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Subsite specificity of the proteinase from myeloblastosis associated virus

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The subsite requirements of the aspartic proteinase from the myeloblastosis-associated virus (MAV) for the cleavage of peptide substrates were studied with a series of synthetic peptides of general structure Ala-Thr-P4-P3-P2-P1•Nph-Val-Arg-Lys-Ala. The residues in positions P4, P3, P2 and P1 were varied and the kinetic parameters for the cleavage of substrates in 2.0 M NaCl were spectrophotometrically determined at pH 6.0 and 37°C. The acceptance of amino acid residues in particular subsites is similar to that observed with the human immunodeficiency virus type 1 (HIV-1) proteinase in our earlier studies on the same substrate series: hydrophobic or aromatic residues are preferable in P1 position, a broad variety of residues are acceptable in P3 whereas the residues occupying P2 plays the decisive role in the substrate cleavage as evidenced by its dramatic influence on both k_{cat} and K_m values. The most remarkable difference between the two enzymes was found in P3 and P4 subsites. In P3, the introduction of negatively charged glutamate increases the substrate binding by the MAV proteinase 12-fold and decreases binding by the HIV-1 proteinase. In P4, Pro in this series is a favourable residue for the MAV proteinase and is strongly inacceptable for HIV-1 the proteinase. The pH profile of the cleavage was studied with a chromogenic substrate and differences between HIV-1 and MAV proteinases are discussed.

Retroviral proteinase; HIV-1 proteinase; MAV; Chromogenic substrate; Subsite specificity

1. INTRODUCTION

The vital importance of retroviral proteinases for correct processing and maturation of viral particles has been shown by several authors (cf. e.f. [1]). These enzymes thus became obvious targets of specific inhibitors with possible therapeutic effect to be used for the treatment of diseases caused by retroviruses. A deep understanding of the substrate specificity of these enzymes should pave the way to a rational design of these inhibitors. A great deal of effort has therefore been devoted to the detailed investigation of the specificity of the proteinase from human immunodeficiency virus-1 (HIV-1) [3,4–6]. On the other hand, much less is known about the other members of the retroviral proteinase family. The aspartic proteinase of the myeloblastosis associated virus (MAV) is an example of a viral enzyme encoded in the first open reading frame of the retroviral genome [1]: the enzyme is therefore expressed in high levels and even a lower activity is sufficient for a correct processing of viral polyproteins. Both X-ray analysis [7] and kinetical studies [8–10] revealed certain features of MAV proteinase different from those of the HIV-1 enzyme: a lower activity and a more promiscuous specificity are among the most remarkable ones. It has been reported recently that the introduction of a *p*-nitrophenylalanine residue into the P1′ position of the proper peptide sequence could produce a sensitive chromogenic substrate for the HIV-1 [2–4] or MAV [9,13] proteinases. As a rule, good substrates of the HIV-1 proteinase are poor substrates of the MAV proteinase and vice versa [9,10]. An exception, however, represents the chromogenic peptide Ala-Thr-His-Gln-Val-Tyr★Nph-Val-Arg-Lys-Ala, which was shown to be a reasonably good substrate for both MAV and HIV-1 proteinases [9,12]. It was designed initially upon the sequence spanning the cleavage junction *pol* P63/P32 of the Rous sarcoma virus (RSV). Nph was introduced into P1′ and the residues in position P2, P2′ and P4′ were replaced to comply with the consensus sequence of scissile bonds in polyproteins of avian sarcoma and leukemia viruses. In our previous study, we have synthesized a series of analogues of this sequence by varying amino acid residues in P4–P1 and studied the kinetic parameters of such substrates with the HIV-1 proteinase [1,12]. The use of a similar set of substrates for the investigation of the subsite preferences and pH dependence of cleavage of the MAV proteinase is reported in this paper.

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Abbreviations: E_t, total enzyme concentration; k_{cat}, rate constant according to equation $V_{max} = k_{cat} \cdot E_t$; PheSta, phenylstatine; MAV, myeloblastosis-associated virus; Nle, norleucine; Nph, 4-nitrophenylalanine. The amino acid residues surrounding the cleaved bond are depicted according to Schechter and Berger [11], i.e. P4-P3-P2-P1★P1′-P2′-P3′-P4′ the scissile bond being indicated by an asterisk.

2. MATERIALS AND METHODS

Peptide substrates summarized in Table I were synthesized by the solid-phase method and purified by HPLC [2]. Aqueous stock solutions of approximately 5 mM were prepared and kept frozen.

