

Studies on the Symmetry and Sequence Context Dependence of the HIV-1 Proteinase Specificity*

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Two major types of cleavage sites with different sequence preferences have been proposed for the human immunodeficiency virus type 1 (HIV-1) proteinase. To understand the nature of these sequence preferences better, single and multiple amino acid substitutions were introduced into a type 1 cleavage site peptide, thus changing it to a naturally occurring type 2 cleavage site sequence. Our results indicated that the previous classification of the retroviral cleavage sites may not be generally valid and that the preference for a residue at a particular position in the substrate depends strongly on the neighboring residues, including both those at the same side and at the opposite side of the peptide backbone of the substrate. Based on these results, pseudo-symmetric (palindromic) substrates were designed. The retroviral proteinases are symmetrical dimers of two identical subunits; however, the residues of naturally occurring cleavage sites do not show symmetrical arrangements, and no obvious symmetrical substrate preference has been observed for the specificity of HIV proteinase. To examine the role of the asymmetry created by the peptide bonds on the specificity of the respective primed and nonprimed halves of the binding site, amino acid substitutions were introduced into a palindromic sequence. In general, the results suggested that the asymmetry does not result in substantial differences in specificity of the S_3 and S_3' subsites, whereas its effect is more pronounced for the S_2 and S_2' subsites. Although it was possible to design several good palindromic substrates, asymmetrical arrangements may be preferred by the HIV proteinase.

The specificity of retroviral proteinases has been studied intensively using both polyproteins and oligopeptides as substrates (for review, see Refs. 1–3). These studies have provided a basis for the rational design of potent, selective inhibitors. Various proteinase inhibitors are now in clinical trials or ap-

proved for therapy (for review, see Refs. 4–6). Comparison of cleavage site sequences of human immunodeficiency virus type 1 (HIV-1)¹ and type 2 (HIV-2) suggested that the enzyme had a broad specificity and lacked consensus substrate sequence (7). Initially three types of cleavage sites were proposed for HIV-1, HIV-2, and simian immunodeficiency virus (8). Subsequently, two major types of cleavage sites were proposed for retroviral proteinases, type 1 having -Tyr(Phe)*Pro- and type 2 having hydrophobic residues (excluding Pro) at the site of cleavage (9–11). These two types of cleavage sites were proposed to have different preferences for the P_2 and P_2' positions, where the peptide bond between P_1 and P_1' is cleaved (notation is according to Ref. 12). Our studies with type 1 substrates indicated a preference for small residues like Cys or Asn at the P_2 position and a preference for β -branched Val or Ile at the P_2' position (10). The lower catalytic constants with P_2 β -branched residues were predicted to be due to steric collision with P_1' Pro (10). On the other hand, using a series of peptides based on a type 2 cleavage site, β -branched residues, especially Val, were found to be favorable at P_2 , whereas Glu was preferred at P_2' (11). Interestingly, Griffith *et al.* (11) found Glu as the preferred P_2' residue in a peptide series, when the P_2 - P_1' sequence of a type 1 cleavage site (-Asn-Tyr*Pro-) was substituted into a type 2 substrate. Although some of the differences of the subsite specificity in type 1 and type 2 cleavage sites were explained by molecular modeling, most of the dependence of the specificity of proteinases on the sequence context is unexplored.

The HIV-1 proteinase is a dimer of two identical subunits. It exhibits an exact crystallographic, 2-fold rotational (C_2) symmetry in the structure without inhibitor (for review, see Ref. 4). Based on this symmetry, the potential advantages of C_2 symmetric HIV-1 proteinase inhibitors including high selectivity, potency, and stability were proposed, and structurally symmetric HIV-1 proteinase inhibitors were designed containing two amino-terminal halves of a putative substrate (13). Crystal structures of HIV-1 proteinase with inhibitors can be either symmetric or asymmetric (4). The symmetry or asymmetry was initially thought to arise from the symmetry or asymmetry of the inhibitor, but even crystal structures of HIV proteinase with symmetric inhibitors can have asymmetric proteinase subunits (14).

Considering the symmetry of the HIV proteinase, a symmetrical preference for substrate residues would be expected for

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¹ The abbreviations used are: HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2; MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein. The nomenclature of viral proteins is according to Leis *et al.* (36).

naturally occurring cleavage sites, since the high mutation rate could readily evolve such sequences. However, there is no obvious preference for symmetrical sequences in HIV proteinase cleavage sites (listed in Ref. 15) or in other retroviral proteinase cleavage sites (see Ref. 1). Using a series of oligopeptides containing single amino acid substitutions in a naturally occurring type 1 cleavage site peptide, the respective P and P' positions (for example, P₂ and P₂') appeared to be similar, but substantial differences were also found. For example, the peptide containing P₂ Asn was a very good substrate; however, Asn at P₂' resulted in a poor substrate (10). It was not clear whether these differences were because of the asymmetrical interactions of the peptide amides and carbonyl oxygens in the substrate or intramolecular interactions of the substrate side chains (10).

To explore further the dependence of HIV proteinase specificity on the sequence context of its substrates we introduced single or multiple substitutions into a type 1 cleavage site peptide and changed it to a naturally occurring type 2 cleavage site sequence. Based on the results obtained, we designed a pseudosymmetric (palindromic) substrate and introduced amino acid substitutions into this sequence to explore the effect of asymmetry created by the peptide backbone on the different specificities of the respective primed and nonprimed proteinase subsites. To study whether the enzyme prefers pseudosymmetric (palindromic) or asymmetric arrangements of the substrate residues, we have also studied the doubly substituted (also palindromic) versions of the starting pseudosymmetric substrate.

