Richard, F. M., & Wyckoff, H. W. (1971) *Enzymes* (3rd Ed.) *4*, 647-806.
Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G., & Rhee, S. G. (1987) *J. Biol. Chem. 262*, 12511-12518.
Salamonczyk, G. M., & Bruzik, K. S. (1990) *Tetrahedron Lett.* 2015-2016.
Sammons, R. D., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 1138-1141.
Seitz, S. P., Kaltenbach, R. F., III, Vreekamp, R. H., Calabrese, J. C., & Perrella, F. W. (1992) *Bioorg. Med. Lett. 2*, 171-174.
Shukla, S. D. (1982) *Life Sci. 30*, 1323-1335.
Snyder, W. R. (1987) *Biochem. Biophys. Acta 920*, 155-160.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468-1472.
Volwerk, J. J., Shashidhar, M. S., Kuppe, A., & Griffiths, O. H. (1990) *Biochemistry 29*, 8056-8062.
Wilson, D. B., Bross, T. E., Sherman, W. R., Berger, R. A., & Majerus, P. W. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4013-4017.
Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J., & Brown, J. E. (1985b) *J. Biol. Chem. 260*, 13496-13501.
Young, R. C., Downes, C. P., Eggleston, D. S., Jones, M., Macphee, C. H., Rana, K. K., & Ward, J. G. (1990) *J. Med. Chem. 33*, 641-646.

Different Requirements for Productive Interaction between the Active Site of HIV-1 Proteinase and Substrates Containing -Hydrophobic*Hydrophobic- or -Aromatic*Pro- Cleavage Sites†

Jonathan T. Griffiths,‡ Lowri H. Phylip,‡ Jan Konvalinka,‡,§ Petr Strop,§ Alla Gustchina,∥ Alexander Wlodawer,∥ Ruth J. Davenport,⊥ Richard Briggs,⊥ Ben M. Dunn,*,⊥ and John Kay‡

Department of Biochemistry, University of Wales College of Cardiff, P.O. Box 903, Cardiff CF1 1ST, Wales, United Kingdom, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, 166 10 Prague 6, CSFR, Macromolecular Structure Laboratory, Frederick Cancer Research and Development Center, National Cancer Institute-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, Maryland 21702-1201, and Department of Biochemistry and Molecular Biology, Box 100245, University of Florida, Gainesville, Florida 32610

Received January 10, 1992; Revised Manuscript Received March 20, 1992

ABSTRACT: The sequence requirements for HIV-1 proteinase catalyzed cleavage of oligopeptides containing two distinct types of junctions (-hydrophobic*hydrophobic- or -aromatic*Pro-) has been investigated. For the first type of junction (-hydrophobic*hydrophobic-) the optimal residues in the P_2 and P_2' positions were found to be Val and Glu, respectively, in accord with recent statistical analysis of natural cleavage sites [Poorman, R. A., Tomasselli, A. G., Heinrikson, R. L., & Kézdy, F. J. (1991) *J. Biol. Chem. 266*, 14554-14561]. For the -aromatic*Pro- type of junction, in the specific sequence context studied here, the value of Glu in the P_2' position was again observed. An explanation for the inefficient cleavage observed for peptides with the sequence -Val-Tyr*Pro- has been provided from molecular modeling of the putative enzyme–substrate complex. A significant effect upon cleavage rates due to the amino acid in the P_5 position has also been documented. While lysine in the P_5 position in one sequence of the -hydrophobic*hydrophobic- type produces a peptide cleaved very efficiently ($k_{cat} > 15$ s^{-1} for Lys-Ala-Arg-Val-Nle*p-nitrophenylalanine-P_2'-Ala-Nle-NH_2, for P_2' = Glu, Gln, Ile, Val, or Ala), for substrates of the -aromatic*Pro- type, the P_5 residue can exert either a positive or negative effect on cleavage rates. These results have again been interpreted in light of molecular modeling. We suggest that interaction of the substrate sequence on the periphery of the active site cleft may influence the match of the enzyme–substrate pair and, hence, control the efficiency of catalysis. Thus, ability of HIV-1 PR to selectively *and* efficiently cleave a variety of totally different sequences may be derived, in part, from extensive interactions at long distances from the actual scissile peptide bond and the inherent flexibility of several key loops of polypeptide structure of the enzyme.

Biochemical characterization of the aspartic proteinase encoded within the genome of the human immunodeficiency virus (HIV)[1] as a dimer of identical subunits (Meek et al., 1989) was rapidly substantiated by crystallographic analysis of native (Navia et al., 1989; Wlodawer et al., 1989) and inhibitor-complexed enzyme (Miller et al., 1989; Swain et al., 1990; Fitzgerald et al., 1990; Erickson et al., 1990; Jaskólski et al., 1991). The proteinase is considered to be responsible for cleavage of (at least) eight sites in the gag and gag-pol

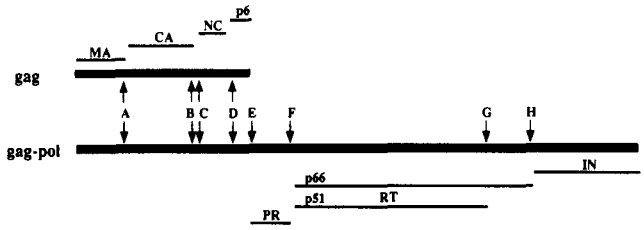
† This work was supported by grants and awards from the NIH (AI28571 to B.M.D.), the National Cancer Institute, DHHS, under contract number N01-CO74101 with ABL (to A.W.), the Czechoslovak Academy of Science (45501 to P.S. and J.K.), and the MRC-AIDS Directed Program (to J.K.).

* Address correspondence to this author.
‡ University of Wales College of Cardiff.
§ Czechoslovak Academy of Science.
∥ NCI-Frederick Cancer Research and Development Center.
⊥ University of Florida.

[1] Abbreviations: HIV, human immunodeficiency virus; HIV PR, HIV proteinase; P_1, P_2, P_1', P_2', S_1, S_2, S_1', S_2', etc., designation of amino acid residues of a substrate or inhibitor and corresponding regions of the enzyme active site involved in a complex according to Schechter and Berger (1967); Nph, *p*-nitrophenylalanine; pepRPC, C_2/C_{18} dual bonded, 100-Å pore size silica-based reversed-phase column; NMR, nuclear magnetic resonance; MHz, megahertz. In all peptides reported here the amino acids are of the L configuration. The cleavage point is indicated in each sequence by an asterisk (*).

0006-2960/92/0431-5193$03.00/0

a.



b.

