Volume 279, number 2, 356–360 FEBS 09429 February 1991
ADONIS 001457919100146F

Studies on the role of the S_4 substrate binding site of HIV proteinases

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Received 19 November 1990

Kinetic analysis of the hydrolysis of the peptide H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 and its analogs obtained by varying the length and introducing substitutions at the P_4 site was carried out with both HIV-1 and HIV-2 proteinases. Deletion of the terminal Val and Gln had only moderate effect on the substrate hydrolysis, while the deletion of the P_4, Ser as well as P_3 Val greatly reduced the substrate hydrolysis. This is predicted to be due to the loss of interactions between main chains of the enzyme and the substrate. Substitution of the P_4 Ser by amino acids having high frequency of occurrence in β turns resulted in good substrates, while large amino acids were unfavorable in this position. The two proteinases acted similarly, except for substrates having Thr, Val and Leu substitutions, which were better accommodated in the HIV-2 substrate binding pocket.

HIV proteinase; Type 1 and 2; Oligopeptide substrate; Enzyme kinetics

1. INTRODUCTION

Since the retroviral proteinase (PR) of the human immunodeficiency virus type-1 (HIV-1), and type-2 (HIV-2) is essential for virus replication [1], the HIV PRs are considered potential targets for chemotherapy [2]. The polyprotein processing by retroviral PRs, including HIV-1 PR, seems to be restricted to the junctions of the protein domains of Gag and Gag-Pol polyproteins [3,4]. However, it is difficult to determine a consensus substrate sequence for the retroviral PRs. Although the substrate specificity of retroviral proteinases may partially depend on the proper substrate conformation, the amino acid sequence may have an important role in determining the cleavage sites. A better understanding of the substrate specificity may help to design potent inhibitors that are effective against both HIV-1 and HIV-2 PRs. Recently, kinetic studies were carried out on oligopeptide substrates with single amino acid substitutions [5–8]. Here we report a kinetic and modeling study on the S_4 site (notation according to Schechter and Berger [9]) of HIV PRs. Variations in substrate length were also analyzed for both proteinases.

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2. MATERIALS AND METHODS

Recombinant HIV-1 PR [10] and chemically synthesized HIV-2 PR [11,12] were used. Active enzyme content was determined by active site titration using a potent transition-state analog inhibitor [13]. Oligopeptides with amide C-termini were synthesized and characterized as described [11]. The assays were performed in 0.25 M potassium phosphate buffer, pH 5.6, containing 7.5% glycerol, 5 mM dithiothreitol, 1 mM EDTA, 0.2% nonidet P-40 and 2 M NaCl. 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 cleavage product peaks were detected by reversed-phase HPLC [11], integrated, and the kinetic parameters were determined at less than 20% substrate turnover by fitting the data to the Michaelis-Menten equation using the Gauss-Newton iteration method. The computer program (Enz 5.0) was written and kindly provided by M. Fivash and J. Racheff from the Data Management Services Inc., NCI-FCRDC, Frederick. The asymptotic standard errors were 5–20%.

The atomic coordinates from the crystal structure of HIV-1 PR with different inhibitors [14–16] were examined on an Evans and Sutherland PS390 molecular graphics system using the program, FRODO [17]. The HIV-2 PR coordinates were obtained by molecular modeling (A. Gustchina and I. Weber, to be published elsewhere). It was possible to place the modeled side chains in identical or closely similar positions to those in the inhibitor co-crystal structures. The amino acid side chains at P_4 were examined in both HIV-1 and HIV-2 PR structures. The conformational flexibility of interacting side chains in substrate and in the enzyme was tested by rotating around single bonds in the side chains of residues to model the process of substrate binding as it might occur in solution. This approach was used to estimate the degree of adjustment required for accommodation of particular residues in the binding site of the enzyme.

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3. RESULTS AND DISCUSSION

A nonapeptide, H-Val-Ser-Gln-Asn-Tyr*Pro-Ile-Val-Gln-NH_2 (designated as SP-211), with the sequence of the cleavage site at the junction of the matrix and capsid proteins in HIV-1 was found to be a good substrate for both HIV-1 and HIV-2 PRs [10–12]. To determine the minimal length of the substrate for the HIV PRs, this peptide was truncated at both the N- and C-termini (Table 1). The most dramatic decrease in catalytic constants (k_{cat}, k_{cat}/K_m) were obtained by the elimination of P_4 Ser or P_3' Val. The minimal substrate requirement for both enzymes was six amino acid residues, from P_4 to P_2' or from P_3 to P_3'. Shorter peptides were not hydrolyzed, and were tested as inhibitors of SP-211 cleavage by HIV-2 PR (Table I). The observed IC_{50} values suggest that some of the shorter peptides could bind to the enzyme with affinity similar to the longer substrates, although only in nonproductive manner.