MATERIALS AND METHODS

Oligopeptide Synthesis and Characterization—Oligopeptides were synthesized by standard tert-butoxycarbonyl or 9-fluorenylmethoxycarbonyl chemistry on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.) or a semiautomatic Vega peptide synthesizer (Vega-Fox Biochemicals). All peptides were synthesized with an amide end. 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—Purified HIV-1 proteinase was prepared as described previously (16). Active site titration for the HIV-1 proteinase was performed with compound 3 (17). The proteinase assays were performed in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, 0.1% Nonidet P-40, 2 M NaCl in the presence of 8–140 nM enzyme. The reaction mixture was incubated at 37 °C for 1 h, and the reaction was stopped by the addition of guanidine HCl (6 M final concentration). The solution was acidified by the addition of trifluoroacetic acid, and an aliquot was injected onto a Nova-Pak C₁₈ reversed-phase chromatography column (3.9 × 150 mm, Waters Associates, Inc.) using an automatic injector. Substrates and the cleavage products were separated using an increasing water-acetonitrile gradient (0–100%) in the presence of 0.05% trifluoroacetic acid. Cleavage products of proteinase-catalyzed hydrolysis for these peptides were identified by amino acid analysis and/or by NH₂-terminal sequencing. Kinetic parameters were determined by fitting the data obtained at less than 20% substrate hydrolysis to the Michaelis-Menten equation by using the Fig. P program (Fig. P Software Corp.). The substrate concentration was 0.01–5.0 mM depending on the approximate K_m values.

Molecular Modeling and Energy Minimization—The structures were examined on Silicon Graphics computers running the program Sybyl (Tripos Inc., St. Louis, MO) or CHAIN (18). The starting model for the HIV-1 proteinase with the substrate Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln (asterisk indicates the site of cleavage) was described previously (19). All the other enzyme-substrate structures were built from this model by altering the side chain(s) of the appropriate residue(s). Each of the side chain torsion angles for substituted residues in the peptide substrate was rotated through 360° in steps of 15° to find the conformation with the smallest nonbonded energy as described (20).

Energy minimization and molecular dynamics of the modified substrates were run using the program AMMP (21), as described previously

(19). Finally, the model structures were examined in the computer graphics system.

RESULTS AND DISCUSSION

Substitution of Amino Acids of a Type 1 Cleavage Site Peptide with Residues of a Type 2 Substrate

Previously, we performed extensive comparisons of the specificities of HIV-1 and HIV-2 proteinases using oligopeptides representing naturally occurring cleavage sites in their Gag and Gag-Pol polyproteins (15). These cleavage sites have been classified as type 1, which contains an aromatic amino acid and Pro at P₁ and P₁', respectively, and type 2, which has mainly hydrophobic residues but not Pro at the site of cleavage. We showed that an oligopeptide (peptide 1 in Table I) representing the cleavage site in p66 of HIV-1 for generating the p51 subunit of the heterodimeric reverse transcriptase of HIV-1 and another peptide (peptide 2 in Table I) representing the homologous sequence in p68 of HIV-2, and therefore proposed to be the cleavage site (22), were substrates of the HIV proteinases (15). These peptides, which match type 2 cleavage site sequences, were the starting points for our design of palindromic substrates since they are partly symmetric with aromatic amino acids at the P₁, P₁', and they contain negatively charged residues at the P₃ and P₃' positions. In addition, peptide 2 also contains Thr at both P₂ and P₂' positions. Furthermore, peptides 1 and 2 with the exception of the P₂' residues share the same sequence in the P₄-P₃' region, which is the major determinant for specificity (23). However, peptide 2 was found to be a much poorer substrate of the HIV proteinases than peptide 1 (see Table I and Ref. 15).

Subsequently Fan *et al.* (24) demonstrated that in fact the sequence of peptide 2 does not represent the actual cleavage site required to be cleaved to produce the smaller subunit of the HIV-2 reverse transcriptase. They found that the real cleavage site has the sequence of AFAM*ALTD and is downstream from the one proposed by Le Grice *et al.* (22). Nevertheless, we in this study and Fan *et al.* (24) have confirmed our initial finding that the HIV-2-derived peptide 2 or its shorter octapeptide homolog is a substrate of the HIV-1 proteinase.

We have also compared the specificity of the HIV-1 and HIV-2 proteinases using a series of oligopeptide substrates containing single amino acid substitutions in the sequence of SP-211 (see peptide 3 in Table I), a peptide that corresponds to the type 1 MA/CA cleavage site in HIV-1 (10, 23). In these studies it was found that substitution of Pro at the P₁' position to any other amino acid tested, including Tyr, formed nonhydrolyzable or very poor substrates of HIV proteinases. These P₁'-substituted peptides inhibited the hydrolysis of SP-211 by HIV proteinase, which suggested that they were able to bind to the enzyme (10). The best inhibition was obtained with the P₁' Tyr-substituted peptide,² suggesting its high affinity for the HIV-1 proteinase. In good agreement with these preliminary findings, both the K_m and k_{cat} values determined in the present study were substantially lower for the P₁' Tyr-substituted peptide compared with the unmodified one (compare peptides 3 and 4 in Table I). Substitution of P₁' Pro of SP-211 with Tyr converts a type 1 substrate to a type 2 substrate. However, the P₁' Tyr-substituted peptide is a poor substrate of HIV-1 proteinase (peptide 4 in Table I). To understand better the specificity of HIV-1 proteinase and the differences of the subsite preference in type 1 and type 2 cleavage sites, further single and multiple substitutions were carried out in the type 1 MA/CA cleavage site substrate (peptide 3), introducing residues characteristic of the type 2 cleavage site peptides 1 and 2

² J. Tözsér and S. Oroszlan, unpublished results.

TABLE I

Assay of substrates having the sequence of naturally occurring cleavage sites and those containing single or multiple substitutions in the cleavage site sequence between the matrix and capsid proteins of HIV-1 by HIV-1 proteinase

