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Effect of pH on the Activities of Penicillopepsin and *Rhizopus* Pepsin and a Proposal for the Productive Substrate Binding Mode in Penicillopepsin†

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ABSTRACT: The pH dependence of kinetic parameters for penicillopepsin and *Rhizopus* pepsin acting on acetylalanyl-alanyllysyl-*p*-nitrophenylalanylalanylalanine amide has been determined. The velocity constants, K_{cat}, show optima between pH 4 and pH 4.5. The Michaelis-Menten constants, K_m, show a strong pH dependence for both enzymes and rise from low values at pH 6.0 (0.08 mM for penicillopepsin and 0.23 mM for *Rhizopus* pepsin) to approximately 8 mM and 1.1 mM, respectively, at pH 2.0. This dependence strongly suggests that for this substrate, with lysine in the P_1 position, binding is controlled by negatively charged carboxyl group(s) on the enzyme. These groups have been tentatively identified in penicillopepsin as aspartic acid-115(114) and glutamic acid-16(13) on the basis of model building and by comparison with the binding of a pepstatin analogue. The S_1 binding site also has hydrophobic character which shows itself in the low K_m (0.004 mM) for the substrate leucylseryl-*p*-nitrophenylalanylnorleucylalanylleucine methyl ester. Tyrosine-75(75), phenylalanine-112(111), and leucine-121(120) are the most likely residues involved in the hydrophobic binding. The binding site for P_1' residues is also hydrophobic and probably involves phenylalanine-190(189), isoleucine-211(213), phenylalanine-295(299), and isoleucine-297(301). In light of the structure of penicillopepsin, now refined at 1.8-Å resolution, the detailed binding mode of a pepstatin analogue also studied at 1.8-Å resolution, and model-building studies, a productive binding mode for the scissile bond to aspartyl proteinases is proposed. Although physiologically the aspartic proteinases show a spread of pH optima from below pH 2 to over pH 7, their pH optima (as expressed in terms of k_{cat}/K_m) lie in a much narrower range when the enzymes act on defined good substrates.

Penicillopepsin and other fungal proteinases have the ability to activate trypsinogen, a reaction that requires specificity for a lysine residue in the S_1 [nomenclature of Schechter & Berger (1967)] binding site (Hofmann, 1963; Sodek & Hofmann, 1970). This lysine specificity has been confirmed kinetically with synthetic lysine-containing peptides (Morihara & Oka, 1973; Hofmann & Hodges, 1982). It is therefore of interest to determine whether or not this specificity is due to an ion pair interaction with a carboxyl group on the enzyme. We present kinetic evidence for such an interaction in penicillopepsin and *Rhizopus* pepsin.

Possible substrate binding modes for a number of different substrates bound to four aspartic proteinases of known structure have been suggested (Andreeva et al., 1981; Blundell et al., 1980; Bott, et al., 1982; Foltmann, 1981; James et al., 1977, 1981; James, 1980). Unfortunately none of these proposals were based on highly refined crystal structures; three were deduced by fitting inhibitors or poor substrate models

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to relatively weak difference electron density maps. The structure of penicillopepsin has subsequently been refined at 1.8-Å resolution to an R factor of 0.136 for 21 962 reflections with $I \geq \sigma(I)$ (James & Sielecki, 1983). The structure of an inhibitor complex, the pepstatin analogue Iva-Val-Val-Sta-OEt[1] bound to penicillopepsin, has been determined and refined at 1.8-Å resolution (James et al., 1982). The very different chemical nature of the inhibitory group in pepstatin at the analogous position to the scissile peptide of a good substrate prevents us from directly extending those results to a productive binding mode. In this paper, we use the new structural results to critically reinterpret our previous proposals on productive substrate binding and to identify those residues involved in the ion pair interaction for a P_1 lysyl residue.

Experimental Procedures

Materials

Penicillopepsin was prepared as described by Hofmann (1976) and was of the highest specific activity obtained so far. *Rhizopus* pepsin was from Miles Laboratories, Elkhart, IN; Leu-Ser-Nph-Nle-Ala-Leu-OMe was from Vega Biochemicals, Tucson, AZ; Z-His-Phe-Trp-OEt was from Bachem Inc., Torrance, CA. Ac-Ala-Ala-Lys-Nph-Ala-Ala amide was prepared as described previously (Hofmann & Hodges, 1982).

Methods

Peptide Synthesis. All amino acids used for the synthesis were in the L configuration. The following peptides were synthesized by using the general procedures for solid-phase peptide synthesis (Erickson & Merrifield, 1976) on a Beckman peptide synthesizer, Model 990: Ac-Ala-Ala-Lys amide and NpH-Ala-Ala amide. 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. The synthesis of Boc-Nph has been described previously (Hofmann & Hodges, 1982).

