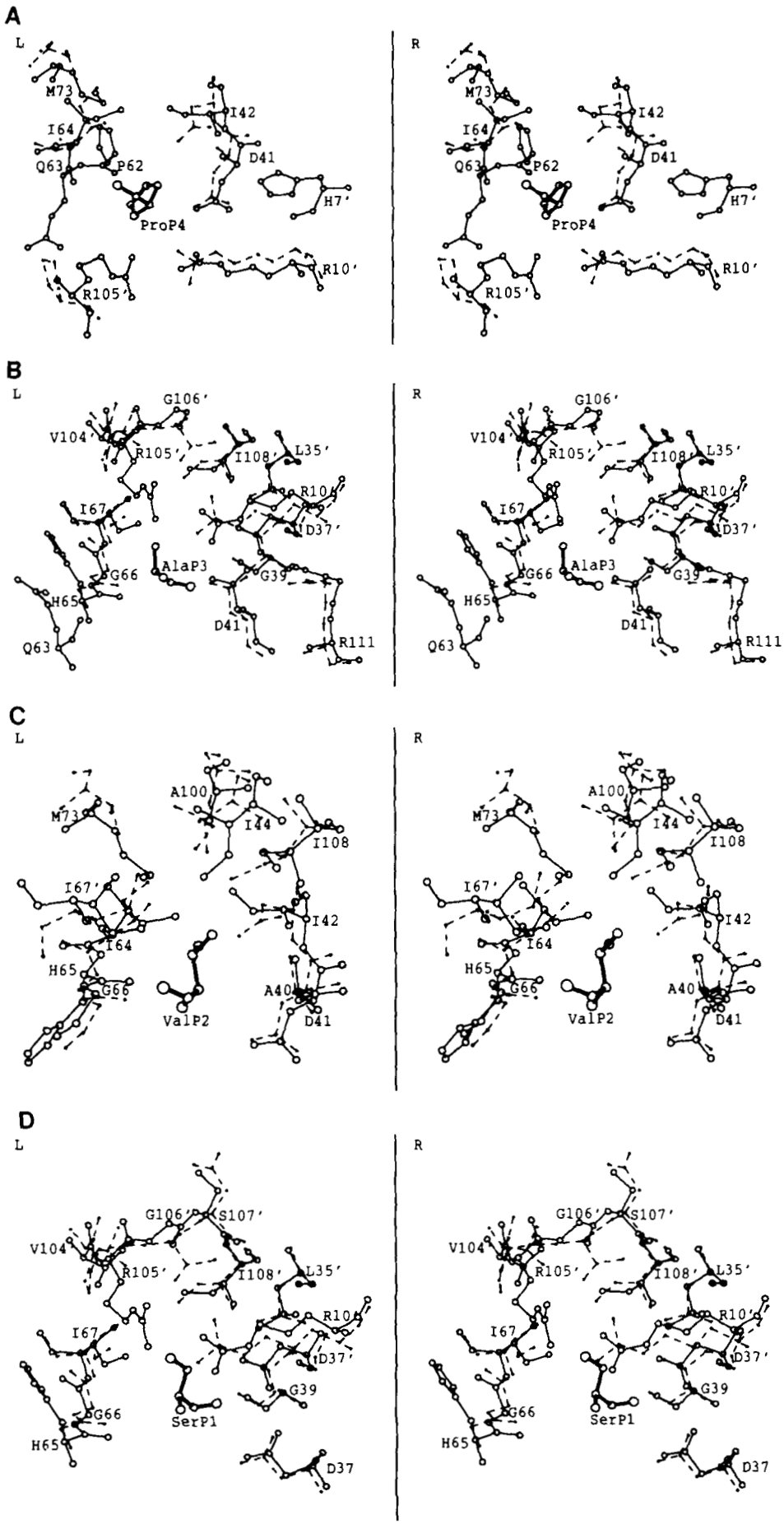
Subsite S3—The S3 subsites of HIV-1 and AMV/RSV PR consist of relatively open and deep pockets that are near the surface (Fig. 2*b*). This allows side chains of amino acids in the P3 position freedom of movement to either interact with hydrophobic residues near the S1 subsite or polar residues at the surface of the enzyme. Thus, the S3 subsite can accommodate both polar and nonpolar residues. This is consistent with the presence of both polar and hydrophobic amino acids in the P3 position of the known AMV/RSV and HIV-1 *gag* and *pol* cleavage sites (Table III). This ability of the S3 subsite to accommodate a variety of residues is also seen with substitutions in the P3 position of the NC-PR peptide (Tables I and II). Replacing Ala with larger amino acids that are charged (His, Arg), polar (Asn), or nonpolar (Phe), resulted in a 2–7.5-fold increase in efficiency of cleavage by AMV/RSV PR, depending upon the amino acid substituted. The increased efficiency was due to both an improvement in binding and an increase in catalytic rate (Table II). In contrast, substitutions of smaller residues, such as glycine or proline, were not tolerated as well.