TYPE		P_5	P_4	P_3	P_2	P_1		P_1'	P_2'	P_3'	P_4'	LOCATION
		~Val	Ser	Gln	Asn	Tyr	*	Pro	Ile	Val	Gln~	A
Aromatic*Pro		~Val	Ser	Phe	Asn	Phe	*	Pro	Gln	Ile	Thr~	E
		~Cys	Thr	Leu	Asn	Phe	*	Pro	Ile	Ser	Pro~	F
		~Lys	Ala	Arg	Val	Leu	*	Ala	Glu	Ala	Met~	B
Hydrophobic*		~Ser	Ala	Thr	Ile	Met	*	Met	Gln	Arg	Gly~	C
Hydrophobic		~Arg	Pro	Gly	Asn	Phe	*	Leu	Gln	Ser	Arg~	D
		~Gly	Ala	Glu	Thr	Phe	*	Tyr	Val	Asp	Gly~	G
		~Ile	Arg	Lys	Val	Leu	*	Phe	Leu	Asp	Gly~	H

FIGURE 1: (a) Schematic diagram to illustrate the relationship between the eight documented cleavage sites in the gag and gag–pol polyproteins from the human immunodeficiency virus. The capital letters (A, B, etc.) indicate the position of a cleavage site, while the labeled bars at top and bottom indicate the size and identity (Leis et al., 1988) of the resulting protein fragment. (b) The amino acid sequences from P_5 to P_4' flanking the eight cleavage sites.

polyproteins produced upon translation of the retroviral genome. Two distinct types (-aromatic*Pro- and -hydrophobic*hydrophobic-) of cleavage site have been identified, and the sequences enclosing these vary considerably (Figure 1). Thus, in contrast to the defined primary structural specificity displayed by most proteinases, the retroviral enzyme has to be adaptable enough to tolerate such variety and yet still be able to accomplish selective cleavage. This has been considered in a number of previous investigations [for example, see Pettit et al. (1991), Poorman et al. (1991), Tomasselli et al. (1991a), Tözsér et al. (1991a,b), and Fitzgerald and Springer (1991)]. In the contributions made to such analyses by our laboratories, the preferences for the P_1, P_2, and P_3 residues has been documented using synthetic peptide substrates (Konvalinka et al., 1990; Richards et al., 1990; Phylip et al., 1990). For example, a β-branched residue (Val/Ile) is preferred in P_2 in the -hydrophobic*hydrophobic- type cleavage junctions (B, C, D, G, and H in Figure 1) whereas Asn is invariably found in this position in junctions of the -aromatic*Pro- type (junctions A, E, and F, Figure 1). In addition, when mutations were introduced into the P_2 and P_1 positions flanking/contributing to cleavage sites in polyprotein substrates (Jupp et al., 1991), comparable effects were observed with these protein substrates to those alluded to above with the synthetic peptides. Thus, the use of peptides as substrates appears to provide an adequate reflection of the processing of protein substrates by this enzyme with respect to amino acid preferences near the cleavage point. Since assays employing peptides are considerably more convenient to perform and permit quantitative data to be derived, we have focused our attention on synthetic peptides mimicking the -Leu*Ala- junction (B in Figure 1) but with replacement of the Ala residue in P_1' with the chromophoric reporter group, *p*-nitrophenylalanine (Nph).

Table I: Kinetic Parameters for the Hydrolysis by HIV-1 Proteinase of a Series of Chromogenic Substrates, Lys-Ala-Arg-Val-Nle*Nph-X-Ala-Nle-NH_2, with Val in P_2 and Systematic Variation of the P_2' Residue[a]

	K_m (μM)	k_{cat} (s^{-1})
Lys Ala Arg Val Nle * Nph **Glu** Ala Nle NH_2	35	32
Lys Ala Arg Val Nle * Nph **Gln** Ala Nle NH_2	160	16
Lys Ala Arg Val Nle * Nph **Ile** Ala Nle NH_2	60	17
Lys Ala Arg Val Nle * Nph **Val** Ala Nle NH_2	100	15
Lys Ala Arg Val Nle * Nph **Ala** Ala Nle NH_2	100	17
Lys Ala Arg Val Nle * Nph **Asp** Ala Nle NH_2	nd	0.5
Lys Ala Arg Val Nle * Nph **Asn** Ala Nle NH_2	nd	0.4
Lys Ala Arg Val Nle * Nph **Thr** Ala Nle NH_2	210	0.4

[a] Determinations were made at pH 4.7 and μ = 0.3 M. nd = not determined.

Table II: Kinetic Parameters for the Hydrolysis by HIV-1 Proteinase of a Series of Chromogenic Substrates, Lys-Ala-Arg-Asn-Nle*Nph-X-Ala-Nle-NH_2, with Asn in P_2 and Systematic Variation of the P_2' Residue[a]

	k_{cat} (s^{-1})
Lys Ala Arg Asn Nle * Nph **Glu** Ala Nle NH_2	0.7
Lys Ala Arg Asn Nle * Nph **Gln** Ala Nle NH_2	0.1
Lys Ala Arg Asn Nle * Nph **Ile** Ala Nle NH_2	0.01
Lys Ala Arg Asn Nle * Nph **Val** Ala Nle NH_2	0.02
Lys Ala Arg Asn Nle * Nph **Ala** Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Nle * Nph **Asn** Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Nle * Nph **Thr** Ala Nle NH_2	<0.0001

[a] K_m parameters were estimated by the competitive substrate assay and/or FPLC analysis to lie in the range of 40–130 μM for these peptides.

In the present study, differences in preferences for individual subsites as well as correlations between a given amino acid appearing in one site with the presence/absence of a specific amino acid in other sites in the substrate are examined in detail. This was accomplished by introduction of an -aromatic*Pro- junction dipeptide into the sequence of residues that normally flanks the -Leu*Ala- cleavage site (Junction B in Figure 1). Systematic replacement of residues in the other flanking positions was then carried out. The data obtained have been interpreted on the basis of the known structures of several inhibitor–HIV proteinase complexes [for reviews, see Fitzgerald and Springer (1991), Wlodawer et al. (1992) and Swain et al. (1991)] and a proposed structure of an active site bound substrate developed from molecular modeling (Tözsér et al., 1991a).

Materials and Methods

Enzyme and Substrate Peptides. Homogeneous preparations of HIV-1 proteinase were obtained, and all peptides were prepared by solid-phase peptide synthesis, as previously described (Richards et al., 1990). All substrates were characterized by amino acid composition and reversed-phase HPLC analysis and were pure by these criteria.

Kinetic Analyses. Kinetic analyses were performed in 0.1 M sodium acetate buffer, pH 4.7, containing 4 mM EDTA and sufficient NaCl to give a final ionic strength of 0.3 M, as described previously (Richards et al., 1990; Konvalinka et al., 1990; Phylip et al., 1990). This involved monitoring of peptide cleavage either by spectroscopic methods by following the decrease in absorbance at 300 nm for substrates containing the -Nle*Nph- cleavage site (Figure 1; Tables I and II) or by reversed-phase FPLC analysis using a pepRPC column (Pharmacia, Milton Keynes, U.K.) for peptides containing the -Tyr*Pro- junction. Initial velocities were measured with at least six different concentrations of each peptide substrate within an appropriate range, and kinetic constants (K_m and V_{max}) were determined therefrom using the ENZFITTER program (Elsevier-BIOSOFT, Cambridge, U.K., by R. J. Leatherbarrow). The estimated error for all determinations