N-Acetyl-Ala-Ala-Lys Amide. The COOH-terminal amino acid, Boc-Lys(Z) (1.5 mmol/g of resin), and DCC (1.5 mmol/g of resin) were added to the copoly(styrene–2% divinylbenzene)benzhydrylamine-resin (0.49 mmol of NH_2/g of resin; Protein Research Foundation, Japan). The coupling (90 min) resulted in a substitution of 0.49 mmol of Boc-Lys(Z)/g of amino acid resin as determined by the picrate monitoring method (Hodges & Merrifield, 1975).

A 90-min treatment with acetic anhydride/benzene/pyridine (1:3:3, 25 mL/g of amino acid resin) was carried out to block any free amino groups on the resin.

The Boc groups were removed at each cycle of the synthesis by two deprotection steps with 50% TFA/CH_2Cl_2 (v/v) for 20 min in the first deprotection and 5 min in the second. Neutralizations were carried out with 5% diisopropylethylamine/CH_2Cl_2 (v/v). Double couplings of Boc-amino acids (3 equiv) in CH_2Cl_2 (5 mL/g of resin) and DCC (3 equiv) in CH_2Cl_2 (2.5 mL/g of resin) were carried out at each subsequent step of the synthesis. The reaction time for the coupling was 90 min. The program used for the attachment of each amino acid has been described (Hodges et al., 1981). The NH_2 group of the completed peptide was acetylated as above. The effectiveness of the acetylation was monitored by the picrate method (Hodges & Merrifield, 1975).

Nph-Ala-Ala Amide. The synthesis was carried out in a similar manner to that described above. The cleavage of the peptides from the resins and removal of blocking groups were carried out in hydrofluoric acid as described previously (Hodges & Hofmann, 1982).

Fo-Nph-Ala-Ala Amide. This peptide was prepared from H-Nph-Ala-Ala amide by formylation with acetic anhydride in 98% formic acid according to the procedure of Sheehan & Yang (1958). Acetic anhydride (47 μL) was added slowly to H-Nph-Ala-Ala amide (20 mg, 57 μmol) in 0.2 mL of 98% formic acid cooled in an ice bath. The reaction mixture was allowed to warm to room temperature. After 3 h, water (50 μL) was added and the solution dried in vacuo over NaOH pellets. The product did not give a ninhydrin reaction and was used without further purification.

Ac-Ala-Ala-Lys-OH. This peptide was isolated from accumulated reaction mixtures of assays of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide with penicillopepsin. High-voltage electrophoresis showed that all the substrate had been hydrolyzed during storage. The solutions were freeze-dried and the residues dissolved in a minimum volume of 0.1 M NH_4HCO_3. The peptides were separated from each other, from traces of penicillopepsin, and from buffer salts on a column (1 × 60 cm) of Sephadex G-25 (superfine) in 0.1 M NH_4HCO_3. The column eluate was collected in fractions of 1 mL and monitored at 220 and 280 nm. Ac-Ala-Ala-Lys-OH eluted at 19 mL. The peptide was identified by amino acid analysis and was shown to be homogeneous by high-voltage electrophoresis at pH 3.6.

High-Voltage Electrophoresis. Peptides were separated on Whatman 3MM paper by high-voltage electrophoresis in volatile buffers of the following compositions: pH 2.1, acetic acid/formic acid/water, 15:1:250 v/v; pH 3.6, acetic acid/pyridine/water, 200:20:1780 v/v; pH 4.7, acetic acid/pyridine/water, 5:5:190 v/v, 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 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, and 4.7.

Amino Acid Analysis. Peptides were hydrolyzed 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 Beckman–Spinco analyzer, Model 120C, fitted with high sensitivity colorimeter cells (18-mm light path; Evans Electroselenium Ltd., Halstead, Essex, U.K.).

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

Enzyme Assays. Enzyme assays were performed and the kinetic constants k_{cat} and K_m determined as described previously (Hofmann & Hodges, 1982). The assays with Leu-Ser-Nph-Nle-Glu-Leu-OMe were carried out spectrophotometrically at 310 nm (Inouye & Fruton, 1967). All assays were at 25 ± 0.5 °C. The following buffers were used; pH 2.0 and 2.5, 20 mM formic acid/HCl; pH 3.0–4.5, 20 mM sodium formate; pH 4.25–5.5, 20 mM sodium acetate; pH

[1] Abbreviations: Ac, acetyl; Fo, formyl; Nle, norleucyl; Nph, *p*-nitrophenylalanyl; OEt, ethyl ester; OMe, methyl ester; Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; DCC, *N,N*-dicyclohexylcarbodiimide; Iva, isovaleryl; Sta, statine residue [(4*S*,3*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]; I, intensity of Bragg reflection from hkl planes; R, the crystallographic agreement factor defined as $\sum_{hkl}||F_o| - |F_c||/\sum_{hkl}|F_o|$ where $|F_o|$ and $|F_c|$ are the structure factor amplitudes measured and calculated, respectively; TFA, trifluoroacetic acid.

