A new chromophoric substrate for penicillopepsin and other fungal aspartic proteinases

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The hexapeptide *N*- α -acetylalanylalanyl-lysyl-*p*-nitrophenylalanylalanylalanylamide has been synthesized and was found to be a good substrate for fungal aspartic proteinases that possess trypsinogen-activating activity, namely penicillopepsin, *Rhizopus* aspartic proteinase, *Endothia* aspartic proteinase and the aspartic proteinases from *Aspergillus oryzae* and *Penicillium roqueforti*. The peptide is rapidly cleaved between the lysine and *p*-nitrophenylalanine residues. Calf chymosin and human renin cleave the same bond, but only very slowly. The cleavage is accompanied by an absorbance decrease with a maximum at 296 nm ($\Delta\epsilon -1800\text{ M}^{-1}\cdot\text{cm}^{-1}$). Pig pepsin and the aspartic proteinases from two *Rhizomucor* species cleave the peptide slowly on the carboxy side of *p*-nitrophenylalanine. For the five enzymes that hydrolysed the peptide rapidly, K_m values range from 0.16 to 0.42 mM and k_{cat} from 6 to 46.6 s^{-1} at pH 4.5 and 25°C. A comparison of the kinetic parameters of the hexapeptide with those of the dipeptide *N*- α -acetyllysyl-*p*-nitrophenylalanylamide obtained with penicillopepsin shows that at pH 6.0 the catalytic rate constant k_{cat} is over 5000-fold greater for the hexapeptide, whereas the K_m values are essentially the same, showing that the catalytic efficiency is strongly dependent on secondary binding. The new substrate with a *p*-nitrophenylalanine residue in the P'_1 position has advantages over previously used substrates for aspartic proteinases in that it offers a more sensitive spectrophotometric assay that is independent of pH up to 5.5 and can readily be used up to pH 7.0. The presence of lysine makes it very water-soluble. Stopped-flow spectrophotometric experiments with penicillopepsin gave clear evidence that the hydrolysis of the substrate by penicillopepsin is not accompanied by a 'burst' release of *p*-nitrophenylalanylalanylalanylamide.

Although a number of aspartic proteinases from fungal sources have been studied in many laboratories (see review by Hofmann, 1974), simple substrates suitable for both rapid assay and detailed kinetic studies have not been generally available. Most commonly a rate assay based on the protein substrates haemoglobin (Anson, 1938), casein (Kunitz, 1947) or serum albumin (Hofmann, 1976a) has been used for the determination of the enzymic activities. Alternatively, the activation of trypsinogen has provided a very sensitive assay for many of the aspartic proteinases (Nakanishi, 1959; Sodek & Hofmann, 1970; Morihara & Oka, 1973). A milk-clotting assay has been used for enzymes from

Endothia parasitica (Whitaker, 1970) and from *Mucor* (*Rhizomucor*) *pusillus* (Arima *et al.*, 1970).

Although the fungal aspartic proteinases are homologous to pepsin and chymosin (Gripon *et al.*, 1977; Foltmann *et al.*, 1979) and show many similarities in their specificities to the mammalian enzymes (Sodek & Hofmann 1970; Mains *et al.*, 1971) most small pepsin substrates are not hydrolysed by penicillopepsin (Mains *et al.*, 1971) and by the aspartic proteinases of *Aspergillus saitoi* (Ichishima, 1970), *Endothia parasitica* (Whitaker, 1970) and by other fungal proteinases. Longer peptides, such as Z-Ala-Ala-Phe-Phe-OPrPy, one of the best synthetic substrates for pig pepsin, are only poorly hydrolysed by penicillopepsin (Mains *et al.*, 1971) and the aspartic proteinase from *Aspergillus oryzae* (Davidson *et al.*, 1975). Furthermore, many of the pepsin substrates have the disadvantages that their hydrolysis must be followed by a cumbersome

Abbreviations used: Ac, acetyl; Nle, norleucyl; NO_2 -Phe, *p*-nitrophenylalanyl; OMe, methyl ester; OPrPy, 3-(4-pyridyl)propoxy; Z, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; DCC, dicyclohexylcarbodi-imide.