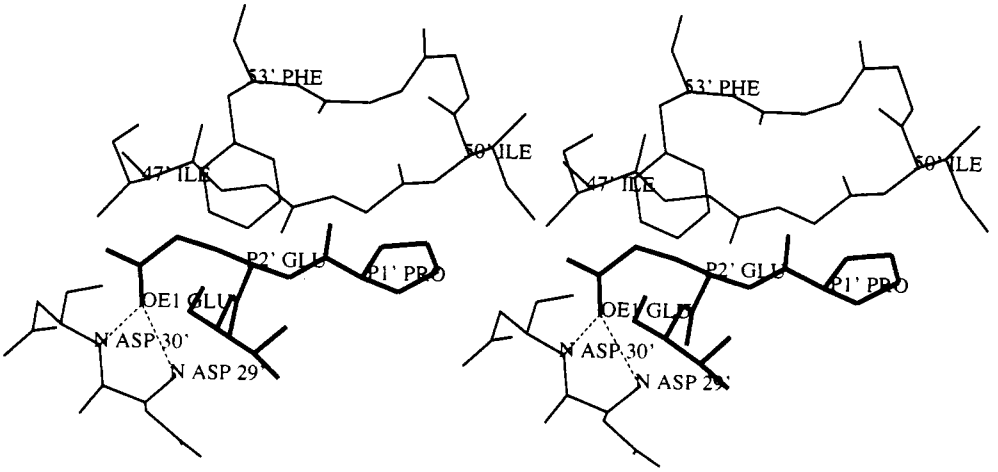


FIGURE 2: Stereo plot illustrating the fit of the P_2' residue in the S_2 region of the active site of HIV proteinase. The side chain of glutamic acid in this structure, modeled on the structure of an inhibitor with Gln in P_2' (Miller et al., 1989), makes acceptable hydrogen bonds to the backbone NH groups of residues 29′ and 30′.

was <20%. Values for k_{cat} were derived from $V_{max} = k_{cat}[Enz]$, where the active concentration of the enzyme was determined by active site titration of HIV-1 proteinase preparations with the tight binding (~0.3 nM) inhibitor, Ro 8959 (Roberts et al., 1990). In the case of some peptides where little or no hydrolysis was detected, an estimate of the affinity of peptide binding to HIV proteinase was derived by analysis of the effect of the peptide acting as a competitive inhibitor (Blake et al., 1989) on the cleavage of a chromogenic substrate.

Proton NMR Spectra. ^1H NMR spectra were obtained at 23 °C on a 7.05-T (300 MHz) Nicolet/Nalorac spectrometer. Approximately 10 mg of each peptide was dissolved in 0.6 mL of 99.9% D_2O, filtered by centrifugation through a 0.2-μm Nylon-66 membrane filter (Rainin Instruments, Woburn, MA), and placed in a 5-mm NMR tube. Spectral acquisition parameters were as follows: 3508 Hz spectral width, 8192 data points (real plus imaginary), 6.17-s relaxation delay, 15-μs rf pulse duration (68 degree flip angle), and 256 acquisitions for Ala-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Nle-NH_2 and 512 acquisitions for Lys-Arg-Gln-Asn-Tyr*Pro-Ile-Ala-Nle-NH_2. The time domain data were multiplied by an exponential function resulting in 0.5-Hz Lorentzian line broadening, zero-filled to 16K data points, and converted to frequency domain spectra by Fourier transformation. Peak assignments were made according to Toma et al. (1978). Peak areas were measured twice for each peptide using the aromatic proton resonances of Tyr both ortho and meta to the hydroxyl group. Since in each case one line of the cis doublet overlapped one line of the trans doublet, yielding three resolvable lines (t; c,t; c), isomer distributions were calculated as both (c/c,t) and (c/(c + t)) or (t/c,t) and (t/(c + t)) ratios.

Molecular Modeling. The crystal structures of complexes of HIV-1 PR with nine different inhibitors (Wlodawer et al., 1992, and references therein) were analyzed on an Evans and Sutherland PS390 molecular graphics system using the program FRODO (Jones, 1985). The atomic coordinates of all inhibitors were taken into account and used as a base for the modeling of the substrate with the sequence, Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (Tözsér et al., 1991b). The residues Lys at P_5, Val/Ile at P_2, and Glu at P_2' were also modeled at the respective positions, in order to facilitate interpretation of the kinetic data.

Results and Discussion

Since we have previously shown that the sequence Lys-Ala-Arg-Val-Nle*Nph-Glu-Ala-Nle-NH_2 is an excellent substrate ($K_{cat}/K_m \approx 10^6$ s^{-1} M^{-1}) for HIV PR (Richards et al., 1990), this template was utilized as the basis for a new series of peptides in which the P_2' residue was replaced systematically (Table I). While the native Glu residue yields the most efficient cleavage (k_{cat} = 32 s^{-1}, Table I), Gln, Ile, Val, and Ala are all acceptable substituents, with k_{cat} values about half of that for the parent sequence. In contrast, introduction of Asp, Asn, or Thr in the P_2' position diminished k_{cat} to values that were very low. In comparison to these major changes observed in k_{cat} (Table I and see below), only small variations were determined in the K_m parameter. Thus, for the sake of brevity, subsequent discussion will be focused largely on those aspects that might significantly affect the turnover number.

The preference for Glu (or, to a lesser extent, Gln) in P_2' may be derived from hydrogen bonding of the side chain carboxyl/carboxamide group(s) to the backbone NH of residues 29′ and 30′ in the enzyme (Figure 2). Conversely, the counterpart Asp or Asn residues that are shorter by a single methylene group would not appear to be long enough to make such favorable hydrogen bonding contacts. These observations are in keeping with recent reports that Glu is the preferred residue in the P_2' position for efficient hydrolysis at -hydrophobic*hydrophobic- cleavage sites in a variety of viral and nonviral proteins (Hui et al., 1990; Tomasselli et al., 1991a,b). Furthermore, a statistical analysis of a collection of viral polyproteins and nonviral proteins that were hydrolyzed by HIV PR indicated the high frequency with which Glu (and, to a lesser extent, Gln) was found in the P_2' position adjacent to the bond being cleaved (Poorman et al., 1991). By contrast, a separate statistical analysis of proteinases from 10 distinct retroviruses and 46 known sites processed by them revealed no such preference for the P_2' subsite (Pettit et al., 1991). This predilection would thus appear to be a feature of the HIV-1 and -2 proteinases (Poorman et al., 1991).

The theoretical analysis of Poorman et al. (1991) also indicated that Val, Ile, Leu, and Ala were found in the P_2' position adjacent to the scissile peptide bond in naturally-occurring protein substrates but with a considerably lower likelihood than Glu (and slightly less than Gln). Thus, our experimentally-derived findings of comparable k_{cat} values for Ile, Val, Ala, and Gln in P_2' in synthetic peptide substrates (Table I) are directly compatible with the theoretical considerations which appeared during the course of collection of our data. The fit of Ile, Val, and Ala undoubtedly reflects the generally hydrophobic nature of the S_2' region of the active site cleft of HIV PR (Figure 2). The extra polarity introduced