Table I: Kinetic Parameters for the Hydrolysis of Three Peptides by Penicillopepsin[a]

substrate	pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Ac-Ala-Ala-Lys-Nph-Ala-Ala amide	5.5	0.078 ± 0.012 (18)	36 ± 2.8	460
Ac-Ala-Ala-Lys-Nph-Ala-Ala amide	3.5	0.440 ± 0.100 (15)	28.8 ± 4	65
Leu-Ser-Nph-Nle-Ala-Leu-OMe	5.5	0.004	11.4 ± 2	2850
Leu-Ser-Nph-Nle-Ala-Leu-OMe	3.5	0.01	6.4	1000
Ac-Lys-Nph amide	5.5	0.220 ± 0.01 (3)	0.010 ± 0.0003	0.047
Ac-Lys-Nph amide	3.5	≥6	≥0.026	0.0044

[a] Assays were performed at 25 °C as described under Methods in 20 mM sodium acetate buffer (pH 5.5) or 20 mM sodium formate buffer (pH 3.5), both adjusted to I = 20 mM.

5.5–7.0, 10 mM sodium phosphate. All buffers were adjusted to an ionic strength of 20 mM with NaCl. Enzymic rates measured at pH 5.5 and higher were corrected for the partial deprotonation of the α-amino group of the product Nph-Ala-Ala amide whose pK_a = 6.65 at 25 °C (Hofmann & Hodges, 1982).

Model Building. All of the conformational studies for possible substrate binding at the active site of penicillopepsin were done with a molecular model of a tetrapeptide Ac-Ala-Lys-Tyr-Ala-NH_2. This model was constructed from a dictionary of standard amino acid stereochemistry (Sielecki et al., 1979). A tyrosyl residue replaces the *p*-nitrophenylalanyl residue in the P_1' position of the substrate studied kinetically. The model of the tetrapeptide and that of the penicillopepsin molecule were displayed on an MMS-X interactive graphics system (Barry et al., 1976). The tetrapeptide was animated and manipulated with the operating system M3, devised and implemented on the MMS-X graphics by Colin Broughton (Sielecki et al., 1982).

Global rotations and translations of the whole tetrapeptide were used to guide it into the penicillopepsin binding cleft. The experimentally observed orientation of the bound inhibitor, Iva-Val-Val-Sta-OEt assisted in positioning the Ac-Ala and part of the lysyl residue relative to penicillinopepsin. Only rotatable bonds, ϕ and ψ for the main chain of the tetrapeptide, and the side-chain χ^1 angles for the lysyl and tyrosyl residues were modified. The conformations of the residues lining the binding cleft of penicillopepsin were not altered from their observed position in the crystal structure of the pepstatin analogue bound to penicillopepsin (James et al., 1982). Throughout all of the manipulations a continual check was made on nonbonded contact distances that were less than the sum of the corresponding van der Waals radii. This feature of the M3 program greatly assisted in eliminating the unallowed regions of conformational space.

Results and Discussion

pH Dependence of K_m for Penicillopepsin and Rhizopus Pepsin. The pH dependencies of K_m of penicillopepsin and *Rhizopus* pepsin for the hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide are shown in Figures 1 and 2, respectively. Both enzymes show a large increase of K_m with decreasing pH. The curve for *Rhizopus* pepsin is sigmoidal and obeys the Henderson–Hasselbalch equation for an ionizing group with pK = 3.8. This suggests that substrate binding depends on a single carboxyl group required for interaction with the lysine residue in position P_1 of the peptide. [Evidence for the assumption that K_m is proportional to the true substrate binding constant for pepsin has been discussed by Clement (1973) and Fruton (1980). We suggest that this assumption also holds for penicillopepsin.] K_m for penicillopepsin shows an even more marked dependence on pH which below pH 3.0 rises to values that for technical reasons could not be determined accurately. The steepness of the curve suggests that the pH dependence