Although AMV/RSV PR exhibited increased activity with many of the P3-modified peptides, HIV-1 PR retained about the same level of activity with all the substitutions (Table I). These results may be explained, in part, by the fact that the HIV-1 S3 subsite is more open than that of AMV/RSV due to the presence of Pro-81 in HIV-1 PR, as compared with Arg-105 at the structurally identical position in the avian enzyme (Fig. 2*b*). This difference would reduce the possibility for additional van der Waal interactions in the HIV-1 S3 subsite, thus precluding changes in catalytic efficiency. The 6-fold 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. Val is at the top of the HIV-1 S3 subsite pointing into the pocket so that it could sterically interfere with the binding of a large amino acid residue such as Phe (12). This steric hindrance would not be a problem 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. However, the charged groups of these amino acids are probably not providing strong ionic interactions with substrate. This is inferred from the fact that calculation of the relative energy of interaction from the steady state kinetic data for the P3-substituted NC-PR peptide substrates indicates that there is only a 1–1.3 kcal/mol improvement in the energy of interaction for the best substrates. Ionic interactions would contribute an additional 4–5 kcal/mol of energy to the interaction of this substrate with PR, which would correspond to increases in the catalytic efficiency of 40–50-fold (26, 27). Such large changes in the catalytic efficiencies are not observed (Table II). The magnitude of change observed here is more consistent with small perturbations in side chain van der Waal or weak hydrogen bond interactions. This suggests that 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 our results, Konvalinka *et al.* (28) found that HIV-1 PR will accept a variety of residues in the P3 position, whereas Strop *et al.* (29) report that the AMV enzyme has a preference for large polar or nonpolar residues at this position.

It is worth noting that in our kinetic studies of PR, we have not discussed the differential free energy of binding as described by Fersht (27). These calculations assume that there is no significant conformational change of the enzyme upon substrate binding. This is not the case for the retroviral PR, where comparison of the crystal structures of HIV-1 PR in the absence and presence of inhibitor (5) shows that the flaps are very flexible. They apparently must open to allow the polyprotein substrate to enter the binding site and then close to dock the substrate and restrain the scissile bond prior to catalysis (30). After cleavage, the flaps again move to release the products. In addition, we have exchanged small residues in the natural NC-PR substrate, in many cases, for larger ones. This may cause a sufficient rearrangement of the side chains in the PR substrate-binding pocket that it invalidates assumptions made by Fersht (27) in derivation of the relationship between catalytic efficiency and free energy of binding.

Subsite S2—The S2 subsites of both PRs (Fig. 2*c*) are sterically restricted, as compared with S4 and S3 and are predicted to accommodate hydrophobic residues (Table IV). Mostly small or intermediate sized hydrophobic residues are found in the P2 position of natural *gag* and *pol* cleavage sites (Table III), with the exception of Phe in one AMV/RSV site and Asn in four of the HIV-1 sites. This ability of HIV-1 PR to accommodate a more polar residue may be related to the presence of Asp-30 in the S2 subsite of HIV-1, as compared with Ile-42 at the analogous location in AMV/RSV PR. Also, the presence of Val-32 in HIV-1 PR, as compared with Ile-44 in AMV/RSV PR, allows for a larger side chain at P2.