Table III: Effect of Variations in the P_5 and P_2' Positions on Kinetic Parameters for the Hydrolysis by HIV-1 Proteinase of a Series of Peptides Containing -Val-Tyr*Pro- as the Scissile Peptide Bond[a]

		k_{cat} (s^{-1})
Lys Ala Arg Val Nle * Nph Glu	Ala Nle NH_2	32
Lys Ala Arg Val Tyr * Pro **Glu**	Ala Nle NH_2	0.5
Ala Arg Val Tyr * Pro **Glu**	Ala Nle NH_2	0.08
Nle Ala Arg Val Tyr * Pro **Glu**	Ala Nle NH_2	1.4
Lys Ala Arg Val Tyr * Pro **Gln**	Ala Nle NH_2	<0.0001
Lys Ala Arg Val Tyr * Pro **Ile**	Ala Nle NH_2	<0.0001
Lys Ala Arg Val Tyr * Pro **Val**	Ala Nle NH_2	<0.0001
Lys Ala Arg Val Tyr * Pro **Ala**	Ala Nle NH_2	<0.0001
Lys Ala Arg Val Tyr * Pro **Asn**	Ala Nle NH_2	<0.0001
Lys Ala Arg Val Tyr * Pro **Thr**	Ala Nle NH_2	<0.0001

[a] K_m parameters for the -Tyr*Pro- containing peptides were estimated at pH 4.7 by the competitive substrate assay and/or FPLC analysis to lie in the range of 600–1250 μM.

by the OH group of Thr must account for the 30-fold reduction observed in the cleavage of the Thr-containing peptide by comparison with that measured for the Val-containing analog (Table I). Indeed, Thr, Asp, and Asn were scored extremely low if at all in the statistical analysis of cleavage sites in proteins (Poorman et al., 1991). The present demonstration of the significance of the residue occupying P_2' taken together with our earlier substantiation of the importance of the P_2 residue (Konvalinka et al., 1990; Phylip et al., 1990) emphasizes the importance for efficient cleavage of interactions made by residues *immediately flanking* the amino acids contributing the scissile peptide bond.

The significant role of the P_2 and P_2' residues was further substantiated by the data obtained with the series of peptides (Table II) in which the β-branched residue in P_2 was replaced by Asn. All of the peptides containing this simple replacement were very poorly cleaved at the adjacent scissile bond between P_1 and P_1'. Despite the generally low values for k_{cat}, once again, the presence of the Glu residue in P_2' resulted in a k_{cat} value that was significantly higher than all of the others obtained. For this class of substrate then, it would appear that the sequence -Val-hydrophobic*hydrophobic-Glu- presents the optimal juxtaposition of the target C=O of the scissile peptide bond with the catalytic Asp residues, ensuring successful cleavage. The extended active site cleft, present in most proteinases but particularly prominent in those of the aspartic class, has evolved to facilitate the correct positioning of the scissile peptide bond. How then does this account for the different types of cleavage site attacked readily by HIV PR?

Since, as has been shown above, the presentation of the scissile peptide bond can be influenced by the residues which span from P_2 through to P_2', this question was addressed by altering the sequence that was hydrolyzed optimally (Table I) to replace the -hydrophobic*hydrophobic- cleavage site in P_1*P_1' with one of the -aromatic*Pro- variety (Table III). The resultant peptide, Lys-Ala-Arg-Val-Tyr*Pro-Glu-Ala-Nle-NH_2 was cleaved more than 60-fold more slowly than its analog containing the -Nle*Nph- type cleavage site (Table III). In an initial attempt to evalute the significance of residues in peripheral subsites (see below), the N-terminal Lys was (i) deleted from the sequence or (ii) replaced with norleucine. The latter maintains the aliphatic backbone of the lysine side chain but lacks the positive charge of the ϵ-amino group (Table III). The deletion resulted in a further reduction in the (already low) k_{cat} value whereas replacement with Nle produced a slight elevation. Thus, the presence of a residue with an aliphatic side chain in P_5 enables a significant value of k_{cat} (albeit still low at around 1 s^{-1}) to be achieved for the peptides containing the -Val-Tyr*Pro- sequence described here.

Table IV: Effect of Variations in the P_5 and P_2' Positions on Kinetic Parameters for the Hydrolysis by HIV-1 Proteinase of a Series of Peptides Containing -Asn-aromatic*Pro- as the Scissile Peptide Bond[a]

		k_{cat} (s^{-1})
Lys Ala Arg Val Nle * Nph Glu	Ala Nle NH_2	32
Lys Ala Arg Asn Tyr * Pro **Glu**	Ala Nle NH_2	~0.06
Nle Ala Arg Asn Tyr * Pro **Glu**	Ala Nle NH_2	1.7
Val Ala Arg Asn Tyr * Pro **Glu**	Ala Nle NH_2	>1.7#
Lys Ala Arg Asn Tyr * Pro **Gln**	Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Tyr * Pro **Asn**	Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Tyr * Pro **Ala**	Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Tyr * Pro **Val**	Ala Nle NH_2	<0.0001
Lys Ala Arg Asn Tyr * Pro **Ile**	Ala Nle NH_2	<0.01
Ac Ser Gln Asn Tyr * Pro Ile	Val	10
Tyr Val Ser Gln Asn Phe * Pro Ile	Val Gln Asn Arg	8
Nle Ser Gln Asn Tyr * Pro Ile	Val Nle NH_2[$]	~2.5

[a] K_m parameters for the -aromatic*Pro- containing peptides were estimated by the competitive substrate assay and/or FPLC analysis to be in the range of 450–1150 μM with one exception (#). In the case, the value, estimated at approximately 3 mM, lay outside the experimentally accessible range of substrate concentrations. The symbol $ indicates that severe product inhibition was encountered with this peptide.

The importance of a Glu residue in P_2' was further emphasized by the data in Table III. Replacement, even by Gln, Ile, Val, or Ala, resulted in peptides that were not cleaved even upon prolonged incubation (16 h) with high concentrations (20 times above normal assay level) of HIV PR. The poor rate of cleavage of the Ile- and Gln-containing derivatives is particularly noteworthy since these residues are found frequently in the P_2' position at -aromatic*Pro- type cleavage junctions (Figure 1).

These observations of very slow cleavage of -Val-Tyr*Pro-P_2'- containing peptides are also in accord with the statistical evaluation of Poorman et al. (1991), as it may be observed from their protein substrate database than when Pro is present in P_1', the P_2 residue is *never* a branched residue such as Val. The structural rationale for this will be considered later.

However, Figure 1 also indicates that -aromatic*Pro- junctions invariably contain not Val but an Asn residue in P_2, suggesting that optimal presentation of -aromatic*Pro- cleavage sites may require a different set of interactions in the P_2–P_2' region. This was investigated by substituting Asn for Val in the P_2 position (Table IV). Once more, however, the peptide Lys-Ala-Arg-Asn-Tyr*Pro-Glu-Ala-Nle-NH_2 was cleaved very poorly. Substitution of the Glu in P_2' by Gln, Ile, Val, Ala, or Asn resulted in even slower rates of cleavage (Table IV), emphasizing once more that, despite the poor absolute magnitude of the k_{cat} value, Glu is still the optimal residue in P_2'. Next, while retaining Glu in P_2', the effect of replacement of the Lys residue in P_5 with a straight chain aliphatic (Nle) or β-branched (Val) side chain in this series was investigated (Table IV). In both cases, k_{cat} values were considerably enhanced over that of the parent Lys-containing peptide. In this instance, therefore, not only is the presence of a residue in P_5 necessary to achieve a (still only) modest value for the turnover number, but a positively-charged ϵ-amino group at the end of the side chain (in Lys) would appear to be detrimental (compared to Nle and Val in P_5). The ca. 28-fold improvement seen in this case may be compared to the 2.8-fold difference between the Lys/Nle-Ala-Arg-Val-Tyr*Pro-Glu-Ala-Nle-NH_2 pair of peptides containing a Val in P_2 (Table III).