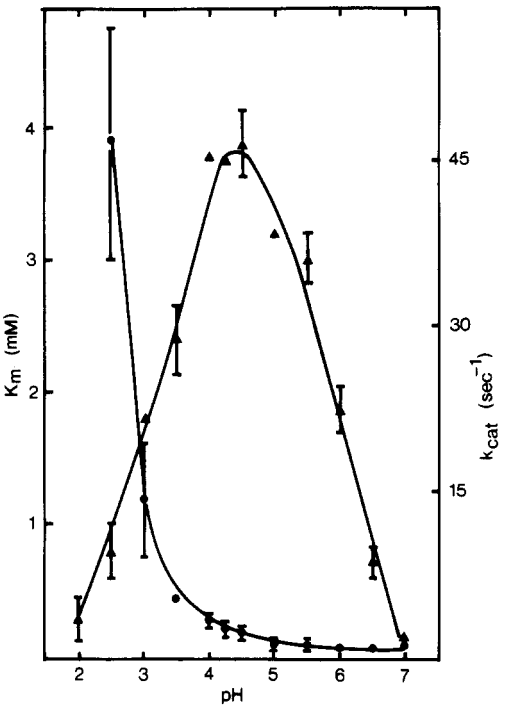

FIGURE 1: pH dependence of k_{cat} and K_m for the hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide by penicillopepsin. Assays were performed in formate–HCl (pH 2–4.5), acetate (pH 4.5–5.5), and phosphate buffers (pH 6.0–7.0), I = 20 mM, as described under Methods. The final enzyme concentration was 20 nM (0.67 μg/mL). The solid lines have no theoretical significance (●), K_m; (▲), k_{cat}. The error bars represent standard deviations of the values obtained from four to eight independent experiments; for clarity some error bars have been omitted. k_{cat} values at pH >5 have been determined from observed rates that were corrected for the incomplete protonation of the α-amino group of Nph-Ala-Ala amide. The K_m value at pH 2.0 is approximately 8 mM and has been omitted from the graph.

Table II: Effect of pH on the Inhibition of Penicillopepsin by Substrate-Related Peptides[a]

inhibitor	K_I (μM) pH 5.5	K_I (μM) pH 3.5
Ac-Ala-Ala-Lys	>10000	ND[b]
Ac-Ala-Ala-Lys amide	~3000	>10000
Nph-Ala-Ala amide	690 ± 70	~2000
Fo-Nph-Ala-Ala amide	1290 ± 160	1420 ± 180
Z-His-Phe-Trp-OEt	2	4

[a] Enzyme assays were performed at 25 °C in 20 mM sodium acetate buffer (pH 5.5) or in 20 mM sodium formate buffer (pH 3.5) adjusted to I = 20 mM with NaCl. Inhibition constants were determined by the method of Dixon (1953). Inhibition was competitive in all cases. [b] Not determined owing to insufficient material.

is not due to the ionization of a single carboxyl group but represents a more complex phenomenon. A strong pH dependence is also found for the K_m of the substrate Ac-Lys-Nph amide (Table I). Further support for the involvement of a

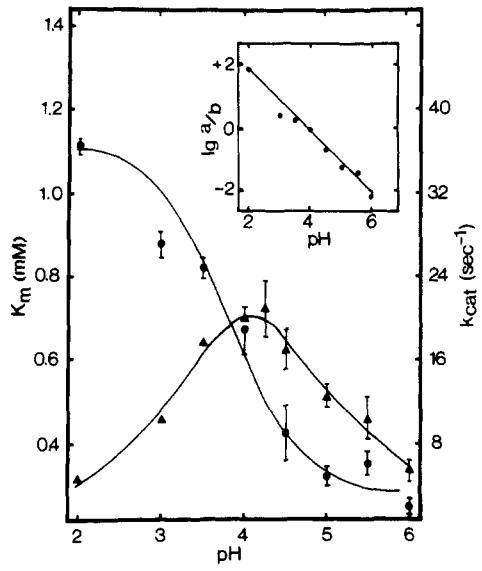

FIGURE 2: pH dependence of k_{cat} and K_m for the hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide by *Rhizopus* pepsin. Assays were under the same conditions as given in Figure 1. The final enzyme concentration was 80 nM. (●) K_m; (▲) k_{cat}. Error bars have the same significance as those in Figure 1. The solid line for the K_m values is a calculated dissociation curve for a carboxyl group with pK_a = 3.8, obtained from the Hill plot shown in the insert. The Hill plot was obtained by assuming limiting values for K_m of 1.12 mM (low pH) and 0.28 mM (high pH). The solid line of the Hill plot has a slope = −1. a/b is the ratio of the protonated and deprotonated forms, respectively, of a proposed carboxyl group on the enzyme.

carboxyl group in the binding of the lysyl side chain comes from the study of the inhibition constants of a series of substrate peptides. This is shown in Table II. Whereas the N-terminal product of hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide, Ac-Ala-Ala-Lys, is a poor inhibitor, probably due to charge repulsion between the carboxyl groups on the peptide and those at the catalytic site, its amide derivative is a good inhibitor and shows a strong pH dependence. The formyl derivative of the C-terminal product, Fo-Nph-Ala-Ala amide on the other hand shows no change in K_I when the pH is lowered. (Nph-Ala-Ala amide, the C-terminal product, does show a pH dependence, owing to the interaction of its ammonium group with the catalytic aspartic acid residues.) The results with these peptides confirm that the pH dependence of substrate binding is due to the lysyl side chain.