The requirement in S2 for small hydrophobic substrate residues is consistent with the results of replacement of Val at the P2 position of the NC-PR peptide substrate with larger hydrophobic residues (Leu or Trp); there is a significant decrease in the catalytic efficiency of AMV/RSV PR (Table II). Substitution of a polar residue (Ser) or a large hydrophobic residue (Trp) in P2 caused an increase in K_M with only a modest change in k_{cat}. In contrast, the Leu-substituted analogue was more efficiently cleaved by HIV-1 PR, reflecting a small improvement in binding. This difference can be explained by the presence of Ile-44 in AMV/RSV, as compared with Val-32 in HIV-1 PR. These residues have been shown to determine the selection of amino acids in the P2 (and P2′) substrate positions by exchanging Val for Ile-44 in AMV/


A
L
M73
I64
Q63
P62
I42
D41
H7'
ProP4
R10'
R105'
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M73
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Q63
P62
I42
D41
H7'
ProP4
R10'
R105'
B
L
G106'
V104'
R105'
I108'
L35'
R10'
I67'
D37'
AlaP3
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H65
D41
Q63
R111
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G106'
V104'
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I64
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G66
A40
ValP2
D41
R
A100
M73
I44
I108
I67'
I42
I64
H65
G66
A40
ValP2
D41
D
L
G106'
S107'
V104'
R105'
I108'
L35'
R10'
I67'
D37'
G39
G66
SerP1
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G106'
S107'
V104'
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D37'
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G66
SerP1
H65
D37