Thus, the results reported in Tables I–IV not only provide evidence for the critical role of both P_2 and P_2' substituents in controlling the rate of cleavage of the -P_1*P_1'- scissile peptide bond by this symmetrical enzyme (Wlodawer et al., 1989) but

Table V: Kinetic Parameters for the Hydrolysis by HIV-1 Proteinase of a Series of Peptides Containing the -Asn-Tyr*Pro-Ile Central Motif

		K_m (μM)	k_{cat} (s^{-1})
1	Lys Ala Arg Asn Tyr * Pro Ile Ala Nle NH_2	780	<0.01
2	Lys Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	180	0.3
2a	Nle Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	420	0.13
2b	Val Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	1200	0.15
3	Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	430	5
4	Ala Gln Asn Tyr * Pro Ile Val Nle NH_2	650	12
5	Lys Ala Gln Asn Tyr * Pro Ile Val Nle NH_2	400	0.5
6	Lys Ala Gln Asn Tyr * Pro Ile Ala Nle NH_2	150	0.1
7	Lys Arg Gln Asn Tyr * Pro Ile Ala Nle NH_2	190	0.3
8	Ala Ser Gln Asn Tyr * Pro Ile Val Nle NH_2	50	9
9	Ala Ser Gln Asn Tyr * Pro Ile Val NH_2	240	7

also suggest that the presence/identity of the residue in a remote subsite (P_5) may have an interconnected effect to the nature of the residues spanning P_2–P_2' of the substrate. The latter stands in contrast to the statistical analysis of Poorman et al. (1991) which revealed no such correlation between subsites. The absolute magnitudes measured for the turnover numbers of the -aromatic*Pro- containing peptides (for example, k_{cat} = <0.01 s^{-1} for Lys-Ala-Arg-Asn-Tyr*Pro-Ile-Ala-Nle-NH_2; Table IV) are nonetheless sluggish and much slower than that for Ac-Ser-Gln-Asn-Tyr*Pro-Ile-Val (10 s^{-1}; Table IV), the latter peptide mimicking an authentic -aromatic*Pro- junction (junction A in Figure 1). This >1000-fold difference in k_{cat} is especially remarkable when it is considered that the four central residues in both of these peptides, namely, -Asn-Tyr*Pro-Ile-, are identical. Indeed, two further -Asn-aromatic*Pro-Ile- containing peptides, Nle-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Nle-NH_2 and Tyr-Val-Ser-Gln-Asn-Phe*Pro-Ile-Val-Gln-Asn-Arg, also generated respectable k_{cat} values of 2.5 and 8 s^{-1} respectively (Table IV). The importance of a β-branched (Ile) residue in P_2' in -Asn-Tyr*Pro-Ile- containing substrates for the maintenance of k_{cat} has been reported previously (Margolin et al., 1990). From these observations, it is clear that the residues *outside* this central P_2Asn-P_1Tyr*P_1'Pro-P_2'Ile- sequence must also play a major role in adjusting the fit of the scissile bond and, hence, the efficiency of catalytic cleavage (Tomasselli et al., 1991a; Tözsér et al., 1991b).

To examine this, a further series of peptides was synthesized (Table V) in which the optimal -Asn-Tyr*Pro-Ile- central motif was retained, with systematic replacement of the residues in the more peripheral positions. Substitution of Ala by Val in P_3' at the C-terminal end of the peptide enhanced k_{cat} (compare values for peptides 1 and 2, 6 and 5 in Table V), but, in absolute terms, the values measured were still poor. In addition, truncation of the peptide to delete the (Nle) residue in P_4' (compare peptides 8 and 9, Table V) had little effect on k_{cat} but did alter the apparent K_m value by about 5-fold. We and others have shown previously (Konvalinka et al., 1990; Tomasselli et al., 1991a; Poorman et al., 1991; Pettit et al., 1991) that the nature of the residue occupying P_3 is of little significance. Indeed, interchange between Arg and Gln did not substantially alter kinetic parameters (compare peptides 3 and 4, Table V). While the present work was in progress, a separate report by Tözsér et al. (1991a) provided evidence that the nature of the residue in P_4 is of considerable importance with a preference for amino acids with a high propensity to form β-turns. Interchange of Ser and Ala in this P_4 position resulted in a 2-fold alteration in turnover number (Tözsér et al., 1991a). Thus, the minimal effect on k_{cat} of replacement of Ala by Ser in our peptides (compare peptides 4 and 8, Table V) is entirely in keeping with these observations. Indeed, the k_{cat} values measured for peptides 3, 4, 8, and 9 in our series (Table V) are directly comparable to those reported by Tözsér et al. (1991a). Dramatically, however, the substitution of residues that resulted in the sequence of peptide 8 (Table V) produced a substrate with a much lower K_m value and thus a k_{cat}/K_m ratio of 0.18 × 10^6 s^{-1} M^{-1}, higher than the values for the other peptides in our series (Table V) and between 3- and 5-fold improved on the best of the substrates described by Tözsér et al. (1991a).

In our series of peptides (Table V), the presence/nature of the residue in the peripheral P_5 position was the major influence by far. Those peptides with a branched/straight aliphatic side chain without (Val/Nle) and with (Lys) an ϵ-amino group all generated k_{cat} values of <0.5 s^{-1} (peptides 1, 2, 2a, 2b, 5, 6, and 7, Table V). In stark contrast, the four which either do not have a P_5 residue (peptides 3 and 4) or have the very short (methyl) side chain of Ala (peptides 8 and 9, Table V) were all cleaved very efficiently with k_{cat} values (between 5 and 12 s^{-1}) comparable to those measured for the authentic site A peptide analogs (see above and Table IV). Although peptides of this size are unlikely to adopt a single conformational state (Dyson & Wright, 1991), the Tyr*Pro peptide bond can eixst as cis and trans conformers. If only one isomer was able to undergo productive binding to the enzyme to permit hydrolytic attack, then an alteration in the cis/trans ratio resulting from the introduction of a (larger) residue in P_5 might produce a diminution in k_{cat}. In order to examine this possibility, advantage was taken of the demonstration by Toma et al. (1978) that splitting of the proton resonances generated by the amino acid adjacent to a proline residue gives a reliable indication of the conformational equilibrium about the Pro peptide bond. Analysis of the Tyr aromatic protons in peptides 7 and 8 (from Table V) provided equivalent estimates of 70% trans/30% cis (±2–6%) for the Pro peptide bond conformation in each peptide. Since there is not much energetic difference between the cis and trans forms (based on this ratio) and since peptides 7 and 8 have identical conformational equilibria yet differ in their susceptibility to cleavage (k_{cat}) by a factor of 30, it would seem unlikely that this is due to an isomerization of this type of the Tyr*Pro containing substrate as a result of the presence of a larger residue (such as the Lys in peptide 7) in P_5.