Number	Sequence ^a	K_m	k_{cat}	k_{cat}/K_m
		mM	s ⁻¹	mM ⁻¹ s ⁻¹
1	AETF*YVDGAA ^b	0.046 ± 0.006	0.42 ± 0.02	9.1
2	GAETF*YTDGS ^c	0.33 ± 0.06	0.21 ± 0.03	0.64
3	VSQNY*PIVQ ^d	0.15 ± 0.03	6.8 ± 0.07	45.3
4	VSQNY*YIVQ	0.020 ± 0.003	0.010 ± 0.001	0.50
5	VSQTY*PIVQ	0.41 ± 0.05	0.70 ± 0.02	1.7
6	VSQNY*PTVQ	0.85 ± 0.20	2.07 ± 0.37	2.43
7	VSDNY*PIVQ	1.77 ± 0.32	2.62 ± 0.15	1.48
8	VSQNY*PIDQ	6.84 ± 0.98	3.09 ± 0.31	0.45
9	VSQTY*YIVQ	0.035 ± 0.005	0.020 ± 0.001	0.57
10	VSQNY*YTVQ	0.026 ± 0.007	0.18 ± 0.01	6.92
11	VSQTY*YTVQ	0.021 ± 0.003	0.050 ± 0.001	2.38
12	VSQNY*YIDQ	<0.02	ND ^e	0.09 ^f
13	VSQTY*YIDQ	0.006 ± 0.001	0.10 ± 0.01	16.67
14	VSQNY*YTDQ	0.20 ± 0.03	0.20 ± 0.02	1.00
15	VSQTY*YTDQ	0.17 ± 0.03	0.10 ± 0.01	0.59
16	VSDTY*YTDQ	0.19 ± 0.03	0.030 ± 0.003	0.16

^a Amino acids substituted in the sequence of peptide 3 are underlined.

^b Peptide representing the determined cleavage site in reverse transcriptase of HIV-1. Kinetic parameters for this peptide were also reported in Ref. 15.

^c Peptide representing a proposed cleavage site in reverse transcriptase of HIV-2 (22).

^d Peptide representing the MA/CA cleavage site of HIV-1 (peptide 3, previously designated as SP-211). Kinetic parameters for this peptide were also reported in Ref. 15.

^e ND, not determined.

^f Determined as competitive substrate with peptide 45 (Table V).

(Table I). The same substitutions were also introduced in the P₁' Tyr-modified (type 2) substrate. This peptide series allowed us to compare the preference for P₂ Asn over Thr as well as P₂' Ile over Thr in different sequence contexts. The ln (k_{cat}/K_m) values are directly related to the free energy of the binding of the transition state by the enzyme (25). If the subsite interactions are mostly independent of each other, the k_{cat}/K_m ratios (equal to $e^{-\Delta\Delta G/RT}$) for substrate pairs having X and Y residues at the same subsite, should be similar and independent of the surrounding sequence, as has been found for *e.g.* trypsin (26) and chymotrypsin (27).

Single substitutions of P₂ Asn and P₂' Ile with Thr (peptides 5 and 6 in Table I, respectively) resulted in substantial increases in K_m and decreases in catalytic constants compared with the unmodified peptide 3. Comparison of kinetic parameters of peptides 1 and 2 as well as of peptides 3 and 6 suggests that β -branched hydrophobic residues such as Val or Ile at P₂' positions are much more favorable than the β -branched but more hydrophilic Thr in these two different sequence contexts. Furthermore, a similar preference was found in two other series of peptides (11). Single substitutions of P₃ Gln and P₃' Val to Asp (peptide 7 and 8 of Table I, respectively) resulted in dramatic increases in K_m values but only moderate decreases in k_{cat} values, as was found previously for cleavage with HIV-2 proteinase (10).

Substituting Thr at P₂ residue for Asn in the P₁' Tyr analog of SP-211 (peptide 9 in Table I) did not yield substantial changes in the kinetic parameters, whereas substitution of P₂' Thr for Ile in the same sequence context (peptide 10) yielded an approximately 10-fold increase in (k_{cat}/K_m). The same substitution was very unfavorable in the P₁' Pro-containing peptides (compare peptides 3 and 6 in Table I), suggesting a strong influence of the P₁' residue on the preference for the P₂' resi-

due. Substitution of Thr at both the P₂ and P₂' positions of peptide 4 was less effective than the single P₂' substitution (compare peptides 10 and 11 of Table I). Interestingly, whereas the P₃' Asp substitution of peptide 4 yielded a substrate that was even less susceptible to hydrolysis (peptide 12 in Table I), a further substitution for P₂ Thr (peptide 13) yielded a substrate with K_m and k_{cat}/K_m values better than those of the naturally occurring type 2 cleavage site peptide 1. As expected from the comparison of peptides 1 and 2, further substitution of P₂' Ile for Thr yielded a substrate with much lower k_{cat}/K_m value because of the substantial increase of the K_m (peptide 14 of Table I).

Enzyme-substrate models were built and energy minimized to explore the possible interactions of the enzyme and the substrates at the molecular level. The substrate lies in an extended β -conformation in the substrate binding site, which puts P₄, P₂, P₁', and P₃' residues at one side and P₃, P₁, and P₂' on the other side (Fig. 1). Adjacent substrate binding sites (like S₂ and S₁') partially overlap (Fig. 1). Molecular modeling suggested that the P₂ Thr-substituted SP-211 analog could not fit well in the S₂ subsite (not shown) because of the predicted interaction with P₁' Pro, as described previously for another β -branched amino acid, Val (10). When the P₁' Pro is changed to Tyr, this restraint is removed. However, comparison of peptides 4 and 9 suggested that this substitution alone is not sufficient to make Thr preferable over Asn in the SP-211 sequence context, but the additional P₃' Val to Asp exchange is required (peptides 12 and 13). However, a further substitution of P₂' Ile to Thr again resulted in Asn over Thr preference at P₂ (peptides 14 and 15, see Table II).