The crystal structures of HIV-1 PR with various inhibitors [14–16,18,19] show that the inhibitor is bound in an extended beta conformation, as was predicted by molecular modeling [20]. The conformation of the inhibitor is maintained by a series of hydrogen bond interactions between the C=O and NH groups of the inhibitor and atoms of the PR dimer (Fig. 1). The amino acid side chains of inhibitors lie in successive subsites of the PR dimer. It was proposed by Gustchina and Weber [21] that the main chain-main chain interactions between PR and the substrate were important for productive binding and catalysis. These interactions are independent of the particular residues forming the substrate or inhibitor peptide. Although there is some symmetry around the scissile bond, the hydrogen bonding pattern is asymmetric (Fig. 1). While Gly-48 NH in the flexible flap of one subunit interacts with the C=O of P_4, Gly-48' NH interacts with P_3' C=O. The loss of these interactions is predicted to be responsible for the dramatic decrease in catalytic efficiency on removing the P_4 and P_3' residues. In contrast to P_4 and P_3', P_5 and P_4' do not appear to be crucial for efficient substrate hydrolysis. These amino acids may interact with the main chain of the enzyme, however, these interactions are not part of the continuous beta sheets (Fig. 1). Our results about the minimum length of substrate are in accordance with those of Darke et al. [22] and Moore et al. [23] for the HIV-1 PR using substrates based on the same cleavage site. Studies in other laboratories have shown efficient cleavage of peptides starting at P_3 [8,24], however, one of these has Arg at P_3 which has many possibilities of additional interactions, and the other was extended to P_5'. The catalytic properties are expected to depend on the specific combination of amino acids forming the substrate, in addition to the interactions involving main chain atoms.

To further explore the role of P_4 in the substrate hydrolysis, various amino acids were introduced into the P_4 position of SP-211. The kinetic parameters obtained with HIV-1 and HIV-2 PRs are listed in Table II. The most obvious effect is that residues at P_4 with high tendency to form beta turns (Ser, Gly, Asp, Asn, Pro; [25]) show high catalytic constants (k_{cat} and k_{cat}/K_m) compared to the others. It has been suggested that the P_4 position in retroviral cleavage sites is preferentially a β turner or helix breaker [26]. These results again suggest that it is important for the substrate to assume an

Table I

Cleavage of oligopeptides representing the cleavage site between the matrix and capsid proteins of HIV-1 by HIV-1 and HIV-2 PRs

Peptide	HIV-1 PR			HIV-2 PR			
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	IC_{50}[b] (mM)
VSQNY*PIVQ[a]	0.15	6.8	45.3	0.18	6.2	34.4	
SQNY*PIVQ	0.78	15.7	20.1	0.61	12.0	19.7	
QNY*PIVQ	0.91	1.6	1.8	1.04	1.1	1.1	
NY*PIVQ		0			0		1.39
Y*PIVQ		0			0		0.55
VSQNY*PIV	0.12	7.9	65.8	0.11	5.0	45.4	
SQNY*PIV	0.53	13.5	25.4	0.23	3.6	15.6	
QNY*PIV	0.86	1.0	1.2	0.19	0.3	1.6	
NY*PIV		0			0		0.30
Y*PIV		0			0		0.13
VSQNY*PI		<0.5			<0.5		
SQNY*PI		<0.5			<0.5		
QNY*PI		0			0		>1.50

[a] Substrate designated as SP-211 [10–12]. Scissile bond is marked with asterisk

[b] IC_{50} values measured as inhibition of the cleavage of SP-211. Smaller peptides which were also tested and were not hydrolyzed: NY*PI; VSQNY*P; SQNY*P; QNY*P; NY*P; Y*P. These peptides (except the dipeptide) were also able to inhibit the cleavage of VSQNY*PIVQ, with $IC_{50} > 1.5$ mM