ninhydrin method and that they are only poorly soluble in the acidic pH range. Organic solvents cannot be used to increase solubility since they are strongly inhibitory even at concentrations of 2% and less (see Clement, 1973) and cause large shifts in the apparent K_m values in penicillopepsin (T. Hofmann & A. L. Fink, unpublished work). The introduction by Inouye & Fruton (1967) of a *p*-nitrophenyl-alanine residue in position P_1 (as defined by Schechter & Berger, 1967) represented a significant advance over the ninhydrin method. The rate of cleavage at the carboxy side of the nitrophenyl-alanine residue can be followed spectrophotometrically at 310 nm owing to the liberation of a negative charge that induces a bathochromic shift. This shift is dependent, however, on the carboxy ionization and therefore the use of these substrates is restricted in pH-dependence studies of the aspartic proteinases. Limited studies have been carried out on fungal proteinases with substrates containing nitrophenyl-alanine in position P_1 (Voynick & Fruton, 1971; Sampath-Kumar & Fruton, 1974; Houmard & Raymond, 1979; Martin *et al.*, 1980). These studies showed that penta- and hexa-peptides were reasonably good substrates, owing to the secondary binding effects.

Moriyama & Oka (1973) reported a study of the action of several aspartic proteinases on a series of peptides of the type $Z-[Ala]_m-Lys-[Ala]_n$, where $m = 0-2$ and $n = 1-4$, and found that the cleavage of these peptides correlated with the ability of the enzymes to activate trypsinogen. They showed that the k_{cat} values (turnover numbers) for the longest substrate, $Z-[Ala]_2-Lys-[Ala]_3$, were comparable with k_{cat} values observed by Sachdev & Fruton (1970) for the best pepsin substrates. However, the hydrolysis of these peptides was followed by a ninhydrin assay, which suffers from large background colours owing to the presence of lysine residues. Penicillopepsin also rapidly hydrolyses $Z-[Ala]_2-Lys-[Ala]_3$ (T. Hofmann, unpublished work). Replacement of the alanine residue after the lysine by a nitrophenylalanine residue led to an excellent chromophoric substrate with higher sensitivity and less restrictive pH dependence than substrates with the nitrophenylalanine in the P_1 position. The action of penicillopepsin and other aspartic proteinases on $Ac-Ala-Ala-Lys-NO_2-Phe-Ala-Ala$ -amide is described in the present paper. Preliminary results have been reported previously (Hofmann *et al.*, 1979; James *et al.*, 1981).

Experimental

Materials

Enzymes. Penicillopepsin was prepared and tested for homogeneity as described by Hofmann (1976a). The preparations used had the highest specific acti-

vities that have been obtained so far with the trypsinogen-activating assay (Hofmann, 1976a). *Rhizopus* aspartic proteinase was from Miles Laboratories, Elkhart, IN, U.S.A.; calf chymosin from Sigma Chemical Co., St. Louis, MO, U.S.A.; pig pepsin (three-times crystallized) from Calbiochem, San Diego, CA, U.S.A.; the aspartic proteinases from *Aspergillus oryzae* and *Penicillium roqueforti* were gifts from Dr. A. Gertler, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovoth, Israel, and Dr. J. C. Gripon, C.N.R.Z., Jouy-en-Josas, France, respectively; *Rhizomucor* rennin, prepared from 'Emporase' (Dairyland Food Laboratories Inc., Waukesha, WI, U.S.A.) and *Rhizomucor miehei* aspartic proteinase, prepared from 'rennilase' (Novo Industri A/S, Copenhagen, Denmark) were gifts from Dr. W. S. Rickert, Department of Statistics, University of Waterloo, Waterloo, Ont., Canada; human and pig kidney renin were generously supplied by Dr. D. H. Osmond, Department of Physiology, University of Toronto; the acid proteinases A and B from *Scatylidium lignicolum* were gifts from Dr. K. Oda, Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Osaka, Japan; *Endothia* aspartic proteinase was prepared from 'Sure-Curd' (Charles Pfizer and Co., Milwaukee, WI, U.S.A.) by Mains (1973).

Peptide synthesis. All amino acids used for the syntheses were in the L-configuration. The following peptides were synthesized using the general procedures for solid-phase peptide synthesis (Erickson & Merrifield, 1975) on a Beckman peptide synthesizer model 990: $Ac-Ala-Ala-Lys-NO_2-Phe-Ala-Ala$ -amide and analogues without the acetyl or amide group and $Ac-Lys-NO_2-Phe$ -amide. For the synthesis of peptides with a free α -carboxy group, the C-terminal amino acid was esterified as the Boc-amino acid caesium salt (Gisin, 1973) to the co-poly(styrene/1% divinylbenzene) chloromethyl resin (0.9 mmol of Cl⁻/g; Pierce Chemical Co., Rockford, IL, U.S.A.), which was brominated to the extent of one bromine atom per benzene ring (Merrifield, 1963). The extent of substitution of Boc-alanine to the brominated chloromethyl resin was 0.29 mmol/g of resin. When the C-terminal amide was desired on the peptide, 0.49 mmol each of Boc-amino acid and DCC/g of resin were added to the co-poly(styrene/2% divinylbenzene) benzhydrylamine resin (0.49 mmol of NH₂/g of resin; Protein Research Foundation, Osaka, Japan). The coupling resulted in a substitution of 0.47 mmol of Boc-alanine/g of amino acid resin. Excess amino groups were terminated by a 90 min treatment with acetic anhydride/benzene/pyridine (1:3:3, by vol.; 25 ml/g of peptide resin) after prewashing for 5 min with the same mixture. The substitution of amino acid on the resin was determined by amino acid