Inhibitors and, probably, substrates (Gustchina & Weber, 1990; Tözsér et al., 1991a) adopt an extended conformation in the active site of HIV PR. Therefore, the positioning of appropriate residues at P_5 and P_4 can make contacts on the periphery of the active site cleft which have a significant influence on catalytic efficiency. Such peripheral contacts that are "wrongly made" may be relayed into the centerpiece of the active site complex with detrimental consequences. For example, whereas the peptides of the -hydrophobic*hydrophobic- type as exemplified by the Nle*Nph variety were cleaved very rapidly (Table I) with a Lys residue at P_5, the presence of this residue caused a major diminution in the efficiency of cleavage of an -Asn-aromatic*Pro- type of substrate (compare peptides 2 and 3, Table V) with an otherwise closely similar sequence. Thus, the two types of cleavage junction have different requirements for optimal alignment in the active site cleft and effective cleavage by HIV-1 PR. The geometry around the

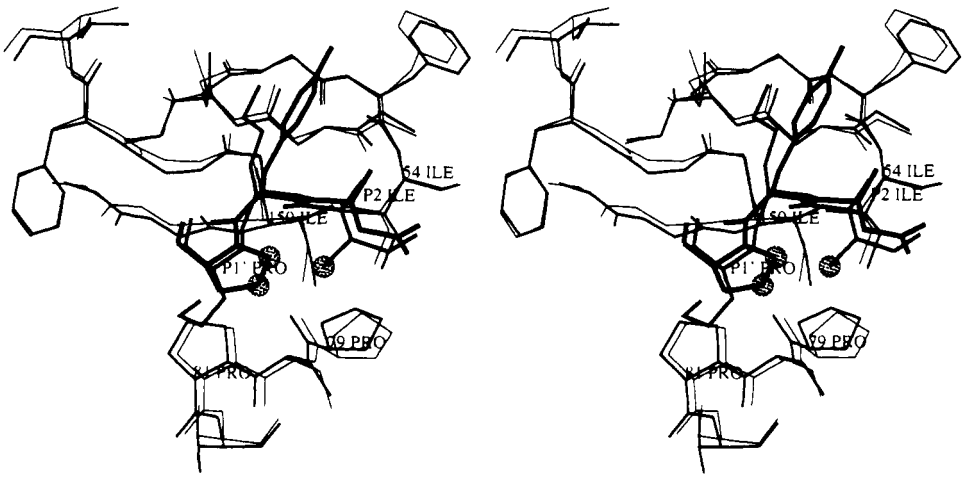


FIGURE 3: Stereo plot illustrating the environment surrounding a -Tyr*Pro- scissile peptide bond in a substrate bound at the active site of HIV proteinase. The structure of a model of an -Ile-Tyr*Pro- containing peptide has been superimposed upon the crystallographically-determined structure of an inhibitor (Miller et al., 1989) which has the sequence, -Asn-NleΨ[CH_2-NH]Nle-, in the region shown. The atoms that would be in potential van der Waals contact in the -Ile(or Val)-Tyr*Pro- structure, two methylenes from Pro and one methyl from Ile/Val, are indicated by the filled spheres.

-hydrophobic*hydrophobic- scissile peptide bond cannot be the same as that of -aromatic*Pro- bonds, particularly with the unique influence of the imido nitrogen of a Pro residue. In an attempt to explain the interactions which such substrates would generate, a model was constructed (Figure 3) for an -aromatic*Pro- containing peptide on the basis of the coordinates of the structures solved for HIV-1 PR complexed with nine inhibitors (Wlodawer et al., 1992), including Ac-Ser-Leu-Asn-PheΨ[CH(OH)CH_2-NH]Pro-Ile-Val-OMe (Swain et al., 1990), the only compound described structurally thus far with proline in the P_1' position.

From this model, it is clear that the γ and δ methylene groups of the proline ring in P_1' occupy space that would not have been filled if the P_1' residue was any other amino acid (Figure 3). Thus, with proline positioned in P_1', if a β-branched side chain were present in the P_2 residue of a substrate or inhibitor, the fork would make unacceptably close contacts with the ring of the Pro (Figure 3). The failure of HIV-1 PR to cleave -Val-Tyr*Pro- containing peptides (Table III) is perhaps then not surprising, and an explanation of why Val/Ile have never been observed in the P_2 position adjacent to -aromatic*Pro- type junctions in viral polyproteins is readily apparent.

The methylene groups within the proline ring may also be involved in interactions with Ile_{50}' at the tip of the loop in the polypeptide strand (called the "flap" and consisting of residues 45–55) contributed by one of the subunits in the dimeric proteinase. In addition, Ile_{54} from the second subunit and the mobile loop containing Pro_{79} and Pro_{81} may reinforce this hydrophobic interaction within the complex (Figure 3). A more extensive asymmetry is thus introduced into the active site by the presence of Pro in P_1' than has hitherto been observed for any other bound ligand (Miller et al., 1989). The net result is a bound structure that differs in subtle but significant interactions from complexes of the -hydrophobic*hydrophobic- type. Thus, hydrophobic interactions between the flexible elements of the protein and the unique structure of a proline, when present in the P_1' position of a substrate, may provide a stronger influence on the structure/conformation around the scissile peptide bond.

Insight into the general pattern of ligand binding in the active site of HIV-1 PR has emerged from studies of the complexes between PR and inhibitors of different chemistries (Fitzgerald & Springer, 1991; Wlodawer et al., 1992; Swain et al., 1991). An energetically significant network of hydrogen bonds stretches over 15–20 Å of the surface of the active site. This network is created by the interaction of every C=O and —NH— of peptide bonds in the bound ligand with appropriate functional groups of the enzyme (Gustchina & Weber, 1990). Indeed, the pattern that has been observed in all complexes of HIV PR with ligands of a peptidic nature so far examined is very consistent, and the interactions might be subdivided into two types: (i) those arising from the "rigid" areas of the enzyme, mainly involving the stable core of the protein or (ii) those from the "flexible" areas which include the two mobile "flaps" and other outer polypeptide loops. In both classes, there are hydrogen bonds that provide interactions that are important in determining the level of catalytic efficiency of the enzyme. However, significant differences between them are to be expected. In class i interactions, the conformational changes which might be caused by alterations of the residues of the substrate at peripheral positions should be localized, since the rigidity of the protein structure prevents substantial movement of those regions of the enzyme. In class ii, however, alterations of residues, even in such peripheral substrate positions as P_5 and P_3, could induce conformational changes throughout the flexible "flaps", including the tips of the "flaps" which are involved in interactions around the scissile bond of the substrate, thus potentially influencing the level of k_{cat} significantly.