Identification of the Probable Cationic Binding Site S_1 in Penicillopepsin. The model-building study that we have done has been directed at two questions. Which carboxyl group(s) form the site of ion pair interaction for a positively charged P_1 lysyl side chain in the fungal aspartic proteinases? Is it possible to deduce the productive binding mode of a good substrate on the basis of the refined atomic coordinates of penicillopepsin and the observed binding mode of an inhibitor molecule, Iva-Val-Val-Sta-OEt? We will address the first question separately, in spite of the fact that the substrate modeling was done with both of these problems in mind.

It is evident from the above kinetic data on the synthetic substrates that the S_1 binding site in penicillopepsin and *Rhizopus* pepsin should have a negatively charged carboxyl group for the P_1 lysine specificity. It has been previously suggested that Asp-38(37)[2] could be the residue providing this negative charge (James, 1980; James et al., 1981). It was proposed that a prior or concomitant conformational rotation about C^{α}–C^{β} of Asp-38(37) was required to form this ion pair interaction. This postulate is not longer tenable if the large conformational change in the flap region of penicillopepsin [Trp-71(71)–Gly-83(83)] that occurs as a result of inhibitor binding (James et al., 1982) would also occur upon substrate binding. The positions of the side chains of Ile-73(73) and Tyr-75(75) in this complexed form of the enzyme preclude any conformational change of the side chain of Asp-38(37) and at the same time prohibit a P_1 lysyl side chain from approaching Asp-38(37).

The side-chain orientation of the Sta residue has been used, in part, to guide the position of the lysyl side chain in the model substrate. First, the Ac-Ala group was fitted to the inhibitor position so that the mean deviation of the 12 common atoms was 0.27 Å. This ensured that no close contacts to penicillopepsin were encountered. The resulting position of the lysyl side chain left Glu-16(13) and Asp-115(114) as the only possible candidates for the negatively charged groups that could form an ion pair interaction. The probable P_1 binding orientation is shown in Figure 3. The N^{η} atom of the P_1 residue is not close enough for direct hydrogen bonding to the O^{δ} atom of Asp-115(114); therefore, we have depicted the interaction as mediated by a hydrogen-bonded water bridge. Alternatively, a small conformational change of Glu-16(13) or Asp-115(114) could facilitate a direct interaction. Although there is no experimental evidence for large conformational changes involving Glu-16(13) and Asp-115(114), these two residues form part of two conformationally mobile stretches of polypeptide chain in the native enzyme (James & Sielecki, 1983).

For the *Rhizopus* enzyme, the evidence of a Hill coefficient of 1 (insert to Figure 2) is consistent with protonation of a single carboxylate group that would disrupt the anionic site for the P_1 lysyl side chain. In contrast for penicillopepsin, the steepness of the curve showing the increase in K_m with decreasing pH (Figure 1) suggests the involvement of additional carboxyl groups than the suggested carboxyl–carboxylate pair, Glu-16(13) and Asp-115(114). [For a discussion of this interaction see Sawyer & James (1982).] Indeed, other carboxyl groups are involved with binding the hexapeptide substrate, Asp-77(77) and possibly Asp-119(118). The latter group is partially buried and hydrogen bonded to His-54(53). Protonation of Asp-119(118) could bring additional conformational changes that would disturb the S_1–P_1 interaction.

The hydrophobic character of the S_1 site in the aspartic proteinases is evident from kinetic experiments. As Table I shows, penicillopepsin readily cleaves Leu-Ser-Nph-Nle-Ala-Leu-OMe, a peptide that is a good substrate for other aspartic proteinases (Martin et al., 1980). Particularly noticeable is the low K_m value. Residues on penicillopepsin contributing to these hydrophobic interactions are tyrosine-75(75), phenylalanine-112(111), and leucine-121(120). These residues are highlighted in Figure 3 and most likely also contribute to the binding affinity of the alkyl portion of the lysyl side chain.

Hydrophobic interactions in position S_1' also contribute to substrate binding, as the K_I values for Nph-Ala-Ala amide and its *N*-formyl derivative show (Table II). Residues involved in the S_1' binding have been proposed previously (James, 1980; James et al., 1981). These strong hydrophobic interactions in both S_1 and S_1' are also responsible for the low inhibition constant of Z-His-Phe-Trp-OEt (Table II), a peptide that is a good substrate for pig pepsin (Hollands et al., 1969) but that is not cleaved by penicillopepsin (Mains et al., 1971).

