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The selectivity of action of the aspartic-proteinase inhibitor IA_3 from yeast (*Saccharomyces cerevisiae*)

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The ability of the aspartic-proteinase inhibitor IA_3 from yeast (*Saccharomyces cerevisiae*) to affect the activities of a range of mammalian and microbial aspartic proteinases was examined. The inhibitor appeared to be completely selective in that only the aspartic proteinase A from yeast was inhibited to any significant extent. IA_3 thus represents the first example of a totally specific, naturally occurring, aspartic-proteinase inhibitor.

INTRODUCTION

Aspartic proteinases participate in a variety of physiological processes, and changes in the levels of activity expressed may be related to the onset of pathological conditions such as hypertension, gastric ulcers and neoplastic diseases. Pepsin, gastricsin, chymosin, renin, cathepsin D and a variety of microbial enzymes all belong to this class of proteinase (for a review, see Kay, 1985). Extensive sequence homologies have been found between these enzymes (particularly around the two catalytic aspartic acid residues), and all are considered to have broadly similar three-dimensional structures (Pearl & Blundell, 1984). Individual enzymes must have evolved subtle distinctions in structure, however, in order to carry out their specific physiological functions within their own environments. All of the enzymes appear to have an extended active-site cleft that can accommodate at least seven amino acids of a substrate peptide. Discrete alterations in some or all of the seven corresponding subsites of the cleft may then explain the differences in specificity and activity displayed by the various enzymes.

Investigations into such differences have been facilitated for other enzymes by exploitation of the ready availability of natural occurring inhibitors. However, naturally occurring inhibitors of aspartic proteinases are relatively uncommon and are found in only a few specialized locations, i.e. peptide inhibitors from (*a*) various species of actinomycetes (pepstatins) (Aoyagi *et al.*, 1971; Umezawa & Aoyagi, 1977; Valler *et al.*, 1985*a*) and (*b*) the pro-part of various pepsinogens (Dunn *et al.*, 1985) and protein inhibitors from (*c*) parasitic worms (*Ascaris lumbricoides*) (Abu-Erreish & Peanasky, 1974), (*d*) renin-binding proteins (Ueno *et al.*, 1981) (although it is not certain whether the interaction of these proteins with renin actually blocks the activity of the enzyme) and (*e*) the inhibitor of proteinase A in yeast (Saheki *et al.*, 1974; Nunez de Castro & Holzer, 1976).

Although much is known of the molecular details of the yeast inhibitor (IA_3; M_r 8000), including its complete amino acid sequence (Biedermann *et al.*, 1980), relatively little information has been produced concerning its efficacy towards other aspartic proteinases. The present paper describes the effects of yeast inhibitor IA_3 on a representative selection of microbial and mammalian aspartic proteinases, including human renin.

MATERIALS AND METHODS

Preparations of individual aspartic proteinases were obtained and shown to be homogeneous as described previously (Kay *et al.*, 1982, 1983; Reid *et al.*, 1984; Dunn *et al.*, 1985). Yeast proteinase inhibitor (IA_3) was purified to homogeneity as described previously (Biedermann *et al.*, 1980). Its concentration was determined by amino acid analysis after hydrolysis with 6 M-HCl. Inhibition assays were performed for all enzymes (with the exception of human renin) at 37 °C in 0.1 M-sodium formate buffer, pH 3.1, using as substrate the chromophoric peptide Lys-Pro-Ile-Glu-Phe-(NO_2)Phe-Arg-Leu. The hydrolysis of the bond between phenylalanine and *p*-nitrophenylalanine [(NO_2)Phe] is monitored at 300 nm and has been shown to be specific for all of the enzymes under investigation (Dunn *et al.*, 1984; Valler *et al.*, 1985*b*; B. M. Dunn & J. Kay, unpublished work).

Renin assays were performed at 37 °C in 0.43 M-Hepes buffer, pH 7.4, with human angiotensinogen as substrate. The proangiotensin ('angiotensin I') generated was measured by radioimmunosssay.

The inhibitor was preincubated with individual enzymes for 5 min at 37 °C before addition of the substrate to initiate reactions. The inhibition constant (K_i) for the tight binding to yeast proteinase A was derived by the method of Goldstein (1943).