analysis of the acid hydrolysate of the amino acid resin and by picrate monitoring (Hodges & Merrifield, 1975a). The amino acid or peptide resin was hydrolysed in a mixture of methanesulphonic acid/propionic acid/water (5:12:3, by vol.) or HCl/acetic acid/phenol (2:1:1, by vol.) as previously described (Hodges & Merrifield, 1975b). All amino groups were protected at the α -amino position with the Boc group and the Z group was used as the side-chain blocking group for lysine.

For the addition of the same and subsequent amino acids, Boc-amino acids (2-fold molar excess over the amino acyl resin) in dichloromethane (5 ml/g of resin) were added to the resin followed by an equivalent amount of DCC in dichloromethane (5 ml/5 g of resin). The reaction time for the coupling was 90 min. Double couplings were performed at each step of the synthesis. The Boc-groups were removed at each cycle of the synthesis by two deprotection steps for 25 min each involving 35% trifluoroacetic acid/dichloromethane (v/v) in the case of the brominated chloromethyl support and 50% trifluoroacetic acid/dichloromethane (v/v) for the benzhydrylamine supports. Neutralizations were carried out with 5% di-isopropylethylamine/dichloromethane (v/v). The programme used for the attachment of each amino acid has been previously described (Hodges *et al.*, 1981). Peptides that were to have an acetyl group at the *N*-terminal were acetylated as above. The completion of couplings and deprotections were monitored by the picrate method of Hodges & Merrifield (1975a).

N- α -*t*-Butoxycarbonyl-*p*-nitro-L-phenylalanine. NO₂-Phe (2.5 g, 12.5 mmol) was suspended in 35 ml of 50% dioxan and Boc azide (2.71 ml, 18.75 mmol) was added. The pH was adjusted to 10.1 with 4M-NaOH and maintained at this pH with automatic titration equipment for 16 h at room temperature. The extent of the reaction was followed by t.l.c. on silica gel [chloroform/methanol/acetic acid (17:2:1, by vol.) as solvent; R_F values: Boc-NO₂-Phe, 0.67; NO₂-Phe, origin]. Boc azide (1 ml) was added and the reaction proceeded for an additional 24 h. The reaction mixture was filtered and the filtrate was extracted three times with 5.0 ml of diethyl ether. The aqueous phase was acidified with citric acid to pH 3.0 on the pH-Stat and extracted with three portions of ethyl acetate (5 ml). The combined organic phase was washed with 50 ml of water, dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The Boc-NO₂-Phe was crystallized by slow addition of hexane. The product was filtered and recrystallized from anhydrous diethyl ether/light petroleum (b.p. 60–80°C). The product was filtered and dried over P₂O₅ for 20 h under high vacuum: yield 2.63 g (68%); m.p. 109–110°C (Found: C, 54.39; H, 6.04; N, 8.80; C₁₄H₁₈O₆N₂ requires C, 54.19; H, 5.85; N, 9.03%).

Cleavage of peptides from resins. The cleavage of the peptides from the resins and removal of blocking groups was carried out in HF at 0°C for 60 min in a type-1 HF apparatus from the Protein Research Foundation, with 10% (v/v) anisole as a scavenger for cations. HF and the bulk of the anisole were then removed under vacuum below 0°C and the residual anisole and by-products were extracted with diethyl ether. The cleaved peptide was extracted from the resin with a 10% solution of acetic acid, which was then freeze-dried.