Comparison with Other Aspartic Proteinases. The model-building experiments described above suggest that the most

[2] Residues of penicillopepsin are given their sequence numbers; the numbers in parentheses are those of the equivalent residues in pepsin.

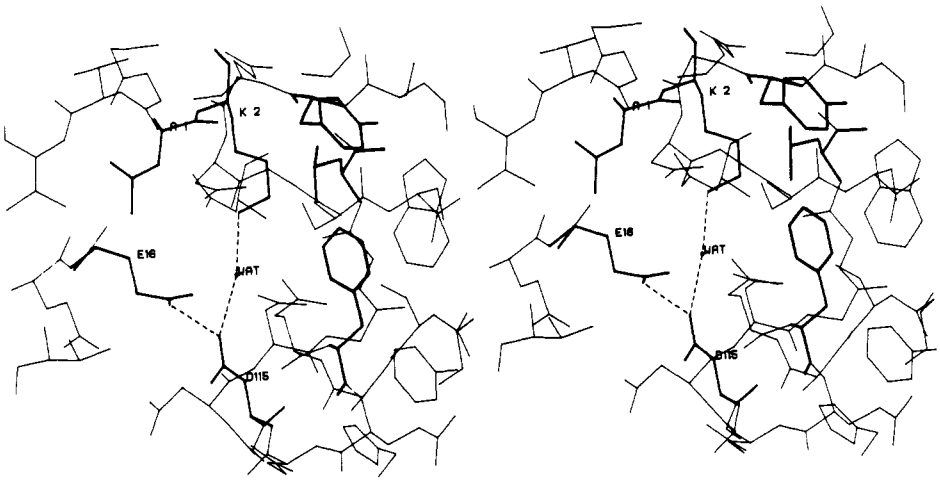

FIGURE 3: Stereoview of the environment of the P_1 lysyl side chain in the model of Ac-Ala-Lys-Tyr-Ala amide bound to penicillopepsin. The positively charged N^{η} atom is in the vicinity of the carboxyl–carboxylate pair glutamic acid-16 and aspartic acid-115 and is shown here with a possible bridging water molecule. The Ac-Ala-Lys portion of the substrate and the residues on penicillopepsin that are involved with binding of the lysyl group [tyrosine-75(75), phenylalanine-112(111), and leucine-121(120)] are drawn in thicker lines.

Table III: Comparison of Amino Acids of Aspartic Proteinases in Positions That Are Homologous to Residues around Glu-16(13) and to Asp-115(114) in Penicillopepsin

enzyme	position no.[a] 14[b] (11)	15[b] (12)	16[b] (13)	17[b] (14)	115 (114)	reference
Enzymes with Specificity for Lysine in Position P_1						
penicillopepsin	Asp	Glu	Glu	Try	Asp	Cunningham et al. (1976)
Rhizopus pepsin	Asp	Ile/Val	Gly	Tyr	?	Gripon et al. (1977)
Endothia pepsin	Asp	Asp	Ala	Tyr	Asp	V. B. Pedersen (personal communication)
aspergillopepsin	Asp	Glu	Glu	Tyr	?	V. M. Stepanov (personal communication)
Penicillium roqueforti Asp proteinase	Asp	Val	Glu	Tyr	?	Gripon et al. (1977)
Enzymes Lacking Lysine Specificity						
Mucor miehei Asp proteinase	Asp/Leu	Glu	Glu	Tyr	Ile	Bech & Foltmann (1981)
pig pepsin	Asp	Thr	Glu	Tyr	Tyr	Tang et al. (1973)
cow pepsin	Asp	Thr	Glu	Tyr	?	Harboe & Foltmann (1975)
human pepsin	Asp	Met	Glu	Tyr	Tyr	Sepulveda et al. (1975)
chicken pepsin	Asp	Ala	Ser	Tyr	Tyr	V. Kostka (personal communication)
calf chymosin	Asp	Ser	Gln	Tyr	Tyr	Foltmann et al. (1979)
mouse submaxillary gland renin	Asn	Ser	Gln	Tyr	Leu	Misono et al. (1982)

[a] Penicillopepsin numbering; pig pepsin numbering in parentheses. [b] The alignment of the aspartic proteinases by homology has some ambiguities in the first 27 residues.

