Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 439-444

HYDROLYSIS OF SYNTHETIC CHROMOGENIC SUBSTRATES BY HIV-1 AND HIV-2 PROTEINASES

Lowri H. Phylip[1], Anthony D. Richards[1], John Kay[1]
Jan Konvalinka[2], Peter Strop[2], Ivo Blaha[2], Jiri Velek[2], Vladimir Kostka[2],
Alison J. Ritchie[3], Anne V. Broadhurst[3], William G. Farmerie[4],
Paula E. Scarborough[4], and Ben M. Dunn[4*]

[1] Department of Biochemistry, University of Wales College of Cardiff, P.O. Box 903, Cardiff, CF1 1ST, Wales, U.K.

[2] Inst. of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Flemingovo namesti 2, 166 10 Prague 6, Czechoslovakia

[3] Roche Products Ltd., P. O. Box 8, Welwyn Garden City, AL7 3AY, U.K.

[4] Department of Biochemistry and Mol. Biology, J. Hillis Miller Health Center, University of Florida, Gainesville, Fl 32610

Received July 23, 1990

Kinetic constants (K_m,k_{cat}) are derived for the hydrolysis of a number of chromogenic peptide substrates by the aspartic proteinase from HIV-2. The effect of systematic replacement of the P_2 residue on substrate hydrolysis by HIV-1 and HIV-2 proteinases is examined.

The proteinase encoded within the genome of human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) plays a crucial role in the production of infectious virions and thus has become a strategic target for inhibitors with substantial potential as drugs for the treatment of AIDS (1). Previous reports have indicated the value of chromogenic peptide substrates (containing a p-NO_2-phenylalanine in the P_1' position as a reporter group) for investigations aimed at characterization of HIV-1 proteinase

* To whom correspondence should be addressed.

Abbreviations
Nle = norleucine; Nph - 4-NO_2-phenylalanine. An asterisk (*) is used to denote the scissile peptide bond in all synthetic peptides.

0006-291X/90 $1.50

(2,3,4). Assays using such substrates facilitate detailed studies into the active site preferences that must be fulfilled for effective interaction. For example, whereas the enzyme-substrate interaction is relatively insensitive to the nature of the residue occupying the P_3 position (1,5), preliminary investigations with HIV-1 proteinase have implied that the residues in P_2 (5) and P_2' (6) may be particularly influential. In this study, we have systematically replaced the P_2 residue in the synthetic peptide Lys-Ala-Arg-Xaa-Nle * Nph-Glu-Ala-Nle-NH_2 and examined the effect of this on the hydrolysis of each peptide not just by HIV-1 but also by HIV-2 proteinase since relatively little has been reported previously on the latter enzyme.

Materials and Methods

Peptides were synthesized and purified by HPLC as described previously (3,5). Hydrolysis of the peptides was monitored spectrophotometrically at 37°C or by reverse phase FPLC (where required) also as described previously. The buffer used was 0.1M sodium acetate, pH 4.7 containing 4mM EDTA and NaCl to give a final ionic strength of either 0.3 or 1.0M. Homogeneous preparations of HIV-1 and HIV-2 proteinases were obtained and their active concentrations were determined by active site titration with compound 17 of ref. 1, as described previously (3,7). In the spectrophotometric assay, final volumes of 800μl and 300μl and active concentrations between 5 - 10 nM and 10 - 15 nM were used for HIV-1 and HIV-2 proteinases respectively. Initial velocities were measured for at least six substrate concentrations and the kinetic parameters (K_m, V_{max}) were derived from a computer fit of the data using the Enzfitter program. Values for k_{cat} were derived from $V_{max} = k_{cat} \times [E]$. In all cases, the values given for K_m and k_{cat} are the means of at least two separate determinations. The precision of each individual estimation was in the range of +/-5-20%.