The recombinant MAV proteinase was obtained as described elsewhere [14]. The active site concentration of its solution was determined by titration with the MAV proteinase inhibitor Pro-Pro-Cys-Val-PheSta-Ala-Met-Thr-Met [13]. The k_{cat} values were calculated from $V_{max} = k_{cat} \cdot E_t$.

The hydrolysis of peptide substrates at pH 6.0 was monitored spectrophotometrically in Aminco DW 2000 Spectrophotometer at 305 nm and 37°C. The buffer used was 0.1 M phosphate containing 4 mM EDTA and 2.0 M NaCl. A final volume of 1000 μl was used and the resulting proteinase concentration varied between 30 and 50 nM. The average extinction coefficient under these conditions was 1200 l/mol · cm.

For those kinetic measurements in distant pH areas where slow hydrolysis rates made the spectrophotometric determination impossible, the time course of proteolysis was followed by reverse-phase HPLC according to Richards et al. [15] using a Gilson chromatograph with a Vydac C18 column. The reaction conditions were the same as those of the spectrophotometric measurement. Total reaction volume of 100 μl was quenched after 5–30 min by addition of 20 μl of 5% trifluoroacetic acid. The conditions are given in detail in the legend to Fig. 1.

The initial rates of hydrolysis were measured for at least 7 substrate concentrations and the kinetic constants were derived from the computer fit of the data using the Enzfitter program. In all cases, the K_m and V_{max} values are the means of at least two separate determinations.

3. RESULTS AND DISCUSSION

When investigating the side chain interactions of the residue occupying the P1 subsite of the peptide substrate Ala-Thr-His-Gln-Val-P1★Nph-Val-Arg-Lys-Ala (Table II) we observed no cleavage with Ser, Arg or Glu in this position. On the other hand, substrates with Tyr, Phe, or Leu were cleaved readily. This finding is in good agreement with our previous findings on the HIV-1 proteinase [12]. This confirms the preference of both proteinases for large, hydrophobic residues in P1. Substrate binding is largely improved when Tyr is replaced by Phe in the scissile bond; hence, the hydroxyl group of tyrosine may be sterically unfavourable. Since, however, the substrate containing Tyr in P1 is readily soluble and shows a high extinction coefficient, further studies were carried out on substrates containing -Tyr★Nph- as the -P1★P1'-residues. Peptide 1 (Table I) Ala-Thr-His-Gln-Val-Tyr★-Nph-Val-Arg-Lys-Ala thus served as a standard in all our kinetic measurements.

A systematic variation of the residue in P2 was carried out in the series Ala-Thr-His-Gln-P2-Tyr★Nph-Val-Arg-Lys-Ala. As shown in Table III valine is the most appropriate residue for P2. The introduction of any other residue leads to a less favourable orientation of the scissile bond as reflected in lower k_{cat} values. Peptides containing Gly and Phe in P2 were not cleaved under the conditions used at all. The introduction of Ile improved substrate binding with a slight decrease in k_{cat} while Leu made both binding and k_{cat} drop significantly. An exception is peptide 8 containing Ala in P2 (Table I). Poor cleavage reflected by a low k_{cat} value is

Table I

Substrates used for subsite preference investigation of MAV proteinase

Number	Sequence
1	Ala-Thr-His-Gln-Val-Tyr ★Nph-Val-Arg-Lys-Ala
2	Ala-Thr-His-Gln-Val-Phe ★Nph-Val-Arg-Lys-Ala
3	Ala-Thr-His-Gln-Val-Leu ★Nph-Val-Arg-Lys-Ala
4	Ala-Thr-His-Gln-Val-Ser ★Nph-Val-Arg-Lys-Ala
5	Ala-Thr-His-Gln-Val-Arg ★Nph-Val-Arg-Lys-Ala
6	Ala-Thr-His-Gln-Val-Glu ★Nph-Val-Arg-Lys-Ala
7	Ala-Thr-His-Gln-Ile-Tyr ★Nph-Val-Arg-Lys-Ala
8	Ala-Thr-His-Gln-Ala-Tyr ★Nph-Val-Arg-Lys-Ala
9	Ala-Thr-His-Gln-Leu-Tyr ★Nph-Val-Arg-Lys-Ala
10	Ala-Thr-His-Gln-Gly-Tyr ★Nph-Val-Arg-Lys-Ala
11	Ala-Thr-His-Gln-Phe-Tyr ★Nph-Val-Arg-Lys-Ala
12	Ala-Thr-His-Glu-Val-Tyr ★Nph-Val-Arg-Lys-Ala
13	Ala-Thr-His-Asp-Val-Tyr ★Nph-Val-Arg-Lys-Ala
14	Ala-Thr-His-Asn-Val-Tyr ★Nph-Val-Arg-Lys-Ala
15	Ala-Thr-His-Arg-Val-Tyr ★Nph-Val-Arg-Lys-Ala
16	Ala-Thr-His-Tyr-Val-Tyr ★Nph-Val-Arg-Lys-Ala
17	Ala-Thr-His-Val-Val-Tyr ★Nph-Val-Arg-Lys-Ala
18	Ala-Thr-His-Pro-Val-Tyr ★Nph-Val-Arg-Lys-Ala
19	Ala-Thr-Pro-Gln-Val-Tyr ★Nph-Val-Arg-Lys-Ala
20	Ala-Thr-Pro-Cys-Val-Tyr ★Nph-Val-Arg-Lys-Ala