RESULTS AND DISCUSSION

The yeast protein IA_3 was confirmed as a tight-binding inhibitor of its endogenous target proteinase from yeast, proteinase A (Table 1). The K_i value obtained at pH 3.1, namely 0.7×10^{-8} M, is comparable with that described by Nunez de Castro & Holzer (1976), 5×10^{-8} M. By contrast, it can be seen that the IA_3 protein at high

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Table 1. Inhibition of various aspartic proteinases by yeast protein IA_3

The peptide Lys-Pro-Ile-Glu-Phe-(NO_2)Phe-Arg-Leu was used as substrate, and all assays were performed after 5 min preincubation of inhibitor and enzyme at 37 °C in 0.1 M-sodium formate buffer, pH 3.1. Human renin was assayed against human angiotensinogen as substrate in 0.43 M-Hepes buffer, pH 7.4.

Enzyme	K_i (nM)
Yeast proteinase A	7
Penicillopepsin	No inhibition at 1200
Endothia parasitica proteinase	No inhibition at 1200
Mucor pusillus proteinase	No inhibition at 1200
Mucor miehei proteinase	No inhibition at 1200
Scytalidium lignicolum proteinase A	No inhibition at 1200
Calf chymosin	No inhibition at 1200
Human pepsin	No inhibition at 1200
Human gastricsin	No inhibition at 1200
Human slow-moving proteinase	No inhibition at 1200
Human cathepsin D	No inhibition at 1200
Human renin	No inhibition at 1200

concentrations showed negligible inhibition of any of the other aspartic proteinases. A pH of 3.1 was chosen to maintain continuity with previous investigations (Reid *et al.*, 1984; Valler *et al.*, 1985*a*, *b*; Dunn *et al.*, 1985), but also since this is close to the optimum pH for assay of most of the enzymes tested. It is not possible to assay renin with angiotensinogen as substrate at this low pH value, so that this enzyme was investigated at pH 7.4.

The striking specificity exhibited by the yeast inhibitor protein is a novel finding. Other aspartic-proteinase inhibitors (naturally occurring and synthetic), although displaying preferences for individual (types of) enzyme(s), nevertheless do show some inhibitory efficacy towards many of the enzymes. For example, within the group of pepstatins (Aoyagi *et al.*, 1971; Umezawa & Aoyagi, 1977), which contain the unusual amino acid statine as a dipeptide analogue (Holladay *et al.*, 1985), isovaleryl-pepstatin has K_i values towards (various) pepsin(s) and cathepsin D of approx. 10^{-10} M (Rich & Bernatowicz, 1982; Knight & Barrett, 1976), but values of 3×10^{-8} M and lower against calf chymosin and other enzymes (Valler *et al.*, 1985*a*; Holdsworth *et al.*, 1985). Pepstatin derivatives such as lactoyl-pepstatin have K_i values ranging from 4×10^{-10} to 6×10^{-6} M towards human pepsin and gastricsin respectively (Valler *et al.*, 1985*a*). Similarly, synthetic peptide inhibitors of renin containing statine (Boger *et al.*, 1983) or other non-hydrolysable analogues (Szelke *et al.*, 1982; Hallett *et al.*, 1985), although they do display very effective inhibition of human renin (K_i values approx. 10^{-9}–10^{-10} M), nevertheless demonstrate weaker interactions (approx. 10^{-8} M) with other aspartic proteinases such as *Endothia parasitica* proteinase (Hallett *et al.*, 1985). In addition, the inhibitor peptide derived from the pro-part of pepsinogen has also been shown to display preferential, but not exclusive, inhibition characteristics (Dunn *et al.*, 1983, 1985).

Thus the singularity of the inhibition demonstrated by the yeast inhibitor is all the more remarkable. Since some degree of inhibition is always observed for every aspartic proteinase with peptides such as pepstatin, this is probably due to the strength of the energy of interaction derived from the first statine (Sta; transition-state analogue) residue in the acyl-Val-Val-Sta-Ala-Sta sequence being oriented in close juxtaposition to the two catalytic aspartic acid residues. This effect would appear to be sufficiently large to dominate other interactions found between side chains of residues occupying the seven different subsites.

The only other well-documented inhibitor of aspartic proteinases is the protein from *Ascaris lumbricoides*. This has an M_r of approx. 17000 and does display very distinct preferences for pepsins and gastricsins of pig/human origin, with virtually no inhibitory effect on other aspartic proteinases (Valler *et al.*, 1985*a*). The greater selectivity of the protein inhibitors and particularly the yeast IA_3 protein suggests that interactions with the proteinase molecule in loci distinct from the active-site cleft (i.e. regions that are unique for individual enzymes) may be of great significance. Peptide inhibitors which have a length sufficient to permit interactions within the active-site cleft alone may not then be able to achieve the absolute specificity demonstrated by the IA_3 protein. A knowledge of the crystal structure of the yeast proteinase A–IA_3 complex would not only be of interest in its own right, but might provide valuable insights for the design of synthetic inhibitors targetted towards individual aspartic proteinases.