Purification of peptides. In general purification of the cleaved peptides was not necessary. A column purification method was developed for the Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide peptide. The cleaved peptide was dissolved in starting buffer and applied to an Aminex 50W (X4; 20–29 μ m) column (1.9 cm $\times$ 50 cm) and eluted with a linear gradient prepared from 300 ml of pyridine acetate buffer (0.1 M in pyridine), pH 5.0, and 300 ml of pyridine acetate buffer (0.5 M in pyridine), pH 5.0, at a flow rate of 55 ml/h. The resin was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. The column effluent was stream-divided with a Beckman 120B amino acid analyser preparative system; a flow rate of 5 ml/h was used for the ninhydrin analytical system. Fractions (8 ml) were collected. Only one peak was detected by the ninhydrin reaction and eluted between 10 and 11 h. The peptide was homogeneous on paper electrophoresis. Amino acid analysis revealed the following molar ratios: Ala, 3.96; NO₂-Phe, 1.03; Lys, 1.01.

High-voltage electrophoresis. Peptides were separated on Whatman 3MM paper by high-voltage electrophoresis at 60 V/cm for 45 min in volatile buffers of the following compositions: pH 2.1, acetic acid/formic acid/water (15:1:250, by vol.); pH 3.6, acetic acid/pyridine/water (10:1:89, by vol.); pH 4.7, acetic acid/pyridine/water (1:1:38, by vol.); pH 6.5, acetic acid/pyridine/water (3:100:900, by vol.), as described by Kurosky & Hofmann (1976). Methyl Green (Stevenson, 1971) and a mixture of amino acids were run as markers. A cadmium/ninhydrin reagent was used for the detection of the peptides (Heilmann *et al.*, 1957). Because of the presence of a lysine residue all *N*-acetylated peptides gave a positive reaction.

Peptide analysis. The identity of the synthetic peptides and of product peptides eluted from paper after electrophoresis was confirmed by amino acid analysis and *N*-terminal analysis, where applicable. The homogeneity of the synthetic peptides was checked by high-voltage electrophoresis at pH 2.1, 3.6, 4.7 and 6.5.

Amino acid analysis. Peptides were hydrolysed under vacuum with 5.7 M-HCl (0.2 ml) at 107°C for 20 h. The amino acid composition was determined by the method of Moore *et al.* (1958) in a

model 120C Beckman-Spinco analyser fitted with high sensitivity colorimeter cells (18 mm light path; Evans Electro Selenium, Halstead, Essex, U.K.) or on a Durrum D-500 analyser. The elution times on the Durrum-D-500 analyser were: phenylalanine, 54 min; *p*-nitrophenylalanine, 62 min. The colour value of the latter was 0.837 relative to phenylalanine.

***N*-Terminal analysis.** Peptides eluted from paper after electrophoresis were analysed for their *N*-terminal residues by the dansyl-Edman technique of Gray (1967).

Enzyme assays. The rate of cleavage of the *p*-nitrophenylalanine-containing peptides was followed spectrophotometrically at 25°C in either a Zeiss Model PMQ II (Zeiss, Toronto) with a model E1101S, Esterline Angus recorder (Esterline Angus Instrument Co., Indianapolis, IN, U.S.A.) or a Cary 118 spectrophotometer (Varian Instruments, Palo Alto, CA, U.S.A.).

Unless otherwise indicated enzyme assays were performed at $25 \pm 0.5^\circ\text{C}$ in 20 mM-sodium acetate buffer, adjusted to an ionic strength of 20 mM with NaCl. Assays were carried out in cells of 2 or 10 mm light paths and at wavelengths of 296, 306 or 320 nm. The cells and wavelengths were chosen so that the initial absorbance of the substrate solution did not exceed 2.0. Rates obtained in absorbance units/s were converted into rates in mM/s with the use of the molar difference absorption coefficients for the appropriate wavelength given in Table 1. The kinetic parameters K_m and k_{cat} were evaluated from initial rates measured from the recorder traces plotted as linear plots in three different ways: $1/v$ versus $1/[S]$, according to Lineweaver and Burk; v versus $v/[S]$, according to Woolf and Hofstee; and $[S]/v$ versus $[S]$, according to Hanes. The three values each for K_m and k_{cat} obtained were averaged with values from duplicate or triplicate experiments.

Table 1. *Spectral parameters of Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide used in enzyme assays*

The molar absorption coefficients were determined with solutions whose concentration was accurately measured by amino acid analysis after hydrolysis in 5.7 M-HCl. Difference absorption coefficients ($\Delta\epsilon$) were determined after complete cleavage between lysine and *p*-nitrophenylalanine. The same values were obtained for the substrates without the acetyl or the amide group respectively. Results are means $\pm$ s.d. (where indicated) for the numbers of observations indicated in parentheses. $\lambda_{max} = 277$ nm; $\Delta\lambda_{max} = 296$ nm.

