J. Enzyme Inhibition, 1985, Vol. 1, pp. 77–82.
8755–5093/85/0101/0077 $25.00/0

Printed in Great Britain.

THE INTERACTION OF ASPARTIC PROTEINASES WITH NATURALLY-OCCURRING INHIBITORS FROM ACTINOMYCETES AND ASCARIS LUMBRICOIDES

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(*Received 11 February 1985*)

Aspartic proteinase participate in a variety of physiological processes and alterations in the level of activity expressed may be associated with the onset of pathological conditions such as hypertension, gastric ulcers and neoplastic diseases. Included among the members of this class of proteinases are enzymes such as pepsin, gastricsin, renin, chymosin, cathepsin D and a variety of microbial enzymes (for a review, see Ref. 1). Extensive sequence homologies have been demonstrated among these enzymes and all are considered to have broadly similar 3-dimensional structures[2]. However, each enzyme must have evolved subtle distinctions in structure and therefore activity from the others in order to carry out its specific physiological function in its own environment. All of the enzymes appear to have an extended active site cleft within which are located two aspartic acid residues that are responsible for operation of the catalytic mechanism[3]. At least seven amino acids of a substrate can be accommodated within the seven corresponding sub-sites of the cleft (usually depicted as S_4–S_3') so that cleavage can occur at the bond (commonly) between two hydrophobic residues occupying the S_1–S_1' sites[4]. The differences in specificity and activity may then be explained by discrete alterations in some or all of the sub-sites in the various enzymes.

In order to investigate such differences, it has been traditional with other enzymes to exploit the availability of naturally-occurring inhibitors whose interactions with their targets may be examined. Synthetic counterparts may then be designed appropriately from the information gained. In the case of aspartic proteinases, however, naturally-occurring inhibitors are found relatively infrequently[5] by comparison with inhibitors of the other classes of proteolytic enzyme.

N-acylated pentapeptides known as pepstatins have been isolated from various species of Actimomycetes. Two forms, differing only in the nature of their *N*-acyl substituent, have been described in most detail[6,7]. These are isovaleryl (A) and acetyl (B)-pepstatins, both of which contain the unusual amino acid statine (4-amino-3-hydroxy-6-methylheptanoic acid).

A. Iva–Val–Val–Sta–Ala–Sta

B. Ac–Val–Val–Sta–Ala–Sta

C. Lac–Val–Sta–Ala–Sta

The first statine residue has been shown to be the essential feature upon which inhibition depends[8,9].

Both of these compounds are poorly soluble in aqueous media whereas by the simple expedient of introducing a hydrophilic lactoyl residue as the acylating group, the resultant blocked tetrapeptide, lactoyl-pepstatin (C) is rendered totally water soluble.

Isovaleryl-pepstatin (and fragments thereof) has been used extensively as a probe of the active site environment and catalytic mechanism of readily available aspartic proteinases such as pig pepsin but little is known about the effects of these inhibitors on a wider spectrum of aspartic proteinases. The present paper considers these interactions with a variety of mammalian and microbial aspartic proteinases together with the specificity exhibited by a protein inhibitor from Ascaris lumbricoides, a parasitic worm found in pig intestine[10].

MATERIALS AND METHODS

Isovaleryl and acetyl-pepstatins were generous gifts from Professors H. Umezawa and S. Murao respectively. Lactoyl-pepstatin was synthesised as described previously[11]. The inhibitor from Ascaris lumbricoides was the kind gift of Drs. R. Peanasky/T. Hofmann. All of the enzymes used in this study were obtained as described previously[5,11–13]. The homogeneity of each preparation was confirmed by SDS-polyacrylamide by SDS-polyacrylamide gel electrophoresis on 8.4% gels or by disc electrophoresis on 5.6% acrylamide gels[14].

The absolute concentrations of the pepstatins and the synthetic peptide substrate for use in the assays were determined by amino acid analysis after hydrolysis in 6 M-HCl at 105°C in sealed evacuated tubes (norleucine was added as an internal standard). The concentration of the Ascaris inhibitor was determined by titration against a solution of pig pepsin of pre-determined concentration. The active concentration of this standard enzyme had been determined, in turn, by titration against a known concentration of isovaleryl-pepstatin.

Kinetic constants (K_i) for the inhibition of the various enzymes were determined at pH 3.1 in 0.1 M sodium formate buffer at 37° using Pro–Thr–Glu–Phe–Nph–Arg–Leu as substrate. Hydrolysis of this chromophoric peptide at the Phe–Nph (nitrophenylalanine) bond can be monitored readily be following the decrease in extinction at 300 nm[5,15]. Previous investigations have indicated that cleavage by all of the aspartic proteinases under study is restricted to this bond alone, (M. J. Valler, J. Kay, and B. M. Dunn – unpublished observations). Enzymes and inhibitors were pre-incubated for 5 min at 37° to allow for possible time dependent inhibition[16] before

initiating the reaction by the addition of substrate. For tight-binding inhibitors, K_i values were obtained by the methods of Green and Work[17] or Goldstein[18] whereas plots of $1/v$ *vs.* [inhibitor] at different substrate concentrations were used to derive the values for weaker-binding inhibitors.