FIG. 2

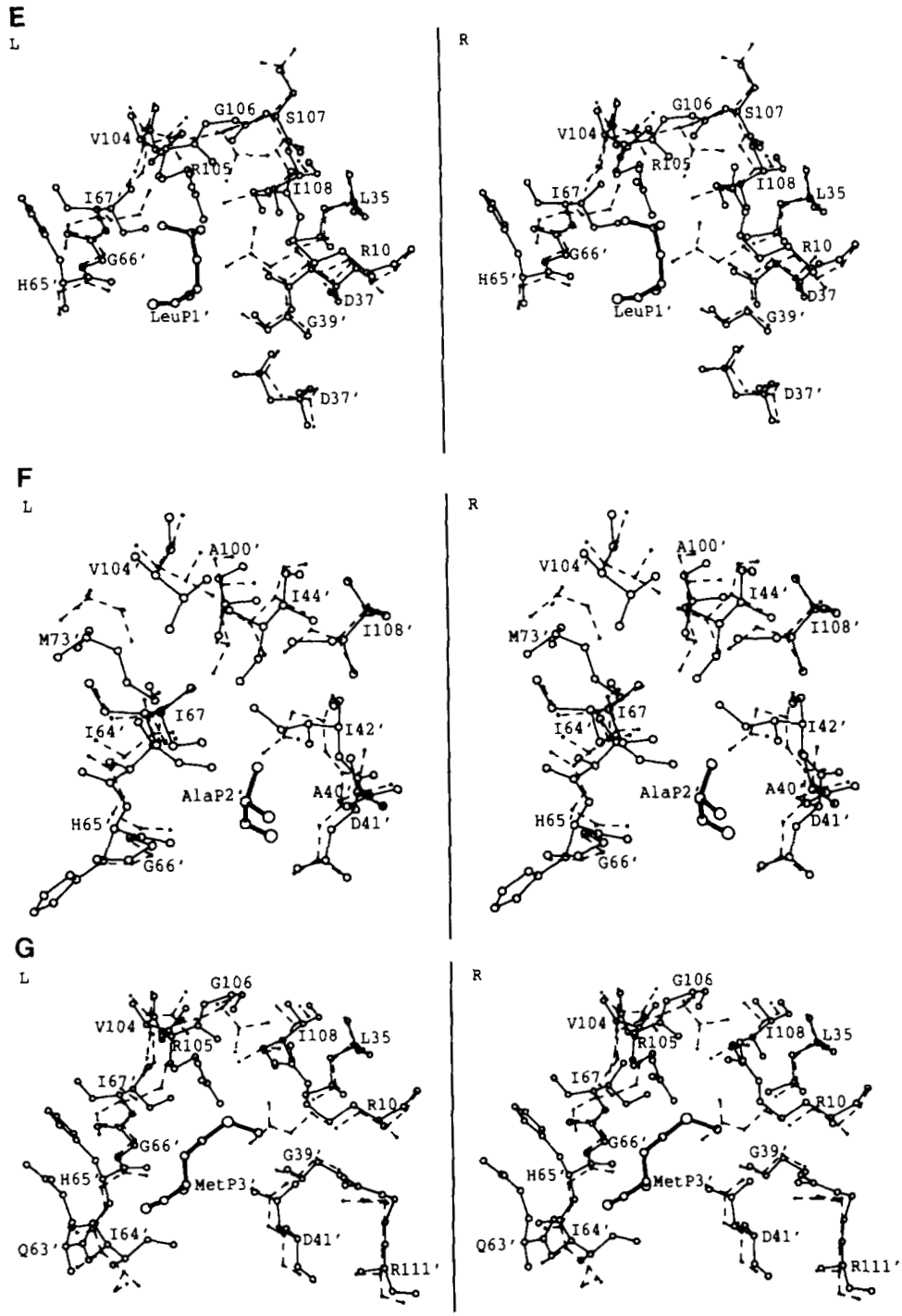

FIG. 2. **Stereo views of subsites S4–S3′ of AMV/RSV and HIV-1 proteases.** The HIV-1 PR atomic coordinates, shown by *dashed lines*, are from the crystal structure 7HVP (19) with inhibitor JG365. The RSV PR coordinates are from the crystal structure of Jaskolski *et al.* (17) with residues 61–69 of the flaps from modeling (12) and are shown in *thin continuous lines*. Note that RSV PR residues His-7, Pro-62, and Gln-63 have no corresponding residues in HIV-1 PR. The model substrate is shown in *thicker lines*. In each view, the substrate side chain is approximately vertical, and the related subsites, S3 and S3′, S2 and S2′, and S1 and S1′, are shown in very similar orientations. *a*, subsite S4 with Pro at P4; *b*, subsite S3 with Ala at P3; *c*, subsite S2 with Val at P2; *d*, subsite S1 with Ser in P1; *e*, subsite S1′ with Leu at P1′; *f*, subsite S2′ with Ala at P2′; *g*, subsite S3′ with Met at P3′.

RSV PR.[2] The wild type AMV/RSV PR prefers valine in the P2 position of the NC-PR substrate, whereas HIV-1 PR and a mutant RSV PR containing Val in the S2 subsite prefer Leu. Thus, 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. This suggests that optimization of van der Waal interactions in the individual enzyme subsites is a primary determinant in selection of amino acids in the different substrate positions. Equine infectious anemia virus PR also shows a preference for small hydrophobic residues at P2, which correlates with the presence of Thr 30 in subsite S2 in the place occupied by Asp-30 in HIV-1 PR.[4] Tozser *et al.* (24)

[4] Weber, I., Tözsér, J., Wu, J., Friedman, D., and Oroszlan, S. (1993) *Biochemistry* **32**, 3354–3362.

have reported kinetic measurements for HIV-2 PR, which has Ile at position 32 instead of the Val present in HIV-1 PR. A similar compensation in substrate for the gain of a methyl group in the amino acid at position 32 in HIV-2 was not observed. This was attributed to an unfavorable steric interaction of Pro in P1′ with the β-branched side chains such as Val in P2.

As with S3 and S4, the S2 subsite of AMV/RSV PR is predicted to be smaller than that of HIV-1 PR due to the presence of Ile-44 and Ile-42 in AMV/RSV PR, as compared with Val-32 and Asp-30 in HIV-1 PR (Fig. 2*c*). Thus, although neither enzyme preferred polar or large hydrophobic residues in P2, the HIV-1 PR was more tolerant of substitutions than AMV/RSV PR. Table II shows lower relative k_{cat}/K_M values for AMV/RSV PR than HIV-1 PR with the same analogues (Table I). The ability of HIV-1 PR to accommodate various amino acids in the S2 subsite, as well as in the S3 and S4 subsites, may explain why previous studies of HIV-1 PR demonstrated an expanded substrate selectivity range, as compared with AMV/RSV PR (20).

Konvalinka *et al.* (28), using a different peptide as a starting point, found a relatively strong preference for Val or Ile in the P2 position for HIV-1 PR. Using the same enzyme and *pol* precursor proteins as substrate, Jupp *et al.* (31) found a preference for Val, as compared with Gly in this position. Strop *et al.* (29), using myeloblastosis-associated virus PR, found a preference for small hydrophobic residues in S2, which is consistent with the preference for Val in P2 of NC-PR.