It is of interest to note that substitution of P₁' Pro to Tyr in various sequence contexts yielded much lower K_m and k_{cat} values (*e.g.* compare peptides 3 and 4, 5 and 9 etc.). Both the substrate backbone and the proteinase residues of the S₁' (and S₁) subsites may move to accommodate the large Tyr at P₁' (and P₁) by moving toward the overlapping and adjacent subsites S₂-S₃' (S₃-S₂') and therefore restrict the space available for other subsites (see Fig. 1). Similar movements of a mutated residue in the substrate binding site have been observed in the crystal structure of an inhibitor-resistant proteinase (28). The restriction of the available space at the neighboring subsites could be a major determinant in the sequence context-dependent preference for P₂ and P₂'. In the -P₂-Tyr*P₂'-Asp- context there is a preference for one larger and one smaller residue at P₂ and P₂' suggesting complementarity of these sites. In the case of Ile at P₂', the smaller Thr is preferred over Asn at P₂ (peptides 12/13 in Table II), whereas in case of Thr at P₂' the larger Asn is preferred over Thr at P₂ (peptides 14/15 in Table II). Conversely, with the larger Asn at P₂ the smaller Thr is preferred at P₂' (peptides 12/14), whereas with the smaller Thr at P₂ the larger Ile is preferred at P₂' (peptides 13/15). In the case of the -P₂-Tyr*P₂'-Val- context the preference is less distinct. In accordance with the suggested P₂-P₂' complementarity, with P₂' Thr there is a preference for the larger Asn at P₂ (peptides 10/11 in Table II), whereas with Asn at P₂ there is a preference for the smaller Thr at P₂' (peptides 4/10 in Table II). However, when P₂ is Thr, P₂' Thr was preferred over Ile (peptides 9/11 in Table II). Apparently the hydrophobic, β -branched Val at P₃' may restrict the type of amino acid which can be accommodated optimally in the S₂' subsite.

In summary, results on the preference of Asn over Thr at P₂ and Ile over Thr at P₂' positions (Table II) suggest a very strong dependence on sequence context. For example, P₂' preference depends not only on the P₁', but also on the P₂ and P₃' residues. Strop *et al.* (29) suggested a context dependence in the P₂-P₂' residues in the specificity of avian myeloblastosis virus protein-

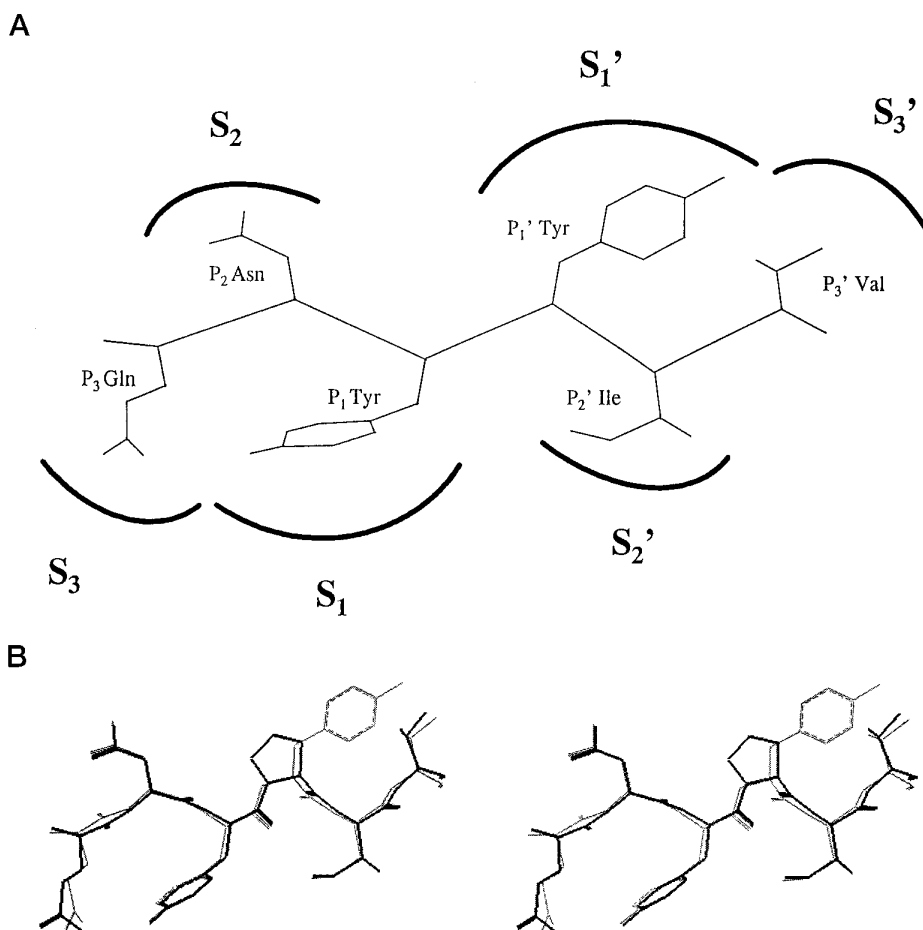


FIG. 1. Panel A, schematic representation of the P_1' Tyr-substituted HIV-1 MA/CA substrate (peptide 4 in Table I) in the S_3 - S_3' subsites of proteinase. The relative size of each subsite is indicated approximately by the area enclosed by the curved line around each substrate side chain. Panel B, stereoview of residues P_3 - P_3' of peptides 1 (thin lines) and 4 (thick lines).

TABLE II
Preference for Asn over Thr at P_2 and Ile over Thr at P_2' positions of substrates

Peptides	Sequence ^a					k_{cat}/K_m ratio
	P_2	P_1	P_1'	P_2'	P_3'	
3/5	<u>Asn</u> /Thr	Tyr	Pro	Ile	Val	26.6
4/9	<u>Asn</u> /Thr	Tyr	Tyr	Ile	Val	0.9
10/11	<u>Asn</u> /Thr	Tyr	Tyr	Thr	Val	2.9
12/13	<u>Asn</u> /Thr	Tyr	Tyr	Ile	Asp	0.005
14/15	<u>Asn</u> /Thr	Tyr	Tyr	Thr	Asp	1.7
3/6	Asn	Tyr	Pro	<u>Ile</u> /Thr	Val	18.6
4/10	Asn	Tyr	Tyr	<u>Ile</u> /Thr	Val	0.07
9/11	Thr	Tyr	Tyr	<u>Ile</u> /Thr	Val	0.24
12/14	Asn	Tyr	Tyr	<u>Ile</u> /Thr	Asp	0.09
13/15	Thr	Tyr	Tyr	<u>Ile</u> /Thr	Asp	28.25

^a Only P_2 - P_3' segment of the substrates are shown; the outer residues were identical. The preferred residue is underlined.

ase, which was also demonstrated by Ridky *et al.* (30). Our results indicate that P_3' , and likely other outer distal residues like P_4 and P_3 , may also substantially influence the preference for a subsite. The change of preference at the P_2' position as a function of P_1' and P_3' residues (Table II) also suggests that the context dependence of the specificity of HIV-1 proteinase is not restricted only to residues located at the same side of the peptide backbone. Molecular modeling and inspection of the crystal structure of HIV proteinase-inhibitor complexes suggest that the peptide backbone of the substrate does not occupy a rigid position, and depending on the substitutions, it could move toward the S or S' sites. SP-211 contains the relatively small Pro at P_1' , which may allow a relatively larger S_2' subsite, and this peptide showed Ile over Thr preference (peptide 3/6 in Table II). A large hydrophobic residue at P_1' , such as Tyr,

could force the peptide backbone toward the S_2' site, making it smaller. This could be the reason for the Thr over Ile preference at P_2' in the case of the P_1' Tyr-substituted peptides (see peptides 4/10 in Table II).