The side chain of the P_3 residue of a bound ligand is positioned snugly between Phe_{53} of one of the mobile "flaps" and Pro_{81}' of an outer loop from the second subunit (Figure 4). With ligand bound as an extended β-strand (Gustchina & Weber, 1991), the side chain of the P_5 residue would then be positioned on the same side of the cleft as that of P_3. Extended inhibitors show a tendency to "curl up" at their extremity away from the body of the enzyme due to the twist inherent in an extended β strand (Wlodawer et al., 1992). This could result in strong interaction with the outer structure of the "flap" regions, in effect "returning the passionate embrace" provided by the closing of the two "flaps" of the enzyme down around the ligand positioned in the active site. Modeling suggests that favorable hydrophobic interactions could be generated by an appropriate P_5 residue if the side chain were positioned between the side chains of residues Phe_{53} and Met_{46} on the "flap". In the case of a Lys in P_5, the four methylene groups of the side chain can make acceptable hydrophobic contacts while the

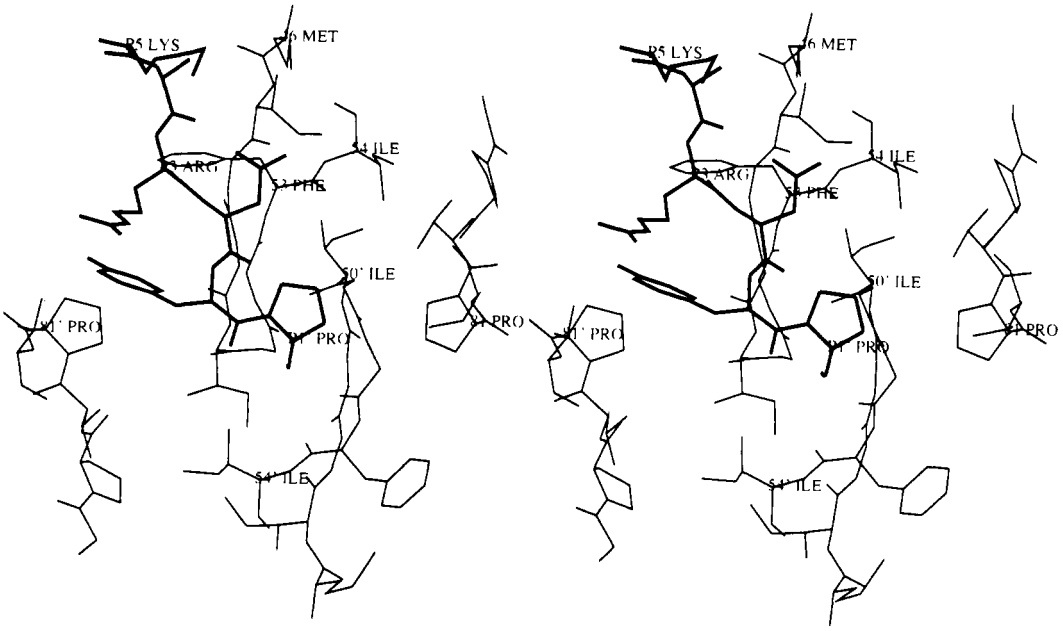


FIGURE 4: Stereo plot of the region surrounding the P_5–P_1' portion of a -Tyr*Pro- containing substrate built by modeling into the active site of HIV PR. This view illustrates the position of the P_3 Arg side chain between residues Phe_{53} and Pro_{81}' of the enzyme, as well as a possible interaction between a P_5 Lys side chain and enzyme residues Phe_{53} and Met_{46}. The packing of Ile_{50}' around the P_1' Pro as well as the further interaction of Ile_{54} with Ile_{50}' and the proximity of the loops including Pro_{81} may also be noted in this view.

Table VI: Effect of P_5 Substitution on k_{cat} Values Measured for Three Groups of Cleavage Site Sequences

	k_{cat} (s^{-1})
Ala Arg Val Tyr * Pro Glu Ala Nle NH_2	0.08
Lys Ala Arg Val Tyr * Pro Glu Ala Nle NH_2	0.5
Nle Ala Arg Val Tyr * Pro Glu Ala Nle NH_2	1.4
Lys Ala Arg Asn Tyr * Pro Glu Ala Nle NH_2	~0.06
Nle Ala Arg Asn Tyr * Pro Glu Ala Nle NH_2	1.7
Val Ala Arg Asn Tyr * Pro Glu Ala Nle NH_2	>1.7
Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	5
Lys Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	0.3
Nle Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	0.13
Val Ala Arg Asn Tyr * Pro Ile Val Nle NH_2	0.15

charged ϵ-amino group is directed into bulk solvent (Figure 4). Such interactions with the "flap" may be relayed, because of the intrinsic mobility of this region and because of the interdigitation of the two mobile "flaps" and loops around the Pro in P_1', in such a way as to perturb the hydrogen bonding interactions around the scissile peptide bond and the precise alignment that is necessary for effective cleavage at the -Tyr*Pro- type cleavage junction.

The opportunity to form such alternative potential interactions on the periphery of the active site arises from having an enzyme with sufficient built-in flexibility to reposition "flap" and loop features. The subtleties of these available options are emphasized by collation (in Table VI) of the data obtained for some of the peptides described earlier in Tables III–V. In the first subset, the -Tyr*Pro- cleavage site is flanked by Val in P_2 with the Glu in P_2' that was demonstrated (Table I) to be optimal for cleavage at -hydrophobic*hydrophobic- junctions. In the case of the -aromatic*Pro- junction, the introduction of a residue (either Lys or Nle) in P_5 resulted in a substantial improvement in k_{cat}. When the central motif was adjusted to position an Asn in P_2 while retaining the Glu in P_2', in the resultant set of peptides a Lys in P_5 was not beneficial while the aliphatic side chains of Nle or Val facilitated cleavage by some 30-fold. In this instance, the positioning of the positive charge on the ϵ-amino group of the lysine residue would appear to be counterproductive to the potentially beneficial contacts that can be made by the methylene groups in the otherwise equivalent side chains of Nle and Lys. In the final subset (Table VI) where the central four residues are -Asn-Tyr*Pro-Ile-, little difference was detected with variation of the P_5 residue by comparison with the major influences observed when no residue was present in P_5. This result is highly significant since junction A (Figure 1) in the viral polyprotein has the sequence Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val (Val in P_5 and Ile in P_2'). Thus, although six of the nine residues in Val-Ala-Arg-Asn-Tyr*Pro-Ile-Val-Nle-NH_2 are identical to those in the polyprotein junction with five of those located between P_2 and P_3' around the (potentially) scissile peptide bond, the measured k_{cat} value still falls into the low range. In this case, then, residues in P_4, P_3, or P_4' might be concluded to exert a negative influence on the positioning of the scissile peptide bond.

The above comparisons underscore the conclusion that, while the proteinase possesses the ability to adapt to a variety of cleavage site sequences, the entire active site cleft must be involved in interaction with the ligand. It is our contention that the selectivity evidenced by this retroviral enzyme is the inevitable consequence of an extensive network of interactions not only within the seven subsites (from S_4 to S_3') known to be important for HIV PR (Darke et al., 1989; Moore et al., 1989; Tözsér et al., 1991a), as well as for the archetypal aspartic proteinase (Dunn et al., 1986), but extending outside this region as well. Furthermore, we must conclude that this interactive network is correlated such that a sequence difference at any point in the substrate can lead to changes in hydrogen bonding interactions that can in turn cause significant alterations in catalytic cleavage efficiency. Such multiple, interacting determinants for substrate recognition culminate in different requirements for the optimal sequence flanking the two types of cleavage junction. These conclusions have implications for the design of inhibitors directed against retroviral enzymes such as HIV-1 PR. Current strategies of design are focused on producing the smallest possible structure that retains high affinity for the active site cleft of the target enzyme without affecting comparable aspartic proteinases of the human host and, yet, will possess some metabolic stability in vivo. However, such compounds, targeted at subsites S_3

to S_3' at most, are not likely to take advantage of the "extra" specificity derived from interactions of an extended peptide with the "flap" regions. This specificity may have evolved to provide stronger interactions with the extended sequence present in the polyprotein substrate with which the enzyme is designed to interact. Accessing this "Achilles heel" of the retroviral proteinases while maintaining bioavailability of the drug remains a major challenge for targeted drug design of the future.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the contributions to this work made by our colleagues in Hofmann LaRoche/Roche Products, Ltd. and by the late Herve Hirel, from Rhone-Poulenc in Vitry-sur-Seine, France.