Subsite S1—The S1 subsites of both HIV-1 and AMV/RSV PR, like the S3 subsites, consist of relatively large and deep pockets (Fig. 2*d*). They also include the catalytic aspartic acid residues. However, the S1 subsites are mainly hydrophobic and embedded inside the protein. It is therefore not surprising to find hydrophobic residues at this position in the natural *gag* and *pol* polyprotein cleavage sites (Table III). Also, similar to the more open S3 subsite, substitution of large hydrophobic amino acids in the P1 position increases the turnover rate of substrate. For instance, exchanging Trp, Phe, or Tyr for Ser in the P1 position of the NC-PR substrate resulted in 26, 17, and 19-fold increases, respectively, in efficiency of cleavage of these substrates by AMV/RSV PR (Table II). These increases are due to favorable changes in both binding and rate of catalysis. Among the different NC-PR analogues analyzed, substitution of amino acids in P1 produced the largest increases in catalytic efficiency. This probably reflects the large size of the S1 subsite and its closeness to the scissile bond. The finding that substitution of AMV/RSV Val-104 with HIV-1 Thr or AMV/RSV Ser-107 with HIV-1 Asn significantly increased the rate of catalysis by the AMV/RSV PR without changing the substrate specificity[2] is consistent with the above argument. The HIV-1 PR also showed an increase in activity with Trp substituted in P1, but only 8-fold, whereas Leu in this substrate position resulted in a 13-fold increase in the rate of catalysis. Thus the HIV-1 enzyme prefers medium-sized hydrophobic residues in P1. This preference can be explained by the presence of Val-82 in HIV-1 PR, as compared with Gly-106 in AMV/RSV PR (12). The Val side chain points down into the subsite pocket. Thus, in contrast to the S4, S3, and S2 subsites, the S1 subsite of HIV-1 PR is smaller than that of AMV/RSV PR (see Fig. 2*d*). Several reports have noted the importance of having a large hydrophobic residue, particularly a Leu, in P1 (21, 29–31). This feature is also reflected in the choice of naturally occurring P1 residues in the case of HIV-1 (Table III).

Interestingly, polar or charged residues substituted at the P1 position (His, Arg, and Glu) resulted in better substrates for AMV/RSV PR than for the HIV-1 enzyme. This may reflect the fact that the AMV/RSV S1 subsite is more polar than that of HIV-1 PR due to the presence of Arg-105 and His-65. In contrast, Gly substitution was tolerated far better by HIV-1 PR.

Subsites S1′–S3′—Because of the symmetrical structure of the PR homodimer, the S1′–S3′ subsites utilize many of the same amino acids that form the S1–S3 subsites. Thus, one would expect the properties of the "prime" subsites to mimic those described for S1–S3. However, the peptide substrate is asymmetric and binds in an asymmetric manner. Therefore, the properties of the prime subunit of the enzyme are different from those of the "nonprime" subunit. One difference appears to be that the S1′–S3′ subsites are smaller. This can be seen directly by comparing the stereo picture of the subsite S3 to that of S3′ and of subsite S2 to that of S2′ (Fig. 2, *b–g* and *c–f*, respectively). In the case of S1 and S1′, measurements were made of the distance between the C_α atoms of residues 105 and 106 in AMV/RSV PR and 81 and 82 in HIV-1 PR to the corresponding substrate positions. It was found that these distances were 0.7–1.5 and 1.3–1.6 Å closer to P1′ than to P1, respectively. Thus, changes in the P1′–P3′ amino acids to larger residues usually resulted in decreased catalytic efficiencies for both PRs (Tables I and II). The effect of the size difference between the related prime and nonprime subsites was also observed in the analysis of a AMV/RSV p2–p10 peptide substrate/inhibitor (20). Its ability to inhibit the PR activity was related, in part, to the steric properties of amino acid side chains in the P2′ and P3′ positions and their ability to fit into the relatively small S2′ and S3′ subsites (20).

None of the amino acids substituted in the P1′ position of the NC-PR substrate improved the efficiency of cleavage by either PR, as compared with wild type. This is in contrast to the large increases in the activity observed for both PRs when the same substitutions were placed in the P1 position. The amino acids tested included polar (Arg, Gln, Glu), large hydrophobic (Phe), or no side chain (Gly). AMV/RSV PR tolerated polar residues in the P1′ position of the NC-PR substrate (such as Arg) better than HIV-1 PR (Tables I and II). This is also seen in the amino acids found in the natural *gag* and *pol* cleavage sites listed in Table III, in which three of the avian sites contain either Ser or Thr. Like the structurally related S1 subsite, the avian S1′ subsite is more polar due to the presence of His-65′ and Arg-105, as compared with Gly-48′ and Pro-81 in HIV-1 PR. In referring to PR, the prime amino acids are derived from the second subunit of the PR dimer. Substitution of His-65 and Arg-105 with Gly and Pro, respectively, produces AMV/RSV PRs that no longer tolerate polar amino acid residues in the P1′ position of the NC-PR peptide substrate (12).