Substitutions in a Palindromic Substrate Sequence

S_3 and S_3' Substitutions—Peptide 16 (Table I) contains a palindromic sequence at the P_3 - P_3' positions.

Elimination of P_5 Val and substitution of P_4' Gln for Ser resulted in a completely palindromic substrate (peptide 17 in Table III). It is important to note that this shorter peptide showed 10-fold higher k_{cat} and k_{cat}/K_m values than peptide 16. The effect of residues P_5 and P_4 on substrate hydrolysis has been reported previously (11, 23). To compare the specificity of the S_3 and S_3' substrate binding pockets in an identical sequence context, the Asp residues of the palindromic peptide 17 were substituted to Gly, Gln, Phe and Leu residues (Table III). Although the Gly and Gln residues were also doubly substituted, kinetic measurements were not possible for the doubly substituted Phe and Leu peptides because of low solubility (peptides 26 and 29 in Table III). Substitution of the P_3 and P_3' Asp to Gly (peptides 18 and 19, respectively) resulted in similar K_m and k_{cat} values and only a 2-fold decrease in k_{cat}/K_m values. Interestingly, changing both P_3 and P_3' Asp residues to Gly yielded a much higher K_m value (peptide 20). Single substitutions to Gln also resulted in moderate changes in the kinetic parameters, whereas the double substitution yielded a substantial reduction of the k_{cat} value. Whereas substitution to P_3 Phe resulted in a 4-fold decrease in K_m and k_{cat} (peptide 24), the P_3' Phe substitution did not cause such a big effect (peptide 25). Substitution of P_3 or P_3' Asp to Leu resulted in a substantial decrease in catalytic constants.

Modeling showed that the same residues of the enzyme interact with the appropriate subsite at both sides of the scissile bond (Table IV). S_3 and S_3' subsites are near the surface, and they are formed by hydrophobic residues and two charged residues (Table IV). In the starting palindromic substrate (peptide 17) the P_3 and P_3' Asp residues can interact with Arg-8' and Arg-8, respectively (Fig. 2) and compensate for the lower van der Waals energy compared with Phe in P_3 and P_3' positions. Surprisingly, the catalytic constants of the Gly-substituted peptides are comparable to those obtained with Phe and Asp substitutions. Apparently, P_3 and P_3' in this sequence context do not have a substantial effect on the efficiency of hydrolysis. The largest differences between the P_3 and P_3'

TABLE III
Assay of P_3 and P_3' substituted peptides based on a palindromic sequence by HIV-1 proteinase

Number	Sequence ^a	K_m mM	k_{cat} s^{-1}	k_{cat}/K_m $mM^{-1} s^{-1}$
17	SDTY*YTDS	0.21 ± 0.04	0.34 ± 0.02	1.62
18	SGTY*YTDS	0.50 ± 0.06	0.46 ± 0.02	0.92
19	SDTY*YTGS	0.35 ± 0.03	0.29 ± 0.01	0.83
20	SGTY*YTGS	2.88 ± 0.44	0.62 ± 0.04	0.22
21	SQTY*YTDS	0.62 ± 0.09	0.43 ± 0.04	0.69
22	SDTY*YTQS	0.36 ± 0.06	0.21 ± 0.02	0.58
23	SQTY*YTQS	0.22 ± 0.03	0.060 ± 0.003	0.27
24	SFTY*YTDS	0.052 ± 0.017	0.08 ± 0.01	1.54
25	SDTY*YTFS	0.24 ± 0.05	0.22 ± 0.02	0.92
26	SFTY*YTFS	Not determined ^b		
27	SLTY*YTDS	0.250 ± 0.065	0.010 ± 0.002	0.04
28	SDTY*YTLS	0.082 ± 0.015	0.014 ± 0.001	0.17
29	SLTY*YTLS	Not determined ^b		

^a Amino acids substituted in sequence 17 are underlined.

^b Low solubility of the substrate did not allow the kinetic measurements.

TABLE IV
Residues forming the subsites of HIV-1 proteinase

Residues interacting with the modeled peptide 17 (Table V) are listed. Amino acid residues in the second subunit of the dimer are indicated by a prime.

Subsite	HIV-1 proteinase residues
S_3	Arg-8', Leu-23', Asp-29, Gly-48, Gly-49, Phe-53, Pro-81', Val-82'
S_2	Ala-28, Asp-29, Asp-30, Val-32, Ile-47, Gly-48, Gly-49, Ile-50', Leu-76, Ile-84
S_1	Arg-8', Leu-23', Asp-25', Asp-25, Gly-27, Gly-48, Gly-49, Ile-50, Thr-80', Pro-81', Val-82', Ile-84'
S_1'	Arg-8, Leu-23, Asp-25, Asp-25', Gly-27', Gly-48', Gly-49', Ile-50', Thr-80, Pro-81, Val-82, Ile-84
S_2'	Ala-28', Asp-29', Asp-30', Val-32', Ile-47', Gly-48', Gly-49', Ile-50, Leu-76', Ile-84'
S_3'	Arg-8, Leu-23, Asp-29', Gly-48', Gly-49', Phe-53', Pro-81, Val-82