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REFERENCES

Abu-Erreish, G. M. & Peanasky, R. J. (1974) J. Biol. Chem. **249**, 1566–1578

Aoyagi, T., Kunimoto, S., Morishima, H., Takeuchi, T. & Umezawa, H. (1971) J. Antibiot. **24**, 687–694

Biedermann, K., Montali, U., Martin, B., Svendsen, I. & Ottesen, M. (1980) Carlsberg Res. Commun. **45**, 225–235

Boger, J. S., Lohr, N. S., Ulm, E. H., Poe, M., Blaine, E. H., Fanelli, G. M., Lin, T.-Y., Payne, L.-S., Schorn, T. W., Lamont, B. I., Vassill, T. C., Stabilito, I. I., Veber, D. F., Rich, D. H. & Bopari, A. S. (1983) Nature (London) **303**, 83–86

Dunn, B. M., Lewitt, M. & Pham., C. (1983) Biochem. J. **209**, 355–362

Dunn, B. M., Kammermann, B. & McCurry, K. R. (1984) Anal. Biochem. **138**, 68–73

Dunn, B. M., Parten, B., Jimenez, M., Rolph, C. E., Valler, M. J. & Kay, J. (1985) in Aspartic Proteinases and their Inhibitors (Kostka, V., ed.), pp. 221–244, Walter de Gruyter, Berlin

Goldstein, A. (1943) J. Gen. Physiol. **27**, 529–580

Hallett, A., Jones, D. M., Atrash, B., Szelke, M., Leckie, B. J., Beattie, S., Dunn, B. M., Valler, M. J., Rolph, C. E., Kay, J., Foundling, S. I., Wood, S. P., Pearl, L. H., Watson, F. E. & Blundell, T. L. (1985) in Aspartic Proteinases and their Inhibitors (Kostka, V., ed.), pp. 467–478, Walter de Gruyter, Berlin

Holdsworth, R. J., Baker, T. S., Powell, M. J., Titmas, R. C., Rolph, C. E., Valler, M. J. & Kay, J. (1985) Biochem. Soc. Trans. **13**, 1145

Holladay, M. W., Salituro, F. G., Schmidt, P. G. and Rich, D. H. (1985) Biochem. Soc. Trans. **13**,

Kay, J. (1985) in Aspartic Proteinase and their Inhibitors (Kostka, V., ed.), pp. 1–18, Walter de Gruyter, Berlin

Kay, J., Afting, E.-G., Aoyagi, T. & Dunn, B. M. (1982) Biochem.J. **203**, 795–797
Kay, J., Valler, M. J. & Dunn, B. M. (1983) in Proteinase Inhibitors: Medical and Biological Aspects (Katunuma, N., Umezawa, H. & Holzer, H., eds.), pp. 153–163, Springer-Verlag, Berlin
Knight, C. G. & Barrett, A. J. (1976) Biochem. J. **155**, 117–125
Nunez de Castro, I. & Holzer, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. **357**, 727–734
Pearl, L. H. & Blundell, T. L. (1984) FEBS Lett. **174**, 96–101
Reid, W. A., Vongsorasak, L., Svasti, J., Valler, M. J. & Kay, J. (1984) Cell Tissue Res. **236**, 597–601
Rich, D. H. & Bernatowicz, M. S. (1982) J. Med. Chem. **25**, 791–796
Saheki, T., Matsuda, Y. & Holzer, H. (1974) Eur. J. Biochem. **47**, 325–335
Szelke, M., Leckie, B. J., Hallett, A., Jones, D. M., Sueiras, J., Atrash, B. & Lever, A. F. (1982) Nature (London) **299**, 555–557
Ueno, N., Miyazaki, H., Hirose, S. & Murakami, K. (1981) J. Biol. Chem. **256**, 12023–12029
Umezawa, H. & Aoyagi, T. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A. J., ed.), pp. 637–662, Elsevier/North Holland Biomedical Press, Amsterdam
Valler, M. J., Kay, J., Aoyagi, T. & Dunn, B. M. (1985*a*) J. Enzyme Inhib. **1**, in the press
Valler, M. J., Kay, J. & Dunn, B. M. (1985*b*) Biochem. Soc. Trans. **13**, 1144

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