Table II

Kinetic parameters for cleavage of P1 substrate analogues of general structure Ala-Thr-His-Gln-Val-P1★Nph-Val-Arg-Lys-Ala by MAV proteinase

Substrate number	P1	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m rel. to No. 1
1	Tyr	75	5	1.0
2	Phe	31	3	1.5
3	Leu	52	4	1.2
4	Ser		not cleaved	
5	Arg		not cleaved	
6	Glu		not cleaved	

The peptide numbers relate to Table I. k_{cat}/K_m values are relative, given for comparison with prototype substrate 1. All details are specified in section 2.

Table III

Kinetic parameters for cleavage of P2 substrate analogues of general structure Ala-Thr-His-Gln-P3-Tyr★Nph-Val-Arg-Lys-Ala

Substrate number	P2	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m rel. to No. 1
1	Val	75	5	1.0
7	Ile	42	2	0.7
8	Ala	9	0.5	0.8
9	Leu	124	0.2	0.02
10	Gly		not cleaved	
11	Phe		not cleaved	

All experimental details are given in section 2 and in the legend to Table II.

compensated by excellent binding, yielding a relatively high k_{cat}/K_m ratio compared to the Val-containing analogue 1 (Table I). The same replacement in the P2 position significantly increased K_m and decreased k_{cat} when assayed with the HIV-1 proteinase. This may reflect a difference in the processing sites cleaved by the two proteinases; Ala, which is frequently found in P2 position of the sequences cleaved in avian retrovirus polyproteins [9], does not occur in the P2 position of HIV-1 polyprotein cleavage sites.

Our findings thus point to the desisive role of the amino acid residue in P2. Substrate binding and cleavage are strongly enhanced by a β-branched residue (i.e. Val, Ile) in this position.

It has been proved that the minimal peptide chain length required by the MAV proteinase for proper binding is 4 amino acids in its N-terminal and 3 in its C-terminal part of the substrate [8,9,16]. Therefore, we also investigated the structure requirements for P3 and P4 positions (Table IV). The data obtained indicate a remarkably high tolerance of the enzyme in P3. One replacement only, Gln/Pro in P3 (peptide 18) led to a peptide totally resistant to cleavage, probably due to the distortion of the peptide backbone resulting in a non-productive orientation of the scissile bond. All other variations in P3, involving acidic, basic, aromatic or hydrophobic residues, gave peptides which were mostly bound better and cleaved faster than 'parent peptide' No. 1. This is particularly striking in the case of peptide 12, where introduction of negatively charged Glu in P3 resulted in a 12-fold increase in binding. This may be explained by electrostatic interaction between the glutamate in P3 and the positive charge of the residue in S3 binding pocket of the enzyme. Supporting evidence for this assumption was provided by molecular modeling of peptide 12 in the binding cleft of RSV suggesting possible ionic interaction between Glu in P3 and Arg-10 and Arg-105 in the binding cleft (Konvalinka, J. and Cooper, J., unpublished results). In contrast, a similar K_m improvement was not observed with the HIV-1 proteinase. In this case the Gln/Glu substitution in P3 led to a 2-fold increase in K_m [12]. A deeper discussion of this discrepancy is rather difficult in view of the difference in assay conditions (lower pH and salt concentration).

Table IV

Kinetic parameters for cleavage by MAV proteinase of peptides of general structure Ala-Thr-P4-P3-Val-Tyr★Nph-Val-Arg-Lys-Ala with variations in the P3 and P4 positions

Substrate number	P4-P3	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m rel. to No. 1
1	-His-Gln-	75	5	1.0
12	-His-Glu-	6	5	12.5
13	-His-Asp-	25	1	0.6
14	-His-Asn-	17	2	1.8
15	-His-Arg-	24	3	1.9
16	-His-Tyr-	26	11	6.3
17	-His-Val-	14	6	6.4
18	-His-Pro-		not cleaved	
19	-Pro-Gln-	9	5	8.3
20	-Pro-Cys-	8	3	5.6

All details are given in section 2 and in the legend to Table II.