mutated peptides were obtained with Leu substitutions. Also, these peptides had the lowest k_{cat}/K_m values. Previously, we had proposed that the interaction between the hydrophobic P_3' residue of SP-211 (see peptide 3 in Table I) and Phe-53 of the enzyme may play a crucial role in proper closing of the flexible flap (10). In a molecular dynamics simulation of the flap movement, Phe-53 and its symmetry counterpart were among the few residues suggested to be involved in the "triggering" event (31). In the palindromic sequence context, although peptides with hydrophobic residues at P_3' have lower K_m values than those with polar residues or Gly (Table III), the differences are not as dramatic as in the case of SP-211, where at least 10-fold differences were found (peptides 3 and 8 in Table I; Ref. 10). In our palindromic sequence context, the effect of a hydrophobic residue at P_3' resembles that of P_3 in SP-211 in which only a moderate increase of K_m was obtained by introducing nonhydrophobic residues at P_3 (10). A P_1 Tyr residue in the pseudosymmetric context may partially occupy the overlapping S_3' pocket as P_1 Tyr can partially occupy the S_3 (Fig. 3), resulting in less dependence on P_3' hydrophobicity for the proper closing of the flap.

Only moderate differences were found in the kinetic parameters of substrates having identical substitutions at the P_3 and P_3' sites, except for the Leu substitutions. These results suggest that the asymmetry of the peptide backbone does not play a major role in the large differences of the specificities of the respective S_3 and S_3' subsites we had previously established (10). Instead, the specificity differences are a consequence of the different sequence context of the substrates.

S_2 and S_2' Substitutions—The P_2 or P_2' Thr residues of peptide 17 (Tables III and V) were substituted with Gly, Glu, Cys, Ala, Ile, and Leu residues (Table V). Because of the low solubility, the doubly substituted Ile and Leu substrates were not assayed (peptides 44 and 47, respectively). Single substitutions of the Thr residues with Gly resulted in substantial increases in K_m values and decreases of k_{cat} and k_{cat}/K_m values compared with peptide 17, whereas the doubly substituted peptide was not hydrolyzed (peptides 31–33 in Table V). Previous studies showed that Gly in these positions does not form good substrates, independently of the sequence context (10, 32). Substitution of P_2 Thr to Glu resulted in a large increase in K_m value; however, this peptide showed the highest k_{cat} value among all of the substituted peptides (peptide 33 in Table V). P_2' Glu substitution (peptide 34) resulted in a less dramatic increase in K_m and unchanged k_{cat} values. P_2' Glu was found to be optimal in both type 1 and type 2 sequence context by Griffith *et al.* (11). This preference was predicted to be because of hydrogen bonding of the side chain carboxyl to the backbone NH of residues 29' and 30' of the enzyme (11). Also, P_2' Glu is frequently found in viral and cellular protein cleavage sites of HIV-1 proteinase (33). At the P_2 - P_2' region the type 1 substrate used by Griffith *et al.* (11) contained residues (-Asn-Tyr*Pro-Ile-) identical to those in SP-211; however, P_2' Glu substitution

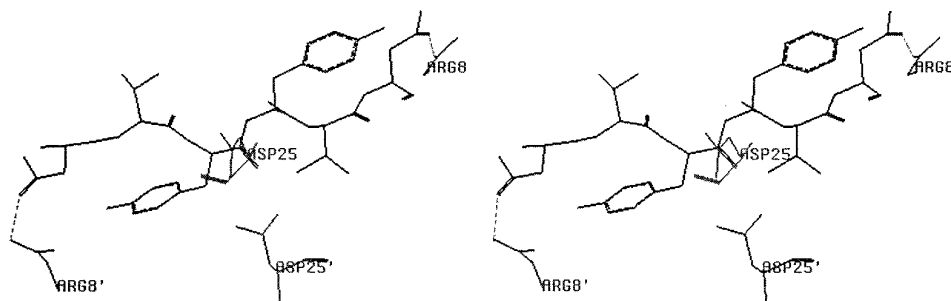


FIG. 2. Stereoview of residues P_3 - P_3' of the palindromic peptide 17 (Table V). Hydrogen bond interactions between P_3 Asp and Arg-8' as well as P_3' Asp and Arg-8 in the enzyme-substrate complex are shown by dashed lines.

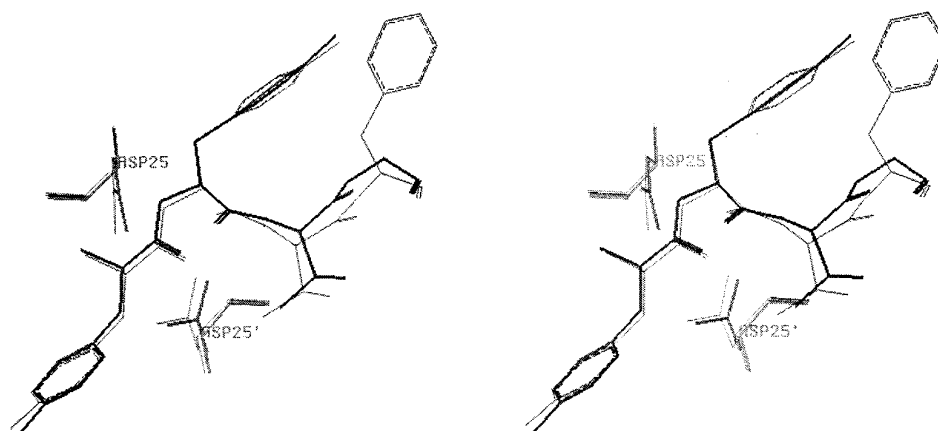


FIG. 3. Stereoview of residues P_1 – P_3' of the substituted derivatives of peptide 17 (Table V containing Gly at P_3' (peptide 19 of Table V, thick lines) and Phe at P_3' (peptide 25 of Table V, thin lines).