Results and Discussion

We have previously demonstrated that HIV-1 proteinase will readily catalyze hydrolysis of synthetic peptide substrates containing - Yaa * Nph - residues as the -P_1 * P_1'- scissile peptide bond (3,5). Corresponding data for HIV-2 proteinase at pH 4.7 are presented in Table 1 for a series of 10 synthetic peptide substrates. These data are comparable to those reported previously for HIV-1 enzyme (3). In the P_1 position, Met, Leu, Nle, Tyr and Phe (peptides 1,2,4,6,7 and 8) were all readily accommodated, but just as with HIV-1 enzyme (3) when the isomeric beta-branched Ile residue was introduced in this position, the peptide (Peptide 3) was essentially resistant to attack by HIV-2 proteinase. Truncation of the substrate at the N-terminus by removal of two residues (peptide 5) had very little effect on the kinetic

parameters. Thus, HIV proteinases would appear to require only three residues in the P_3-P_2-P_1 positions for effective catalysis when the substrate contains four residues in P_1'-P_4'.

Initial replacement of the residues occupying P_3 and P_2 (peptides 8 - 10) produced only minor alterations in the kinetic parameters. Thus, all of the peptides (except peptide 3) in Table 1 are effective substrates for HIV-2 proteinase. One consistent feature to emerge from comparison of these data with those reported previously under identical conditions for HIV-1 proteinase (3,5) is that each k_{cat} measured for HIV-2 is approximately half of the value derived with each peptide and the HIV-1 enzyme. Peptide 4, containing Nle in the P_1 position, gave the highest value for k_{cat}, for both HIV-2 (Table 1) and HIV-1 proteinase (3).

An additional series of substrates containing -Nle * Nph- as the scissile peptide bond with systematic replacement of the P_2 residue was then examined using both enzymes. The data were obtained (Table 2) using conditions equivalent to those of Table 1 and those reported earlier (3,5), with the exception that the final ionic strength was lowered to 0.3M in order to be more compatible with physiological values.

When the P_2 residue was varied, relatively minor changes in

Table 1. Kinetic parameters for the hydrolysis of several chromogenic peptides by HIV-2 proteinase

	Peptide Number and Sequence	K_m (μM)	k_{cat} (sec^{-1})
1.	Lys-Ala-Arg-Val-Met*Nph-Glu-Ala-Nle-NH_2	20	7
2.	Lys-Ala-Arg-Val-Leu*Nph-Glu-Ala-Nle-NH_2	90	10
3.	Lys-Ala-Arg-Val-Ile*Nph-Glu-Ala-Nle-NH_2		~0
4.	Lys-Ala-Arg-Val-Nle*Nph-Glu-Ala-Nle-NH_2	50	20
5.	Arg-Val-Nle*Nph-Glu-Ala-Nle-NH_2	65	11
6.	Lys-Ala-Arg-Val-Tyr*Nph-Glu-Ala-Nle-NH_2	25	15
7.	Ala-Thr-His-Gln-Val-Phe*Nph-Val-Arg-Lys-Ala	50	7
8.	Ala-Thr-His-Gln-Val-Tyr*Nph-Val-Arg-Lys-Ala	90	5
9.	Ala-Thr-His-Gln-Ile-Tyr*Nph-Val-Arg-Lys-Ala	85	7
10.	Ala-Thr-His-Arg-Val-Tyr*Nph-Val-Arg-Lys-Ala	85	3

All assays were performed in a final volume of 300 μl in 0.1M sodium acetate buffer, pH 4.7 containing 4 mM EDTA and sufficient NaCl to ensure a final ionic strength of 1M.

Table 2. Kinetic parameters for the hydrolysis of chromogenic peptides of general structure Lys-Ala-Arg-Xaa-Nle*Nph-Glu-Ala-Nle-NH_2 by HIV-1 and HIV-2 proteinases

Peptide Number	Xaa	HIV-1		HIV-2	
		K_m (μM)	k_{cat} (sec^{-1})	K_m (μM)	k_{cat} (sec^{-1})
4	Val	35	32	90	20
11	Ile	45	35	70	19
12	Leu	25	6	85	13
13	Ala	45	19	135	11
14	Asp	20	1.9^+	35	0.9^+
15	Asn	40	0.7^+	70	0.3^+
16	Gly		~0		~0
17	Pro		0		0

All reactions were carried out at 37° in 0.1M sodium acetate buffer, pH 4.7 containing 4 mM EDTA and sufficient NaCl to give a final ionic strength of 0.3M.