The more hydrophobic nature of the HIV-1 S1′ subsite presumably accounts for the activity of the NC-PR peptide with Phe substituted in P1′ (Table I). However, HIV-1 PR is less active with this substrate than with wild type, which contains Leu in this position. The reduced activity of the HIV-1 PR may be due to steric hindrance between Val-82 and Phe in P1′ (Fig. 2*e*). Substitution of Gly in P1′ resulted in a 20-fold and at least 1000-fold decrease in efficiency of cleavage of the NC-PR substrate by HIV-1 and AMV/RSV PRs, respectively. This result verifies the importance of side chain interactions at P1′.

The S1′ subsites for both enzymes are predicted to accommodate medium-sized hydrophobic residues best. Although few such residues were tested in the context of the NC-PR peptide, they were tested in the context of the AMV/RSV p2–

p10 peptide substrate (PYVG-SGLY) (20). The substitution of Ser in P1′ with Ala produced an active substrate with an efficiency of cleavage equivalent to the wild type NC-PR peptide substrate (20). Substitution of the larger Leu in P1′ improved the efficiency of specific cleavage by the AMV/RSV PR an additional 2-fold, due primarily to an increase in the rate of catalysis.

Changes in k_{cat} seem to be more evident for substitutions on the prime side relative to the nonprime side of both the NC-PR and the p2–p10 (20) peptide substrates. The kinetic mechanism for HIV-1 PR described by Hyland *et al.* (32, 33) proposes the existence of several potentially rate-limiting steps after substrate binding. This includes conformational changes, such as opening and closing of the PR flaps, formation of the amide-hydrate intermediate, or product release itself. Our studies cannot identify the rate-limiting step. Therefore, when the k_{cat} varies, it is not clear if this reflects modulation of the rate of the existing rate-limiting step or a change to a different rate-limiting step in the reaction.

The S2′ subsite, like S2, is predicted to accommodate small hydrophobic residues (Fig. 2*f*). The same series of amino acids tested in the P2 position of the NC-PR substrate were introduced into the P2′ position. Every substitution listed in Tables I and II resulted in a significant loss in the efficiency of cleavage by both AMV/RSV and HIV-1 PRs. Although AMV/RSV PR preferred the wild type Ala over any other substitution, HIV-1 PR was somewhat more tolerant than AMV/RSV of changes in P2′ to Ser, Gly, or Leu. Trp, however, is too large for either PR to fit in the S2′ subsite. Tozser *et al.* (24) observed that Val and Leu, medium-sized hydrophobic residues, produced the best substrates for HIV-1 and -2 PRs when substituted in P2′, in qualitative agreement with the results shown here.

Substitution of the small hydrophobic residues Ala or Val for Gly in the P2′ position of the AMV/RSV p2–p10 peptide, produced substrates that were 2–3-fold more efficiently cleaved by AMV/RSV PR than a P2′ Leu-substituted p2–p10 peptide (20). This is consistent with the hypothesis that the S2′ subsite prefers small hydrophobic residues. Similar results were obtained by Margolin *et al.* (34) when substitutions were made in the P2′ position of the HIV-1 MA-CA processing site, *i.e.* a preference for hydrophobic residues, and low or no cleavage when substitution of Gly or Trp was made in this position. With their substrate, however, Ile had a higher k_{cat} than Ala or Leu, which further demonstrates the effect of context on substrate selection by PR. In studies with the myeloblastosis-associated virus PR, Strop *et al.* (29) reported an apparently symmetrical requirement for small hydrophobic residues in both P2 and P2′ and large hydrophobic residues in P1 and P1′. Although our results indicate similar preferences, the substrate side chain specificities are not identical in either the S2 subsite, as compared with S2′ or in the S1 subsite, as compared with S1′.

The AMV/RSV PR, but not the HIV-1 PR, was stimulated when Tyr replaced Met in the P3′ position of the NC-PR substrate. As presented in the discussion of the S3 subsite, this difference is probably related to the presence of Gly-106 in AMV/RSV PR and Val-82 in HIV-1 PR (Fig. 2*g*). The other substitutions tested at P3′, Leu, Asn, Gly and Asp, caused large reductions in activity for both enzymes.