TABLE V
Assay of P_2 and P_2' substituted peptides based on a palindromic sequence by HIV-1 proteinase

Number	Sequence ^a	K_m mM	k_{cat} s^{-1}	k_{cat}/K_m $mM^{-1} s^{-1}$
17	SDTY*YTDS	0.21 ± 0.04	0.34 ± 0.02	1.62
30	SDGY*YTDS	1.41 ± 0.28	0.06 ± 0.01	0.04
31	SDTY*YGDS	1.82 ± 0.28	0.09 ± 0.01	0.05
32	SDGY*YGDS	Not hydrolyzed ^b		
33	SDEY*YTDS	4.86 ± 1.04	3.66 ± 0.53	0.75
34	SDTY*YEDS	0.91 ± 0.16	0.34 ± 0.04	0.36
35	SDEY*YEDS	5.14 ± 0.86	0.79 ± 0.09	0.15
36	SDCY*YTDS	0.09 ± 0.006	0.44 ± 0.01	4.89
37	SDTY*YCDs	0.12 ± 0.02	1.04 ± 0.06	8.67
38	SDCY*YCDs	1.00 ± 0.20	2.78 ± 0.20	2.78
39	SDAY*YTDS	0.098 ± 0.004	1.30 ± 0.02	13.27
40	SDTY*YADS	0.17 ± 0.04	0.82 ± 0.03	4.82
41	SDAY*YADS	0.24 ± 0.03	3.16 ± 0.17	13.17
42	SDIY*YTDS	0.10 ± 0.02	1.02 ± 0.08	10.20
43	SDTY*YIDS	0.03 ± 0.001	0.55 ± 0.03	18.33
44	SDIY*YIDS	Not determined ^c		
45	SDLY*YTDS	0.51 ± 0.05	0.48 ± 0.03	0.94
46	SDTY*YLDs	0.06 ± 0.02	0.57 ± 0.09	9.50
47	SDLY*YLDs	Not determined ^c		

^a Amino acids substituted in sequence 17 are underlined.

^b Not hydrolyzed when incubated with 140 nM HIV-1 proteinase for 1 h at 37 °C.

^c Low solubility of the substrate did not allow the kinetic measurements.

was an unfavorable substitution in the SP-211,² suggesting the importance of residues outside the P_2 – P_2' determining P_2' preference, as we have also found with the multisubstituted peptides (Table II). Therefore, preference for Glu at P_2' position of type 1 and type 2 cleavage sites is also strongly dependent on the surrounding substrate residues. Single substitutions of the palindromic peptide 17 with small hydrophobic residues such as Cys (peptides 36–37) and Ala (peptides 39–40) at P_2 and P_2' resulted in somewhat better K_m and k_{cat} values, as well as higher k_{cat}/K_m values. It is interesting to note that the double Cys substitution (peptide 38) resulted in a substantially higher K_m value and a less efficient substrate than the single substituted ones. Also, the double Ala substitution (peptide 41) did not improve the substrate over the P_2 Ala-substituted one, although P_2' Ala was also favorable over Thr in single substitution. These findings suggest that symmetrical (palindromic) arrangements do not necessarily give better substrates than

asymmetrical arrangements. The substitutions with Ile gave lower K_m and increased catalytic constants (peptides 42–43). Only P_2' Leu was a preferable substitution, whereas P_2 Leu substitution was not (peptides 45 and 46, respectively).

The S_2 and S_2' subsites of HIV-1 proteinase are also hydrophobic, but they are smaller than the S_3 and S_3' sites. The P_2 side chain is surrounded by the hydrophobic Val-32, Ile-47, Ile-50, Leu-76, and Ile-84 residues. The same amino acid residues of the other subunit form the S_2' . Comparing the kinetic parameters of the P_2 and P_2' substituted substrates, the small hydrophobic Ala was a better substitution at P_2 than at P_2' , whereas the larger Cys, Ile, and Leu residues were more preferred at P_2' , suggesting that the S_2' subsite may be somewhat larger than the S_2 . Interestingly, the largest differences in k_{cat}/K_m values were found between peptides having Leu substitutions in P_2 and P_2' (compare peptides 45 and 46 in Table V) as well as P_3 and P_3' (compare peptides 27 and 28 of Table III). Because of the asymmetry of the peptide bonds, the pseudosymmetry center of the substrates is shifted approximately by 0.5 Å, positioning the carbonyl oxygen rather than the center of the scissile bond into the line of the C_2 symmetry axis of the proteinase dimer. This deviation puts the P_2' residue farther from the center of the enzyme compared with P_2 (Fig. 4), toward the open end of the binding site, which may be a reason why larger residues are better at P_2' than at P_2 . The effect of this deviation is stronger close to the center, which may be the reason for larger differences obtained for the P_2 and P_2' substitutions than for the P_3 and P_3' substitutions. Furthermore, whereas S_3 , S_3' subsites are exposed at the surface, the S_2 , S_2' subsites are more restricted because they are smaller and inside the proteinase.

Hydrolysis of Substrates Having Palindromic Sequences

The hydrolysis of peptide 17 and its doubly substituted derivatives in Tables III and V showed that peptides with completely palindromic sequences could be substrates of HIV-1 proteinase. Based on mirroring the NH_2 -terminal side of the sequence of the naturally occurring MA/CA (VSQNY*PIVQ) and CA/NC cleavage site (TATIM*MQRGN), we synthesized other palindromic peptides, and these were also found to be substrates of the enzyme (peptides 48 and 49 of Table VI). Simultaneous substitution of P_2 and P_2' residues of peptide 49 to Ala (peptide 50) produced little change in k_{cat}/K_m . Since Tyr is better in P_1 than Met in both type 1 (10) and type 2 cleavage site sequences (2), Tyr was substituted for Met at both P_1 and P_1' , which resulted in the most efficient palindromic substrate in our series (peptide 51). These results suggest that various palindromic substrates could be designed for HIV-1 proteinase,

FIG. 4. Comparison of the interactions of P₂ Leu-S₂ and P₂' Leu-S₂' of the substituted palindromic peptides. Stereoview of the P₂ Leu residue of peptide 45 (Table VI) with the residues of S₂ (shown in thin lines) rotated 180° and superimposed on P₂' Leu residue of peptide 46 (Table VI) with the residues of S₂' (shown in thick lines).