probable anionic site for the ϵ-ammonium group of lysine is the carboxyl–carboxylate pair of glutamic acid-16(13)–aspartic acid-115(114). Because of the extensive homology among the aspartic proteinases, it is to be expected that all those enzymes that have been shown to cleave on the C-terminal side of lysine residues would have a similar anionic site whereas those without this specificity would not. Table III gives a list of those aspartic proteinases for which sequence information is available. Therein, we compare the residues in positions 14–17 (11–14) and in 115 (114) for the enzymes with the ability to activate trypsinogen and cleave the lysine peptide (Morihara & Oka, 1973; Hofmann & Hodges, 1982). The N-terminal sequences of the enzymes listed in Table III have been aligned on aspartic acid-14(11) because this residue has been shown to be involved in an interaction with a conserved basic residue in position 304 (308) which is a lysine in penicillopepsin (Hsu et al., 1977) and on tyrosine-17(14) because this also appears to be a highly conserved residue. We assume that sequentially homologous amino acids occupy analogous positions in the tertiary structure. On this basis it appears that glutamic acid-16(13) cannot be a determinant for the lysine specificity since it is present in some enzymes in both groups. However, aspartic acid-115(114) is unique to the two lysine-specific enzymes for which information is available. Unfortunately no information is available for position 115 in *Rhizopus* pepsin. If we assume that this enzyme has an aspartic acid in that position, then we have a basis for the difference in the pH dependences of K_m for penicillopepsin and *Rhizopus* pepsin. In the latter the lysine side chain would interact only with aspartic acid-115(114) because there are no acidic amino acids in positions 15 and 16. The pH dependence of K_m shows therefore a simple titration curve. Furthermore, it is likely that because of sequence differences between the two enzymes, the structures are not identical and the distance between the ammonium group of P_1 lysine and the carboxylate could be less in *Rhizopus* pepsin and make a closer specific one-to-one interaction possible. In penicillopepsin, on the other hand, the model building suggests a more distant interaction, possibly mediated via a water molecule. The interaction is, however, with a carboxyl–carboxylate pair. One of the two carboxyl groups will have a considerably lower pK than the other. In addition there may be small pH-dependent conformational changes that affect the K_m values, as the small difference between K_m at pH 5.5 and K_m at pH 3.5 for Leu-Ser-Nph-Nle-Ala-Leu-OMe indicates (Table I). A conformational effect is also suggested by the small difference in K_I for Z-His-Phe-Trp-OEt which is higher at pH 3.5 than at pH 5.5 (Table II). These factors are presumably responsible for the

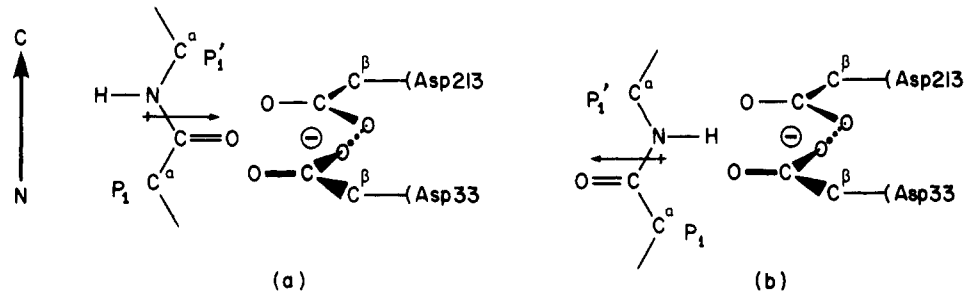

FIGURE 4: (a) Diagrammatic sketch of the approach of the substrate scissile bond to the catalytic aspartyl residues Asp-33(32) and Asp-213(215). The dipole moment of the peptide bond is indicated by a small arrow, with the arrow tip corresponding to the negative end of the dipole. The direction of the substrate polypeptide is also shown. (b) As in (a) but with the peptide dipole of the scissile bond approaching the two catalytic aspartyl residues from an alternative direction. The positive end of the peptide dipole is directed in toward Asp-33(32) and Asp-213(215). These two possible binding modes are detailed in stereo in Figure 5.

more complex pH dependence of K_m.

So far all aspartic proteinases of fungal origin show lysine specificity, with the exception of the *Mucor miehei* enzyme. This enzyme has an extra residue between the conserved aspartic acid-14(11) and tyrosine-17(14) (Table III). This implies that the structure in this region will be different from that of the other enzymes. This difference and the lack of an aspartic acid at position 115 can readily explain the lack of lysine specificity. None of the vertebrate enzymes have been shown to have lysine specificity.

The lysine specificity of aspartic proteinases differs profoundly from that of the trypsin family of enzymes where the aspartic acid residue that determines the lysine and arginine specificity (aspartic acid-189) is located in a well-developed deep pocket and provides an absolute specificity for P_1.