λ (nm)	$\Delta\epsilon$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	ϵ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)
277		10970 ± 170 (6)
296	-1800 ± 40 (4)	8200
306	-1450 ± 30 (4)	5760
320	-735 ± 25 (4)	2240

The standard deviations of these averaged values did not exceed $\pm 15\%$. We did not have sufficient data for a more rigorous statistical treatment. A limited number of analyses were carried out in which complete progress curves were recorded, either at low enzyme levels in a Cary 118 spectrophotometer fitted with a data logging device that was processed by computer with a curve-fitting program kindly supplied by Dr. D. Kells, Department of Biochemistry, University of Toronto, Toronto, Ont., Canada, or under second-order conditions in a stopped-flow spectrophotometer (see below). The values obtained from these progress curves agreed well with those obtained from the initial rate measurements.

Spectral analysis of substrate (Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide) and product (NO₂-Phe-Ala-Ala-amide). The molar absorption coefficients of the substrate and its two analogues with free *N*-terminal and C-terminal groups respectively were determined from their absorption spectra. The values obtained are given in Table 1. The peptide NO₂-Phe-Ala-Ala-amide in its fully protonated form (pH 4.0) has an absorbance maximum at 272 nm, with an absorption coefficient $\epsilon = 10500 \pm 600 \text{ M}^{-1} \cdot \text{cm}^{-1}$. In its fully deprotonated form (pH 8.8) its spectrum is similar to, but not identical with, that of the substrate, with $\lambda_{max} = 279$ nm. The molar difference absorption coefficient $\Delta\epsilon$ between the two forms is $2500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 296 nm and 25°C and is thus significantly higher than the difference between the substrate and the product (Table 1). The dissociation constant $pK_a = 6.65$ of the α -amino group was determined in 20 mM-sodium phosphate buffers between pH 5.2 and 7.9. The concentrations of the solutions used were determined by amino acid analysis. Difference spectra between substrate and product were obtained on several occasions in a Cary 118 spectrophotometer. Substrate solutions (1 ml, 0.1–0.2 mM) at pH 3.5–4.5 were placed in both reference and sample cells. A small amount of penicillopepsin (1–2 μg) was added to the sample cell. Spectra were recorded repeatedly until no further change was observed. They were used to calculate the difference absorption coefficients listed in Table 1.

Results and discussion

Enzymic hydrolysis of Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide

The ability of several aspartic proteinases to hydrolyse the hexapeptide Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide at pH 3.4 was tested qualitatively after prolonged incubation by analysis of the products on high-voltage electrophoresis at pH 3.6. The results are shown in Table 2. Penicillopepsin, *Rhizopus* aspartic proteinase, *Endothia* aspartic proteinase and the aspartic proteinases from *Penicillium*

Table 2. *Effect of various aspartic proteinases on Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide*

Assays were performed in 20 mM-ammonium formate, pH 3.4, with 50 μ l of substrate (1 mM). Incubations were at 25°C for 10 min, 4 h and 20 h. The reactions were stopped by immersion of the tubes into a boiling-water bath for 1 min. The reaction mixture (20 μ l) was applied to Whatman 3MM paper for high-voltage electrophoresis at pH 3.6. Peptides were identified after elution by amino acid analysis and, where applicable, by N-terminal analysis.

Enzyme	Trypsinogen-activating ability	Reference	[Enzyme] (μ g/50 μ l)	Products formed	Remarks
Penicillopepsin	+	Hofmann & Shaw (1964)	0.5	Ac-Ala-Ala-Lys and NO ₂ -Phe-Ala-Ala-amide	Reaction complete in 10 min
<i>Rhizopus</i> aspartic proteinase	+	Graham <i>et al.</i> (1973)	1.0		
<i>Endothia</i> aspartic proteinase	+	Mains (1973)	1.0		
Aspartic proteinase from <i>P. roqueforti</i>	+	Zevaco <i>et al.</i> (1973)	1.0		
Aspartic proteinase from <i>A. oryzae</i>	+	Davidson <i>et al.</i> (1975)	1.0		
Calf chymosin*	?		2.0	Ac-Ala-Ala-Lys and NO ₂ -Phe-Ala-Ala-amide	Little conversion after 20 h
Human renin*	?		2.0		
Pig pepsin	0	Moriyama & Oka (1973)	2.0	Ac-Ala-Ala-Lys-NO ₂ -Phe and Ala-Ala-amide	Incomplete conversion after 20 h
Aspartic proteinase from <i>Rhizomucor</i> species	0	Moriyama & Oka (1973)	2.0		
Acid proteinases A and B from <i>S. lignicolum</i>	0	Oda <i>et al.</i> (1976)	1.0	Ac-Ala-Ala-Lys-NO ₂ -Phe and Ala-Ala-amide and other products	Multiple cleavages
Pig renin	?		2.0	No reaction	