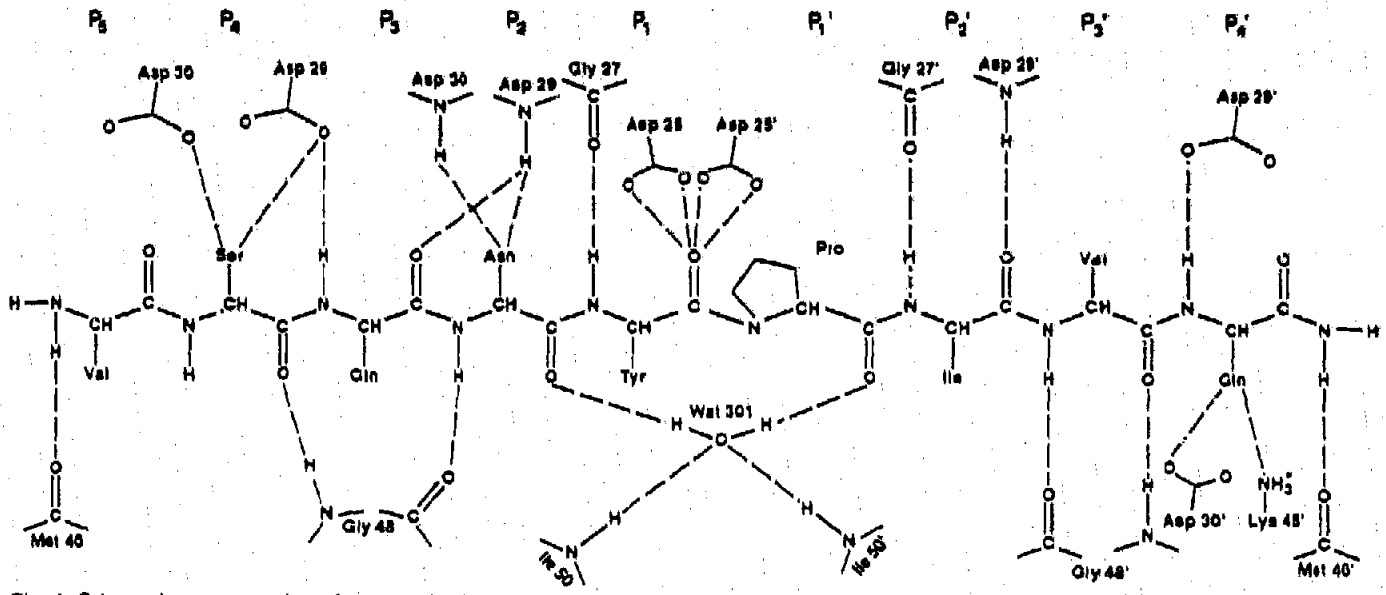

Fig. 1. Schematic representation of the putative interactions of the substrate, VSQNY*PIVQ, (SP-211, P_3–P_3') in the HIV-1 PR binding site. The PR residues that form hydrogen bond interactions with the substrate are shown. Dashed lines indicate hydrogen bond interactions. The residues from the second subunit in the PR dimer are indicated by a prime. Interactions are observed in crystal structures [14–16] with the exception of P4' which was predicted by modeling. Two short continuous β sheets are formed by each C=O and NH group of substrate residues P_4 to P_1 and P_1' to P_3' with the flap residues 48–50 and residues 27–29 of each PR subunit. The peptide C=O and NH groups further from the scissile bond than P_4 and P_3' do not form continuous beta sheet interactions with the flaps.

extended β conformation within the PR binding site, and alter conformation on the surface of the PR dimer in order to connect the folded portions of the polypeptide substrate. The S_4 subsite is a well-defined pocket: Asp-29, Asp-30 and Ile/Val-47 were found to be its most important components (Fig. 2). As was originally predicted from modeling studies [20], S_4 most easily accommodates smaller amino acid side chains. S_4 does not readily accommodate side chains that are branched at the β carbon atom due to the proximity of Ile-47 in HIV-1 PR. This was tested by rotating the side chain of PR residue 47 in order to explore its conformational flexibility (Fig. 2). Ile-47 in HIV-1 in some conformations can interfere with the binding of residues with branched side chains at P_4 (Fig. 2). This explains the poor kinetic values for branched amino acids like Thr, Val and Ile. In the case of Thr, the potential of forming hydrogen bonds similar to those of the OH of Ser may partially compensate for this effect.