+ Slow reaction were analyzed by FPLC for determination of V_{max} values.

K_m were observed with both enzymes. By contrast, substantial differences were detected in the k_{cat} parameter. In contrast to the effect observed when beta-branched residues were present in the P_1 position, substituting Val/Ile into P_2 (Peptides 4,11) produced adequate k_{cat} values for both enzymes. Replacement of Ile by an isomeric Leu residue (peptide 12) lowered k_{cat} approximately six-fold for HIV-1 proteinase whereas comparable values were derived for the enzyme from HIV-2 (Table 2). Alanine was acceptable in this position (peptide 13) for both enzymes although the k_{cat} value obtained for each enzyme was lowered by approximately two-fold in relation to the respective value derived for Ile (peptide 11). As noted above in relation to the peptides in Table 1, the k_{cat} values derived for HIV-2 proteinase were approximately half of those obtained for the same peptide substrate with HIV-1 proteinase with the exception of the substrate containing leucine in P_2 (peptide 12; Table 2). For HIV-1 proteinase, the highest values of k_{cat} were obtained with Ile, Val or Ala in P_2. The "longer" leucine side chain does not appear to fit comfortably in the S_2 subsite. In contrast, HIV-2 proteinase appears to have slightly more adaptability in this subsite.

The presence of Asp or Asn (peptides 14, 15) in the P_2 position resulted in substantial reduction of the catalytic rate constants for both enzymes to values sufficiently low (~1 sec^{-1}) such that they had to be measured using a non-continuous FPLC assay method (8). Furthermore, additional peptides (not shown) containing Glu, Gln or Tyr in the P_2 position were also hydrolysed slowly, i.e. k_{cat} values between 0.2 and 0.6 sec^{-1} (with K_m values < 40 - 65 μM) were obtained with HIV-1 proteinase. Insertion of a Gly or Pro residue (peptides 16, 17; Table 2) into P_2 effectively abolished hydrolysis at the adjacent - Nle * Nph - peptide bond.

The nature of the side chain of the residue in P_2 is therefore important for effective substrate cleavage by both enzymes. Crystallographic investigations (9,10) have revealed that occupancy of the active site cleft is associated with major movements of the two beta hairpin loops termed "flaps" which overhang the active site in the empty enzyme. This "hugging" mechanism may be triggered by the appropriate fit of individual side chains of the substrate leading to an optimal alignment of catalytic residues or to an essential distortion of the scissile peptide bond (as postulated for hydrolysis by archetypal aspartic proteinases; 11). With the symmetry inherent in the homodimer, residues on both sides of the scissile peptide bond may be predicted to be essential for optimal binding and/or catalysis. Furthermore, the known cleavage sites in the HIV gag and gag/pol polyproteins are of two general types: the -Hydrophobic*Hydrophobic- and the -Aromatic*Pro- junctions. At the former junctions, Ile, Val, Leu or Ala are indeed frequently found in the P_2 position while Leu, Gln or Glu occur in P_2'. In the -Aromatic*Pro- type junctions, Asn is found most commonly in P_2 typically paired with Ile/Val in P_2'. Indeed, it has been shown very recently (6) using an FPLC-based assay and an -Asn-Tyr*Pro-containing substrate that the presence of Ile or Val in the P_2' position was essential for effective catalysis by HIV-1 proteinase.

It is apparent that pairing of residues in P_1 and P_1' and in P_2 and P_2' is necessary. The appropriate combination of residues occupying these four positions influences the adoption of the most beneficial conformation and contact with the active site and, ultimately, the most facile cleavage. The design of selective inhibitors should take account of all of these features.

Acknowledgments

It is a pleasure to acknowledge the contributions made by Drs Noel Roberts, Mary Graves, Stuart LeGrice and Herve Hirel.

Supported by awards from the MRC-AIDS Directed Programme, from the NIH (AI28571) and from the Royal Society Joint Scientific Research Programme.

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