* Also tested at pH 6.5, with qualitatively the same results.

roqueforti and *Aspergillus oryzae* rapidly cleaved the peptide at the -Lys-NO₂-Phe- bond. No other cleavages were observed after 4 h at the enzyme levels used in these experiments. After 20 h some faint additional bands appeared on the electrophoretograms. These are discussed below. Calf chymosin and human kidney renin also cleaved the same bond, but at a rate that is approx. 10⁻⁴ times that of penicillopepsin. Pig kidney renin showed no cleavage of the peptide, whereas pig pepsin and the aspartic proteinases from two *Rhizomucor* species cleaved it slowly on the carboxy side of the nitrophenylalanine residue. The acid proteinases A and B from *Scatylidium lignicolum* showed little specificity and rapidly produced several peptide bands that were not further identified. [Although these proteinases have low pH optima, inhibition studies show that they do not react with those covalent and non-covalent inhibitors that characterize aspartic proteinases (Oda *et al.*, 1976). The present results add further to the suggestion that these enzymes belong to a different class of proteinases.]

The overall results clearly show that the ability to hydrolyse the hexapeptide rapidly on the carboxy side of lysine is closely correlated with the ability to activate trypsinogen, a correlation that was previously shown by Moriyama & Oka (1973) for

Rhizopus aspartic proteinase and four other fungal proteinases not used in our study, and with different lysine substrates.

Kinetics of the hydrolysis of Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide

The kinetic parameters k_{cat} and K_m determined as described in the Experimental section for the five fungal enzymes that were qualitatively shown to hydrolyse the hexapeptide rapidly are given in Table 3. These experiments were carried out at pH 4.5, the optimum pH for k_{cat} with penicillopepsin (T. Hofmann, unpublished work). The values of both K_m and k_{cat} are similar for all five enzymes. The replacement of the alanine residue in position P₁' in the substrate used by Moriyama & Oka (1973) by a nitrophenylalanine residue had little effect on k_{cat} of *Rhizopus* aspartic proteinase, the only enzyme available for comparison. Those authors obtained k_{cat} = 30.5 s⁻¹ at pH 4.5 and 40°C, compared with our value of 10.8 s⁻¹ at 25°C. However, there is a large difference in K_m ; Moriyama & Oka (1973) found a value of 6.1 mM, compared with 0.42 mM in the present paper (Table 3). K_m values for the other three enzymes studied by Moriyama & Oka (1973) range from 2.5 to 34 mM and are thus two to three orders of magnitude greater than the values we ob-

Table 3. *Kinetic parameters of the action of various aspartic proteinases on Ac-Ala-Lys-NO₂-Phe-Ala-Ala-amide*
Reactions were carried out in 0.02 M-sodium formate buffer, pH 4.5 at 25°C.

Enzyme	Range of [S] (mM)	[E] (μg/ml)	K _m (mM)	k _{cat.} (s ⁻¹)	k _{cat.} /K _m (s ⁻¹ ·mM ⁻¹)
Penicillopepsin	0.03–1.0	0.67	0.18	46.6	260
<i>Rhizopus</i> aspartic proteinase	0.05–1.4	1.75	0.42	10.8	26
Aspartic proteinase from <i>Aspergillus oryzae</i>	0.05–1.0	0.8	0.21	29	138
Aspartic proteinase from <i>Penicillium roqueforti</i>	0.05–1.0	1.1	0.23	32	140
<i>Endothia</i> aspartic proteinase	0.05–1.0	2.5	0.16	6*	>37

* This value is low because *Endothia* aspartic proteinase was only partially purified.

Table 4. *The effect of chain length and terminal charges on k_{cat.} and K_m of the hydrolysis of homologous substrates by penicillopepsin*

Assays were performed in 0.02 M-sodium phosphate (pH 6.0) or 0.02 M-sodium acetate buffer (pH 4.5 and 5.5) at 25°C. Cleavage occurred in both substrates at the -Lys-NO₂-Phe- bond.