In most respects, the results for HIV-2 PR are similar

Table II

Kinetic parameters determined for substrates having VXQNY*PIVQ structure

Xaa	HIV-1 PR			HIV-2 PR		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
Ser[a]	0.15	6.8	45.3	0.18	6.2	34.4
Thr	1.5	3.0	2.0	1.0	3.1	3.1
Gly	0.6	10.3	17.2	0.8	10.5	13.1
Asp	1.5	10.0	6.6	1.4	14.5	10.4
Asn	1.0	8.1	8.1	1.0	6.6	6.6
Pro	0.28	6.3	22.5	0.40	4.7	11.7
Arg	1.3	2.7	2.1	1.5	2.2	1.5
Lys	1.4	1.2	0.9	1.2	1.3	1.1
Met	0.9	0.9	1.0	1.0	0.9	0.9
Ala	0.7	3.1	4.4	0.8	2.4	3.0
Leu	0.40	0.06	0.2	0.6	0.6	1.0
Ile	N.D.	0.06	N.D.	N.D.	0.10	N.D.
Phe	N.D.	0.14	N.D.	N.D.	0.20	N.D.
Val	N.D.	0.08	N.D.	N.D.	0.50	N.D.

[a] Corresponds to the original substrate (SP-211) representing the cleavage site between the matrix and capsid proteins in HIV-1. N.D. = not determined.

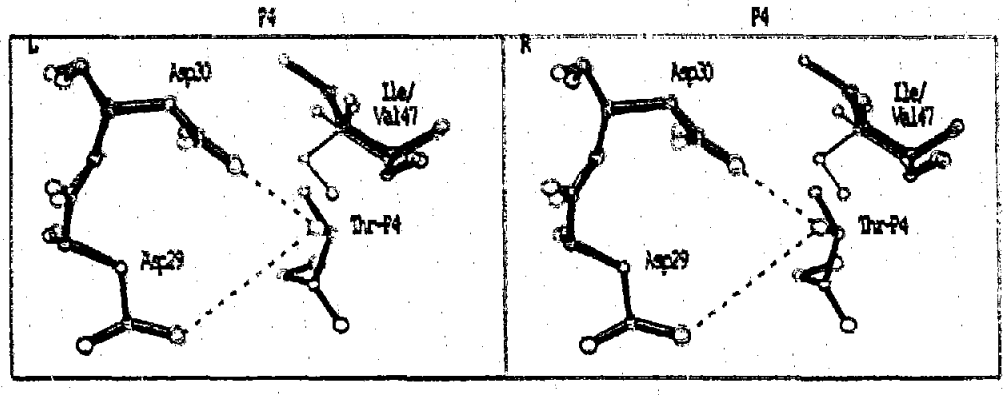

Fig. 2. Stereo view of Thr at P_4 in subsite S_4 of HIV PRs. Solid bonds indicate the residues of HIV-2 PR, open bonds indicate the equivalent residues of HIV-1 PR. Ile-47 is shown also in thin lines to indicate a position in which it would have extremely poor contacts with Thr at P_4. Dashed lines indicate potential hydrogen bond interactions.

to those for HIV-1 PR. Examination of the model of HIV-2 PR showed that the only difference in S_4 is that residue 47 is the smaller Val (Fig. 2), which may explain the better kinetic parameters for branched amino acids. However, the most pronounced difference was obtained with the Leu-containing substrate, which has about 10 times higher k_{cat} for HIV-2 than for HIV-1 PR. Ile/Val-47 is also part of the P_2 substrate-binding pocket, this may explain the better k_{cat} value for a substrate containing Leu and P_2 which has been noted for HIV-2 PR compared with HIV-1 PR [8].

Recently, extensive studies were carried out investigating the preference for the P_2' of HIV-1 PR [5] and $P_1 - P_3$ positions for both PRs [6–8]. The crucial role of the P_2 and P_2' positions were suggested [5,8]. Our results suggest that the P_4 position may also be important for proper catalytic activity, and unfavorable amino acids (or protecting groups in artificial peptides) may result in a substantial decrease of substrate-binding and hydrolysis rate.

Acknowledgements: We wish to thank Dr Alexander Wlodawer for providing us with the coordinates of the protease complexed with the U-85548e inhibitor prior to publication, Cathy Hixson and Suzanne Specht for their help with the amino acid analysis and John M. Louis for providing *E. coli* lysates containing HIV-1 proteinase. Research was sponsored by the National Cancer Institute, DHHS under contract no. NO1-CO-74101 with ABL.

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