RESULTS AND DISCUSSION

The inhibition constants for the interaction of several aspartic proteinases of animal origin with the three pepstatins are shown in Table I. It is clear that the isovaleryl and acetyl-derivatives have equal potency towards all of these enzymes so that the size of the hydrophobic *N*-acyl substituent (which should occupy the S_4 sub-site in the enzymes) appears to be of little significance. However, an entirely different picture emerges when the data for the shorter derivative, lactoyl-pepstatin are examined (Table I). This statine-containing peptide interacts much less tightly than its hydrophobic counterparts (by approx. 30–50 fold) with all of the enzymes tested except pig and human pepsins and pig gastricsin. For these three enzymes then, occupancy of the S_4 sub-site would appear to be relatively unimportant. However, this is in contrast to the findings of Rich and Bernatowicz[16] who demonstrated that, for pig pepsin, shortening of the Iva–Val–Val–Sta sequence to Iva–Val–Sta resulted in a thirty-fold increase in the K_i value. Thus, with the present results, it would seem that the loss of binding energy resulting from vacation of the S_4 sub-site must be compensated for by a more favourable interaction of the hydrophilic lactoyl substituent (by comparison with the hydrophobic valine residue) with the S_3 sub-site. Support for the idea that pig pepsin can tolerate hydrophilic or hydrophobic residues in its S_3 sub-site has come from studies with the pepsin inhibitor peptide derived from the propart of pepsinogen[5,19] and from our earlier investigations which examined the hydrolysis of a series of chromophoric peptide substrates in which the residue in P_3 was replaced systematically[13,20]. It was observed that pig pepsin would accommodate valine, isoleucine or threonine equally readily in the S_3 sub-site since the K_M values obtained with such substrates at pH 3.1 were 60, 80 and 50 μM respectively. Rich and Bernatowicz[16] have observed that by introducing acyl groups in the P_3 position with increasing degrees of chain branching, a ten fold improvement in binding to pig pepsin can be obtained. Thus, the size of the group occupying S_3 may have more critical importance than its hydrophobic/hydrophilic nature. Apparently, the lactoyl substituent must fulfil this criterion such that for these three enzymes, the water-soluble lactoyl-pepstatin has equal potency to the longer but poorly-soluble, isovaleryl and acetyl-pepstatins.

By contrast to these enzymes, the other animal aspartic proteinases (Table I) were all inhibited more poorly by lactoyl-pepstatin than by the hydrophobic derivatives. Clearly, either the nature of the substituent in P_3 is of significance to these enzymes or they require the S_4 sub-site to be occupied (or both). Studies with synthetic substrates indicated previously[20] that although a small reduction in K_M (of the order of 3-fold) could be obtained with these enzymes by placing a valine residue instead of a hydrophilic threonine in the P_3 position, the magnitude of these changes was not comparable to the 30–50 fold differences observed in Table I. Thus, it would appear that the more crucial factor for inhibitor potency towards these enzymes is occupation of the S_4 sub-site (by a hydrophobic residue).

An even greater distinction is observed when the efficacy of these pepstatins towards microbial aspartic proteinases is measured (Table II). These enzymes are

TABLE I
Kinetic constants (K_i) for the inhibition of animal aspartic proteinases by various pepstatins

	Lactoyl -pepstatin	Acetyl -pepstatin K_i (nM)	Isovaleryl -pepstatin
Human pepsin	0.4	n.d.	< 0.5
Pig pepsin	1	1	1
Pig gastricsin	70	18	19
Chicken pepsin	50	1	0.8
Bovine cathepsin D	30	0.8	1.2
Calf chymosin	1900	63	70
Human gastricsin	5800	120	100

All measurements were made in 0.1 M sodium formate buffer, pH 3.1 at 37°C.
The estimated precision of the values is in the range ± 5%.
n.d. = not determined.

TABLE II
Kinetic constants (K_i) for the inhibition of microbial aspartic proteinases by various pepstatins

	Lactoyl -pepstatin	Acetyl -pepstatin K_i (*nM*)	Isovaleryl -pepstatin
Mucor pusillus proteinase	700	18	2
Endothia parasitica proteinase	1000	9	0.5
Penicillopepsin	> 1300	4[a]	0.15[b]
Yeast proteinase A	85	0.8	0.1

All measurements were made in 0.1 M sodium formate buffer, pH 3.1 at 37°. The estimated precision of the values is in the range ± 5%.
[a]Determined using Lys–Pro–Ile–Glu–Phe–Nph–Arg–Leu as substrate.
[b]Value obtained from Ref. 21.

inhibited more strongly by isovaleryl than by acetyl-pepstatin. This suggests that the bulkier isovaleryl substituent occupies the S_4 sub-site in the microbial enzymes more efficiently than the smaller acetyl derivative. Moreover, when this pocket is left vacant and S_3 is filled with a hydrophilic substituent as in lactoyl-pepstatin, inhibitory potency is reduced still further.