Substrate	pH 6.0			pH 4.5			pH 5.5		
	K _m (mM)	k _{cat.} (s ⁻¹)	k _{cat.} /K _m (M ⁻¹ ·s ⁻¹)	K _m (mM)	k _{cat.} (s ⁻¹)	k _{cat.} /K _m (M ⁻¹ ·s ⁻¹)	K _m (mM)	k _{cat.} (s ⁻¹)	k _{cat.} /K _m (M ⁻¹ ·s ⁻¹)
(1) Ac-Ala-Ala-Lys-NO ₂ -Phe-Ala-Ala-amide	0.072	22.1	320 000	0.18	46.6	250 000	0.078	36	460 000
(2) Ac-Lys-NO ₂ -Phe-amide	0.103	0.0039	39	0.70	0.02	29	0.22	0.01	45
(3) ³ H-Ala-Ala-Lys-NO ₂ -Phe-Ala-Ala-amide	—	—	—	—	—	—	0.036	54	1 500 000
(4) Ac-Ala-Ala-Lys-NO ₂ -Phe-Ala-Ala-O ⁻	—	—	—	—	—	—	1–2*	80	

* Owing to insufficient material, no accurate value could be determined.

tained. These findings again emphasize that one of the specificity features of aspartic proteinases is a requirement for an amino acid with a bulky hydrophobic side chain in position P₁.

The results presented in Table 3 show that Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide is an excellent substrate for at least those fungal enzymes that activate trypsinogen. The presence of a lysine residue renders the peptide highly water-soluble (a >20 mM solution can readily be prepared). The nitrophenylalanine in position P₁ upon cleavage has an α-amino group that upon protonation gives rise to an absorption difference with a Δε = -1800 M⁻¹·cm⁻¹, a value nearly twice (and of opposite sign to) that obtained upon release of a carboxylate ion from a substrate with nitrophenylalanine in position P₁ (Raymond & Bricas, 1979). The α-ammonium group has a pK_a = 6.65; hence the hexapeptide substrate can be used for pH studies up to 5.0 without correction and to over 7.0 with correction for the deprotonation of the product. Conversely, the use of substrates with nitrophenylalanine in position P₁ is limited because the chromophoric shift depends on the α-carboxylate ion whose pK = 3.3 (Raymond & Bricas, 1979) and rate assays must be corrected for protonation over most of the pH range in which aspartic proteinases are active.

The advantage of nitrophenylalanine in position

P₁ has been used by Dunn (1981), who synthesized Pro-Thr-Glu-Phe-NO₂-Phe-Arg-Leu and found it to be an excellent and very soluble substrate for pig pepsin.

The effect of chain length on catalytic efficiency

As a result of extensive studies Fruton (1970) proposed that secondary binding of long peptide substrates for pepsin played an important role in the activity. He showed that the interaction of an extended substrate chain in secondary binding sites causes increases in catalytic efficiency of several orders of magnitude. These increases are almost entirely due to increases in k_{cat.}. Our own work on the activation by non-substrates of the cleavage of small substrates with both pepsin and penicillopepsin (Wang *et al.*, 1974; Wang & Hofmann, 1976) showed that the two enzymes were very similar in their action and suggested that the secondary binding effect was transmitted through the enzyme rather than through an induced strain in the substrate. Table 4 shows that at pH 6.0 an increase in chain length from the dipeptide Ac-Lys-NO₂-Phe-amide to the hexapeptide increases k_{cat.} over 5000-fold and has no effect on K_m. This is strictly analogous to the observations made by Fruton (1970) for similar increases in chain length in pepsin substrates. At pH 4.5 the K_m values are significantly

higher and a difference in K_m between the di- and the hexa-peptides becomes apparent. This suggests a difference in the pH dependence of K_m .

The large increase in k_{cat} through secondary binding is not confined to aspartic proteinases, but is a more general phenomenon among proteinases that do not form precise S_1 - P_1 interactions, i.e. that have no rigidly defined primary specificity. This has been shown very elegantly for two chymotrypsin-related proteinases from *Streptomyces griseus* by Bauer (1978), who found that k_{cat} of the fungal enzymes was strongly dependent on the chain length of the substrates, whereas trypsin and chymotrypsin, which form precise S_1 - P_1 interactions, showed little dependence on binding that was remote from the scissile bond.

A surprising result is the effect of the negative charge on the C-terminal alanine in peptide (4) (Table 4). Compared with peptide (1) the K_m at pH 5.5 has increased some 12–24-fold. It would appear that the carboxylate group in the productive binding mode is located in an environment unfavourable to the negative charge. The positive charge on the N-terminal alanine (peptide 3), on the other hand, has little effect on the kinetic parameters.