The binding pockets of HIV-1 and HIV-2 PRs are predicted to contain four conservative differences (25). Crystallographic data for the HIV-2 enzyme are not publicly available at this time. However, it is reasonable to assume that given the higher degree of homology between the HIV-1 and HIV-2 PRs, as compared with that of HIV-1 and AMV/RSV PR, the residues comprising the substrate-binding pocket would be closely related. The few reports that exist (13, 24), as well as our own unpublished data, indicate that the HIV-1 and HIV-2 PRs have very similar substrate specificities.

Contribution of Amino Acid Side Chain Interactions to Binding of Substrate—The peptides with Gly inserted into the different positions were generally poor substrates with either PR. This was the case even for substitutions at the P3 or P1 positions, where the broadest range of side chains were observed. When comparing the two enzymes, however, HIV-1 PR was more tolerant of glycine substitutions than AMV/RSV PR. Apparently HIV-1 PR is somewhat less dependent upon side chain interactions than the larger, and perhaps more flexible, AMV/RSV PR. This difference in dependence on side chain interactions can be seen by comparing the catalytic efficiency of each substrate relative to the catalytic efficiency of the comparable Gly-modified substrate. For example, Leu at P1 is 19.3-fold better than Gly for HIV-1 PR, whereas it is 85-fold better for AMV/RSV PR. Overall, the side chains have a larger contribution to the catalytic efficiency for AMV/RSV than for HIV-1 PR.

General Comments—There is interaction between the adjacent subsites of the enzyme-binding cleft. Residues that produce good substrates at certain positions in one peptide sequence may not work well in other sequences. For example, although Leu and Ser occur in the P2′ position of natural AMV/RSV PR substrates (Table III), the same substitutions are not favored in the context of the NC-PR substrate (Tables I and II). Tritch *et al.* (22) tried to mix features from various efficiently cleaved substrates and found that the combinations tended to be very poor substrates. Thus substrate selection by PR must be based on recognition of all 7 amino acids of the substrate simultaneously.

Drug Design and Viral Resistance—Rational design of inhibitors of HIV-1 PR is aided by structural data from the large number of co-crystal structures that have been determined. In some cases, new inhibitors have been designed based upon structural information (7, 11). Analysis of HIV-1 PR-inhibitor structures has suggested that substrate or inhibitor binding has two components (25). 1) The main chain C=O and NH groups of peptides or peptidic inhibitors have hydrogen bond interactions with PR residues. These are highly conserved among the different crystal structures[5] and are independent of amino acid sequence. Molecular energy minimization calculations suggest that main chain atoms contribute 56 to 68% of the total PR-inhibitor interaction energy.[6] This suggests that PR residues that form hydrogen bond interactions with main chain atoms of the inhibitor will be conserved and are less likely to mutate in selection of drug resistant viruses. Thus, it is important to include potential hydrogen bond donors and acceptors for these interactions in the design of inhibitors. 2) Each side chain of the substrate or peptidic inhibitor lies in successive subsites from S4 to S3′ formed by PR residues. This component is dependent upon the amino acid sequence of a substrate (or inhibitor) and is being studied by the steady state kinetic measurements presented here and by other laboratories. Amino acids that provide tight binding at a particular position and form good PR substrates are predicted to also form effective inhibitors when these amino acids are placed at the same position in an inhibitor. This has been demonstrated with modifications of the AMV/RSV p2–p10 inhibitory peptide (20), in which a 40-fold improvement in K_i was observed when Gly at P1 was replaced by Trp and Tyr at P3 was replaced by Phe. These

[5] A. Gustchina, personal communication.

[6] A. Gustchina and C. Sansom, personal communication.

specific substitutions were chosen based on the kinetic data presented herein.

Finally, alterations in residues forming the PR subsites have been shown to effect the binding of substrates (12, 20). Even a small change in a particular subsite might be sufficient to reduce the affinity for a specific inhibitor. We would thus predict that the residues that we have proposed as being important determinants in substrate selection in the various PR subsites would be potential sites for selection of natural mutations that will produce drug-resistant phenotypes in AIDS patients treated with inhibitors of HIV PR. These residues would include HIV Val-32, Pro-81, and Val-82 and perhaps Asp-30 and/or Gly-48.

Acknowledgments—We thank Dr. A. M. Skalka for critical reading of the manuscript and Jin Wu for making the stereo figures. We also thank Dr. Joe Giam, Case Western Reserve University, for HIV-1 PR, Dr. E. Houts, Molecular Genetic Resources, for AMV PR, and Dawn Humphrey and Dr. Terry Rosenberry for amino acid composition analysis.

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