Thus, it would seem that the distinctive susceptibility of individual enzymes to these inhibitors reflects the different nature of the S_4 and S_3 sub-sites in each active site cleft. Nevertheless, some degree of inhibition was always observed with each pepstatin with every enzyme. It is likely that this results from the energy of interaction derived from the first statine residue in the Acyl–Val–Val–Sta sequence being bound (supposedly as a transition state analogue) in close proximity to the two catalytic aspartic acid residues. Evidence in support of this has been obtained from X-ray crystallographic analyses of complexes between penicillopepsin[21] or *Rhizopus chinensis* proteinase[22] with pepstatin or pepstatin fragments.

For other proteolytic enzymes such as the serine proteinases and their respective inhibitors[23], it has been possible to demonstrate that extensive contacts are made between enzyme and inhibitor at distances remote from the catalytic apparatus[24,25]. Such crystallographic investigations have been made possible by the ready availability of naturally-occurring inhibitors which are, in themselves, proteins. This facility is not readily available for aspartic proteinases since the few inhibitors that are found in

TABLE III
Kinetic constants (K_i) for the inhibition of various aspartic proteinases by the inhibitor protein from Ascaris lumbricoides

	K_i (nM)
Human pepsin	2.0[a]
Pig pepsin	0.5[a]
Pig gastricsin	1.7[a]
Human gastricsin	26
Human cathepsin D	> 700
Calf chymosin	> 700
Endothia parasitica prot.	> 700
Mucor pusillus proteinase	> 1200
Penicillopepsin	> 1200
Yeast proteinase A	> 1200

All measurements were made in 0.1 M sodium formate buffer, pH 3.1 at 37°.
[a]Values taken from Ref. 26.

nature are mostly small peptides. One exception is the inhibitor protein (mol. wt. approx. 17,000) from Ascaris lumbricoides[10]. It was therefore considered of interest to examine the interaction of this inhibitor with aspartic proteinases other than those studied by the original investigators[10,26].

The evolutionary adaptation of this pig intestinal parasitic worm is immediately obvious from the K_i values (Table III) since the protein had virtually no effect on most of the aspartic proteinases tested. Of the non-porcine enzymes, only human gastricsin (and pepsin) was inhibited to any appreciable extent but the interaction was weaker by about one order of magnitude than that with the corresponding enzyme, pig gastricsin. This is in keeping with the qualitative observations made previously by Abu-Erreish and Peanasky[26] and with our own previous investigations[1,13,20] which have indicated a considerable distinction between the gastricsins of human and porcine origin. By contrast, pig and human pepsins resemble each other very closely in most of their properties[1,27], so that their equivalent susceptibility to inhibition is not unexpected[26]. Since none of the other aspartic proteinases tested were affected significantly by this protein, it would appear that the parasite has evolved a defense mechanism to protect itself against the major gastric enzymes that are likely to be encountered in the gastrointestinal tract of its host.

This selectivity of inhibition is displayed also by the only other naturally-occurring protein inhibitor — the inhibitor of (aspartic) proteinase A from yeast[28]. This also appears to be highly specific for its target enzyme and does not inhibit, for example, pig pepsin[28]. It would seem likely that the stringent specificity displayed by the protein inhibitors (mol. wts 8–17,000) may be derived not only from appropriate interactions made within the seven sub-sites of the active site but also from contacts at loci remote from the cleft. This is in contrast to the situation for short peptides which can exert their inhibitory effects only through interactions within the cleft itself. This might explain why peptide inhibitors such as the various pepstatins are (relatively) effective towards a wide range of aspartic proteinases since they are dependent primarily upon the intimate orientation of the statine residue in close juxtaposition to the two catalytic aspartic acid residues which are present in every aspartic proteinase.

ACKNOWLEDGEMENTS

Supported by grants from the Agricultural Research Council (No. AG 72/31) and from the Science and Engineering Research Council (No. GR/C42521). Our international collaboration was fostered by grants from the Wellcome Trust and The Burroughs Wellcome Fund and by Visiting Fellowship awards from the Science and Engineering Research Council (No GR/C42538) and The University of Wales – 50th Anniversary of the British Council.

We are very grateful to our many colleagues who generously supplied us with samples of their purified enzymes and inhibitors.

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