Prolonged action of penicillopepsin on Ac-Ala-Ala-Lys-NO₂-Phe-Ala-Ala-amide

During the stopped-flow experiments we found that at high enzyme concentrations (0.3 mM) the absorbance at 296 nm increased after the initial expected decrease. The possible cause for this was investigated by (a) recording progress curves at 0.3 mM substrate and three different enzyme concentrations and (b) analysing the products of the reaction at different stages by high-voltage electrophoresis at pH 3.6. At the lowest enzyme concentration (2.5 μ g/ml, pH 5.25, 25°C) the decrease in absorbance at 296 nm due to the initial reaction (cleavage of the Lys-NO₂-Phe bond) was complete after 3 min (phase I). After incubation for 4 h a small continuing increase in absorbance was observed. With an enzyme concentration of 50 μ g/ml, phase I was complete within the mixing time. After a lag of some 10 min the absorbance began to increase and continued increasing for about 8 h (phase II) to a level of 85% of the original absorbance. At an enzyme concentration of 500 μ g/ml, phases I and II were complete in 25 min and were followed by another decrease (phase III) of about 14% of the initial decrease of phase I; this reached an equilibrium after about 4 h. The peptides formed during phase I are, as expected, Ac-Ala-Ala-Lys and NO₂-Phe-Ala-Ala amide. No detectable substrate is left at the end of phase I. During phase II NO₂-Phe-Ala-Ala amide disappears and four new bands appear, the most prominent of which is Ala-Ala-amide. The other three represent condensation products of nitro-

phenylalanine; two of these have been identified as the dimer and trimer respectively. We were unable to detect NO₂-Phe-NO₂-Phe-Ala-Ala-amide; this peptide would be expected to be present on the basis of the known transpeptidation reactions (Wang & Hofmann, 1976). The primary product Ac-Ala-Ala-Lys remains constant throughout all three phases.

The fact that the absorbance during phase II rises to 85% of the original indicates (a) the formation of NO₂-Phe oligomers larger than the dimer and (b) the liberation of carboxylate groups on the nitrophenylalanine. Both reactions are associated with increases in absorbance. During the very slow phase III reactions the major product formed is the free amino acid nitrophenylalanine, presumably from the cleavage of the oligomers.

The phase II and III reactions once again highlight the complexity of the transpeptidation reactions that penicillopepsin can catalyse (Wang & Hofmann, 1976). Their mechanism is not yet clearly understood. It is noteworthy, however, that the bond-forming reaction that takes place during phase II, which mechanistically is presumably the reverse of the reaction of phase I (microreversibility), is clearly governed by a totally different specificity, since there is no evidence for any reformation of the original substrate.

It should be stressed that for kinetic work the reactions of phases II and III do not interfere with the primary cleavage reaction, even when complete progress curves are recorded. There is a considerable delay after the end of phase I when no detectable absorbance changes occur. The rate constants for the three phases have been calculated from the initial slopes: phase I, $k' = 29 \text{ s}^{-1}$; phase II, $k' = 0.009 \text{ s}^{-1}$; phase III, $k' = 0.0003 \text{ s}^{-1}$. The reactions of phases II and III are not due to contaminating enzymes, such as serine proteinases, carboxypeptidases S_1 and S_2 (Hofmann, 1976b) or aminopeptidases. The reactions are not inhibited by treatment of penicillopepsin with di-isopropyl fluorophosphate, *p*-hydroxymercuribenzoate and metal chelators, but are completely suppressed when pepstatin is added at the end of phase I.

Stopped-flow experiments

The availability of a substrate where the release of the amino moiety is accompanied by a chromophoric change made it possible to test the penicillopepsin reaction for any 'acyl-intermediate', provided that the rate of breakdown of such an intermediate and not its formation was the rate-limiting step. Stopped-flow experiments were carried out at pH 5.25 in 0.02 M-sodium acetate at 3°C and 20°C at substrate concentrations of 0.03 to 0.3 mM and enzyme concentrations of 0.00125 to 0.3 mM. Under none of these conditions was there any discontinuity in the

progress curves that might indicate a 'burst' reaction. When the experiment was carried out under second-order conditions ($[S] = [E] = 4 \times K_m$) no evidence for an intermediate was observed, although the presence of an intermediate would have been detected even if its rate of formation had been faster than the dead-time of the instrument (about 2 ms).

Kinetic parameters (k_{cat} and K_m) calculated from several of the stopped-flow experiments agreed well with those determined from initial rate assays and from integrated Michaelis-Menten curves. This suggests that no significant substrate and product inhibitions or activations are involved in the catalytic event.

These experiments accord well with the stopped-flow experiments of Sachdev & Fruton (1975), who used fluorescent substrates and found that the rate-limiting step was associated with the cleavage of the scissile bond in the first detectable ES complex.

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