Hydrophobic residues such as Tyr or Val (peptides 16 and 17, Table IV) in P3 significantly enhance substrate binding, and Tyr moreover remarkably improves cleavage (2-fold increase in k_{cat}). On the other hand, the same replacement in P3 (Gln with Tyr or Val) leads with the HIV-1 proteinase to a 2-fold decrease in the k_{cat} value [12].

Insertion of various other residues, such as Asp, Asn and Arg (peptides 13, 14 and 15, Table IV) into P3 also yields well binding substrates, yet with a significant drop in k_{cat}. Hence, the considerable tolerance the MAV proteinase shows towards residue occupying P3 resembles the kinetic behaviour of the HIV-1 proteinase with partial exception of Glu and bulky hydrophobic residues.

Peptides 19 and 20 (Table IV) are examples of variation in P4. Introduction of Pro into the P4 position was

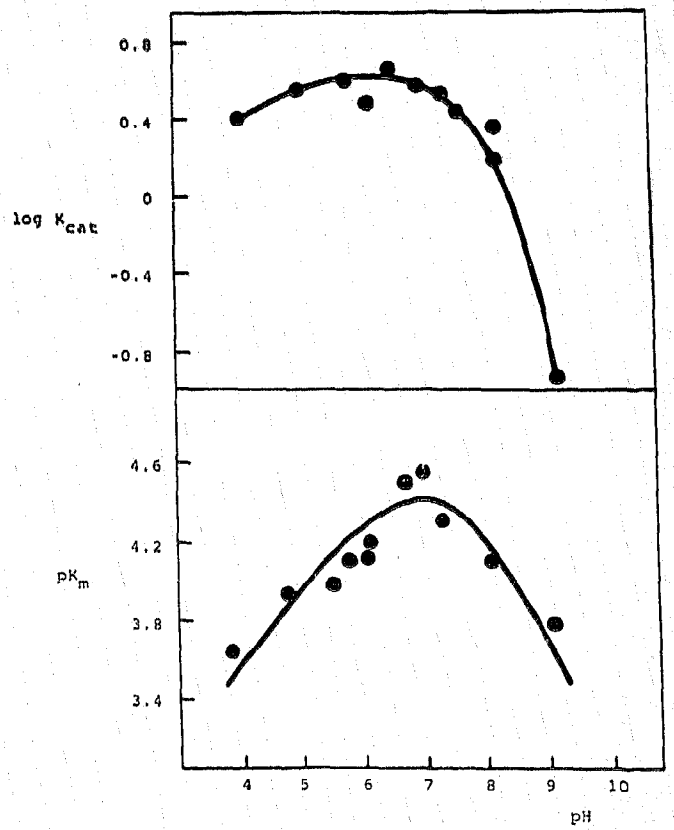

Fig. 1. pH dependence of log k_{cat} (above) and pK_m (below) of the cleavage of peptide Ala-Thr-His-Gln-Val-Tyr★Nph-Val-Arg-Lys-Ala by MAV proteinase. The buffers used contained 4 mM EDTA and 2.0 M NaCl and were sodium acetate (pH 4.0–5.0), phosphate (pH 5.5–7.5) and Tris (pH 8.0–9.0). The determinations of k_{cat} and K_m in the pH range 4.0–7.4 were made spectrophotometrically, the values at pH 8.0 and 9.0 were determined by HPLC using the conditions given in section 2.

not paralleled by distortion of the peptide backbone and unproductive orientation of the scissile bond in the case of MAV proteinase. The same peptide, however, was almost resistant to HIV-1 proteinase, although the binding was sustained [12].

All kinetic experiments with the MAV proteinase were carried out at pH 6.0, which is the apparent pH optimum for the enzyme [9]. The investigation of the pH dependence of kinetic parameters carried out with peptide substrate 1 is given in Fig. 1. Both K_m and k_{cat} values show a bell-shaped pH dependence with optima at pH 6.5 and 5.0–6.5 for K_m and k_{cat}, respectively. The k_{cat} decrease in the basic pH range is substantial (a 30-fold drop between pH 8.0 and 9.0). The HIV-1 proteinase exhibits in contrast minor changes only in k_{cat} between pH 4.0 and 9.0 and a dramatic increase in K_m is observed above pH 6.0 [2]. Although the substrates used for both pH dependence determinations were non-identical, this striking difference in pH profile of two so closely related enzymes must reflect important structure differences in their binding clefts. The residue responsible for the different behaviour of the MAV proteinase may be His-75 or Arg-105 whose counterparts are Gly-48 and Pro-81 in the flap and d' β- chain of the HIV-1 proteinase, respectively [16].

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