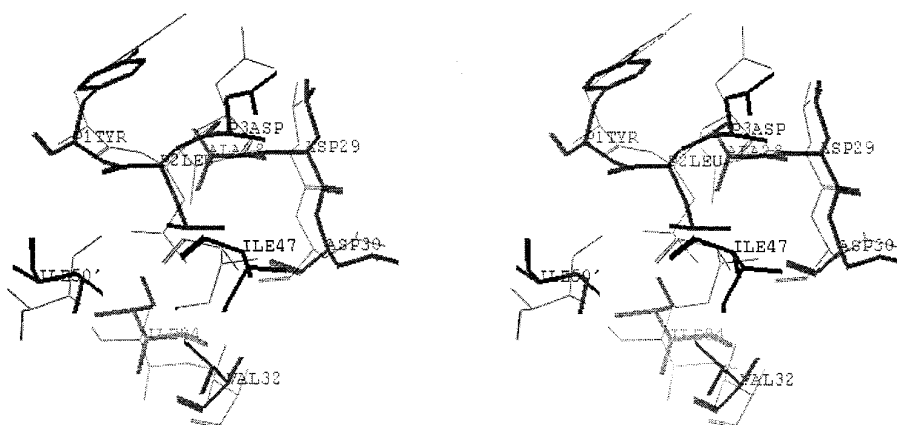


TABLE VI

Assay of peptides containing palindromic or inverted sequences based on the cleavage sites between MA and CA as well as between p1 and NC proteins of HIV as substrates of HIV-1 proteinase

Number	Sequence	K_m mM	k_{cat} s ⁻¹	k_{cat}/K_m mM ⁻¹ s ⁻¹
48	VSQNY*YNQSV	0.013 ± 0.003	0.05 ± 0.01	3.85
49	KATIM*MITAK	<0.05 ^a	ND ^b	1.00 ^c
50	KATAM*MATAK	0.34 ± 0.03	0.14 ± 0.01	0.41
51	KATII*YITAK	<0.05 ^a	ND ^b	27.6 ^d
52	VSQNP*YIVQ		Not hydrolyzed ^e	
53	QVIP*YNQSV		Not hydrolyzed ^e	

^a Increasing substrate concentration above 0.05 mM resulted in a decrease of velocity.

^b ND, not determined.

^c k_{cat}/K_m value was determined as competitive substrate with peptide 17 of Table III.

^d k_{cat}/K_m value was determined as competitive substrate with peptide 40 of Table V.

^e Not hydrolyzed when incubated with 140 nM HIV-1 proteinase for 1 h at 37 °C.

and specificity studies using these substrates could eliminate the differences caused by the sequence context on the preference of the respective S and S' sites.

It is also of interest to note that exchange of P₁ and P₁' residues of SP-211 as well as mirroring the P' residues resulted in nonhydrolyzable peptides (peptides 52 and 53 of Table VI). These results also indicate the nonequivalent nature of the respective S and S' subsites.

CONCLUSIONS

The dependence of the specificity of HIV-1 proteinase on the sequence context of its substrate peptides was studied by making single and multiple substitutions of amino acids in naturally occurring cleavage site peptides, and palindromic substrates were designed. Although it was possible to design palindromic substrates, our results suggest that a symmetric arrangement of residues may not be favorable. The S₃-S₃' region of the HIV proteinase is generally hydrophobic. However, a completely hydrophobic sequence may not provide soluble oligopeptides. Furthermore, the naturally occurring cleavage sites in retroviruses are expected to be in regions connecting folded protein domains of polyproteins, where a high degree of hydrophobicity may be unfavorable for the correct conformation. Besides the hydrophobicity, the size of residues also appears to be important. Our results suggest that in the case of tight packing of S₁ and S₁' with Tyr residues, a relatively larger residue at either P₂ or P₂' is preferable, but not at both positions. These factors may explain why symmetric subsite arrangements have not evolved at the naturally occurring cleavage sites, despite the symmetric nature of the retroviral

proteinases.

Previous studies did not explain whether the rather large differences in specificity of respective S and S' subsites are attributed to the asymmetry introduced by the binding of the substrate and/or the different amino acid sequence context. Design of a palindromic substrate and substitutions in its respective positions allowed us to study these effects. In general, our results suggest that the previously established differences in specificity of P₃ and P₃' positions could be mainly due to the different sequence context: in these positions the asymmetry introduced by the peptide backbone of the substrate does not seem to play an important role. For P₂ and P₂' positions the effect of asymmetry is not negligible; however, the different sequence context could be still the predominant cause of the substantial differences found previously (10).

Our results also show that the earlier classification of retroviral cleavage sites into two types is an oversimplification. Preference of Asn in P₂ and Ile in P₂' in type 1 cleavage sites as well as Ile (Val) in P₂ and Glu at P₂' in type 2 cleavage site substrates seems to be a function of the residues outside of the P₂-P₂' region of the substrate. The enzyme-substrate interaction of the HIV-1 proteinase as well as other retroviral proteinases cannot be described residue by residue, but the neighboring residues of the substrate should also be taken into account. These neighboring residues include not only the residues at the same side of the peptide backbone but also residues at the opposite side as suggested by Strop *et al.* (29) from analysis of avian myeloblastosis virus proteinase specificity. Similar results were obtained by Ridky *et al.* (30) from kinetic analysis of substrates containing double substitutions from P₃ to P₁' in the NC/proteinase cleavage site peptide of Rous sarcoma virus. However, the specificity pattern is even more complex than was originally thought, not only the internal P₂-P₂' residues, but outer residues like P₃' may also substantially influence the preference for a subsite, even for positions separated by four residues in the peptide sequence.

The most important features of the sequence context dependence are the size and β -branch in a residue. P₁' Tyr, unlike Pro, may fill completely the large S₁' subsite and could restrict the space for substrate residues at other subsites. On the other hand, substitution of Pro at P₁' could eliminate unfavorable interactions with β -branched residues at P₂ (10, 30). Even small changes in the substrate sequence might cause rearrangement of the substrate residues, as seen in some enzyme-inhibitor complexes as well as predicted by molecular modeling. This strong sequence context dependence also raises difficulties for predicting cleavage sites. For example, an early prediction method developed by Poorman *et al.* (7) failed to predict the cleavage site in Nef protein (34). New prediction methods that take into account the context-dependent nature

of the specificity (35) might give better results. Also, molecular modeling and improved energy calculation methods may help to develop better methods for predictions.

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