REFERENCES

Blake, R. C., Vassall, R. F., & Blake, D. A. (1989) *Arch. Biochem. Biophys. 272*, 52–68.

Drake, P. L., Leu, C.-T., Davis, L. J., Heimbach, J. C., Diehl, R. E., Hill, W. S., Dixon, R. A. F., & Sigal, I. S. (1989) *J. Biol. Chem. 264*, 2307–2312.

Dunn, B. M., Jimenez, M., Parten, B. F., Valler, M. J., Rolph, C. E., & Kay, J. (1986) *Biochem. J. 237*, 899–906.

Dyson, H. J., & Wright, P. E. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 519–538.

Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X. C., Norbeck, D., Plattner, J. J., Rittenhouse, J., Turon, M., Wideburg, N., Kohlbrenner, W. E., Simmer, R., Helfrich, R., Paul, D., & Knigge, M. (1990) *Science 249*, 527–533.

Fitzgerald, P. M. D., & Springer, J. P. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 299–320.

Fitzgerald, P. M. D., McKeever, B. M., VanMiddlesworth, J. F., Springer, J. P., Heimbach, J. C., Leu, C.-T., Herber, W. K., Dixon, R. A. F., & Darke, P. L. (1990) *J. Biol. Chem. 265*, 14209–14219.

Gustchina, A., & Weber, I. T. (1990) *FEBS Lett. 269*, 269–272.

Gustchina, A., & Weber, I. T. (1991) *Proteins: Struct., Funct., Genet. 10*, 325–339.

Hui, J. O., Tomasselli, A. G., Zürcher-Neely, H. A., & Heinrikson, R. L. (1990) *J. Biol. Chem. 265*, 21386–21389.

Jaskôlski, M., Tomasselli, A. G., Sawyer, T. K., Staples, D. G., Heinrikson, R. L., Schneider, J., Kent, S. B. H., & Wlodawer, A. (1991) *Biochemistry 30*, 1600–1609.

Jones, T. A. (1985) *Methods Enzymol. 115*, 157–171.

Jupp, R. A., Phylip, L. H., Mills, J. S., LeGrice, S. F. J., & Kay, J. (1991) *FEBS Lett. 283*, 180–184.

Konvalinka, J., Strop, P., Velek, J., Cerna, V., Kostka, V., Phylip, L. H., Richards, A. D., Dunn, B. M. & Kay, J. (1990) *FEBS Lett. 268*, 35–38.

Leis, J., Baltimore, D., Bishop, J. M., Coffin, J., Fleissner, E., Goff, S. P., Oroszlan, S., Robinson, H., Skalka, A. M., Temin, H. M., & Vogt, V. (1988) *J. Virol. 62*, 1808–1809.

Margolin, N., Heath, W., Osborne, E., Lai, M., & Vlahos, C. (1990) *Biochem. Biophys. Res. Commun. 167*, 554–560.

Meek, T. D., Dayton, B. D., Metcalf, B. W., Dreyer, G. B., Strickler, J. E., Gorniak, J. G., Rosenberg, M., Moore, M. L., Magaard, V. W., & Debouck, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1841–1845.

Miller, M., Schneider, J., Sathyanarayana, B. K., Toth, M. V., Marshall, G. R., Clawson, L., Selk, L., Kent, S. B. H., & Wlodawer, A. (1989) *Science 246*, 1149–1152.

Moore, M. L., Bryan, W. M., Fakhoury, S. A., Magaard, V. W., Huffman, W. F., Dayton, B. D., Meek, T. D., Hyland, L., Dreyer, G. B., Metcalf, B. W., Strickler, J. E., Gorniak, J. G., & Debouck, C. (1989) *Biochem. Biophys. Res. Commun. 159*, 420–425.

Navia, M. A., Fitzgerald, P. M., McKeever, B. M., Leu, C.-T., Heimbach, J. C., Herber, W. K., Sigal, I. S., Darke, P. L., & Springer, J. P. (1989) *Nature 337*, 615–620.

Pettit, S. C., Simsic, J., Loeb, D. D., Everitt, L., Hutchison, C. A., III, & Swanstrom, R. (1991) *J. Biol. Chem. 266*, 14539–14547.

Phylip, L. H., Richards, A. D., Kay, J., Konvalinka, J., Strop, P., Blaha, I., Velek, J., Kostka, V., Ritchie, A. J., Broadhurst, A. V., Farmerie, W. G., Scarborough, P. E., & Dunn, B. M. (1990) *Biochem. Biophys. Res. Commun. 171*, 439–444.

Poorman, R. A., Tomasselli, A. G., Heinrikson, R. L., & Kézdy, F. J. (1991) *J. Biol. Chem. 266*, 14554–14561.

Richards, A. D., Phylip, L. H., Farmerie, W. G., Scarborough, P. E., Alvarez, A. A., Dunn, B. M., Hirel, Ph-H., Konvalinka, J., Strop, P., Pavlickova, L., Kostka, V. & Kay, J. (1990) *J. Biol. Chem. 265*, 7733–7736.

Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Duncan, I. B., Galpin, S. A., Handa, B. K., Kay, J., Kröhn, A., Lambert, J. R. W., Merrett, J. J. H., Mills, J. S., Parkes, K. E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J., & Machin, P. J. (1990) *Science 248*, 358–361.

Swain, A. L., Miller, M., Green, J., Rich, D. H., Schneider, J., Kent, S. B. H., & Wlodawer, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8805–8809.

Swain, A. L., Gustchina, A., & Wlodawer, A. (1991) in *Structure and Function of the Aspartic Proteinases: Genetics, Structures and Mechanisms* (Dunn, B. M., Ed.) pp 433–441, Plenum Press, New York.

Toma, F., Fermandjian, S., Low, M., & Kisfaludy, L. (1978) *Biochim. Biophys. Acta 534*, 112–122.

Tomasselli, A. G., Howe, W. J., Sawyer, T. K., Wlodawer, A., & Heinrikson, R. L. (1991a) *Chim. Oggi 9*, 6–27.

Tomasselli, A. G., Hui, J. O., Sawyer, T. K., Thaisivongs, S., Hester, J. B., & Heinrickson, R. L. (1991b) in *Structure and Function of the Aspartic Proteinases: Genetics, Structures, and Mechanisms* (Dunn, B. M., Ed.) pp 469–482, Plenum Press, New York.

Tözsér, J., Gustchina, A., Weber, I. T., Bláha, I., Wondrak, E. M., & Oroszlan, S. (1991a) *FEBS Lett. 279*, 356–360.

Tözsér, J., Bláha, I., Copeland, T. D., Wondrak, E. M., & Oroszlan, S., (1991b) *FEBS Lett. 281*, 77–80.

Wlodawer, A., Miller, M., Jaskôlski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science 245*, 616–621.

Wlodawer, A., Swain, A. L., & Gustchina, A. (1992) *Biochem. Pharmacol.* (in press).