Productive Binding of the Scissile Bond in Ac-Ala-Lys-Tyr-Ala Amide. On the basis of the results shown in Figure 3 for the binding of the Ac-Ala-Lys portion of a tetrapeptide substrate, it is possible to extend the model-building study to the P_1' and P_2' sites. In attempting to fit the scissile bond relative to the active site aspartyl residues, Asp-33(32) and Asp-213(215), one is immediately faced with the dilemma of whether or not to mimic the direction of approach observed for the pepstatin analogue to penicillopepsin. Due to the special chemical nature of this inhibitor and its nonproductive mode of binding, it could be argued that its orientation is not necessarily the one adopted by a good substrate. This argument is enhanced by the observation that when the scissile bond is positioned as in the pepstatin analogue complex, i.e., with the carbonyl oxygen atom directed toward Asp-33(32) and Asp-213(215), the negative end of the peptide dipole (Hol et al., 1978) is directed toward these two carboxyl groups (Figure 4a). This is electrostatically unfavorable as there is, presumably, a net negative charge at the active site [only one proton shared by the two carboxyl groups; see James & Sielecki (1983)]. The energetically favorable approach would have the positive end of the peptide dipole directed in toward the carboxyl side chains (Figure 4b). These two possible modes of binding have been examined in detail, and the results are displayed in Figure 5.

The model of Ac-Ala-Lys-Tyr-Ala-NH_2 depicted in Figure 5a represents our interpretation of the productive binding mode in spite of the fact that, at first sight, it is electrostatically the less favorable peptide bond orientation. This electrostatic component will of course be offset by the many favorable peptide–enzyme interactions. Indeed, the intricate hydrogen-bonding network that surrounds the two carboxyl groups of Asp-33(32) and Asp-213(215) could diminish and dissipate the net negative charge tentatively allocated to this site (James & Sielecki, 1983). Therefore, the carbonyl oxygen atom of the scissile bond (Lys-2 in Figure 5a) points in toward the two carboxyl groups of Asp-33(32) and Asp-213(215). Its position is within 0.6 Å of the experimentally observed location of the OH group on the 3-carbon atom in the pepstatin analogue. The carbonyl oxygen atom of Lys-2 is also close to the site of a bound solvent molecule in native penicillopepsin (James & Sielecki, 1983).

Table IV: Hydrogen-Bonding Interactions between Penicillopepsin and the Proposed Productive Binding Mode for Ac-Ala-Lys-Tyr-Ala-NH_2

enzyme	substrate	distance (Å)
Thr-217(219) NH · · ·	O=C of *N*-acetyl	3.1
Asp-77(77) COO^- · · ·	HN of P_2 Ala	2.6
Asp-77(77) NH · · ·	O=C of P_2 Ala	3.2
Gly-215(217)C=O · · ·	HN of P_1 Lys	3.1
Gly-76(76) NH · · ·	O=C of P_1' Tyr[a]	3.1
Gly-35(34) C=O · · ·	HN of P_2' Ala[a]	3.3

[a] These two hydrogen-bonded interactions are postulated on the basis of model building. The first four are analogous to those observed in the Iva-Val-Val-Sta-OEt binding.

Hydrogen-bonding interactions that stabilize the binding between substrate and enzyme are listed in Table IV. The first four entries in this table correspond to those hydrogen bonds also observed in the binding of Iva-Val-Val-Sta-OEt to penicillopepsin (James et al., 1982). The two hydrogen-bonded interactions proposed for the amino portion of the substrate are from the main-chain NH of Gly-76(76) on the "flap" region to the C=O of P_1' tyrosine and from the NH of the P_2' residue (Ala-4 in the present case) to the carbonyl oxygen atom of Gly-35(34). The approximate 2-fold symmetric relationship of the carbonyl oxygen atoms of Gly-35(34) and Gly-215(217) is strengthened by their hydrogen-bond acceptance potential in fixing the orientation of the scissile bond.

The nonbonded interactions between enzyme and this proposed productive binding mode can be appreciated in Figure 5a. We have discussed the hydrophobic contacts important for binding P_1 residues to penicillopepsin (see also Figure 3). The hydrophobic residues with which the P_1' tyrosyl side chain (or another hydrophobic group at P_1') interacts are Phe-190(189), Ile-211(213), Ile-293(297), Phe-295(299), and Ile-297(301). The strand of polypeptide chain containing these latter three residues (293–297) partially surrounds the P_1' side chain of the substrate (Figure 5a) and forms a hydrophobic wall in close proximity to the active site Asp-213(215).

Figure 5b shows the result of reversing the scissile peptide bond between P_1 Lys and P_1' Tyr (to correspond to the case of Figure 4b) and of readjusting the model to best preserve the interactions deemed important to substrate binding as described above. The six hydrogen bonds listed in Table IV and the hydrophobic interactions of S_1 and S_1' have all been

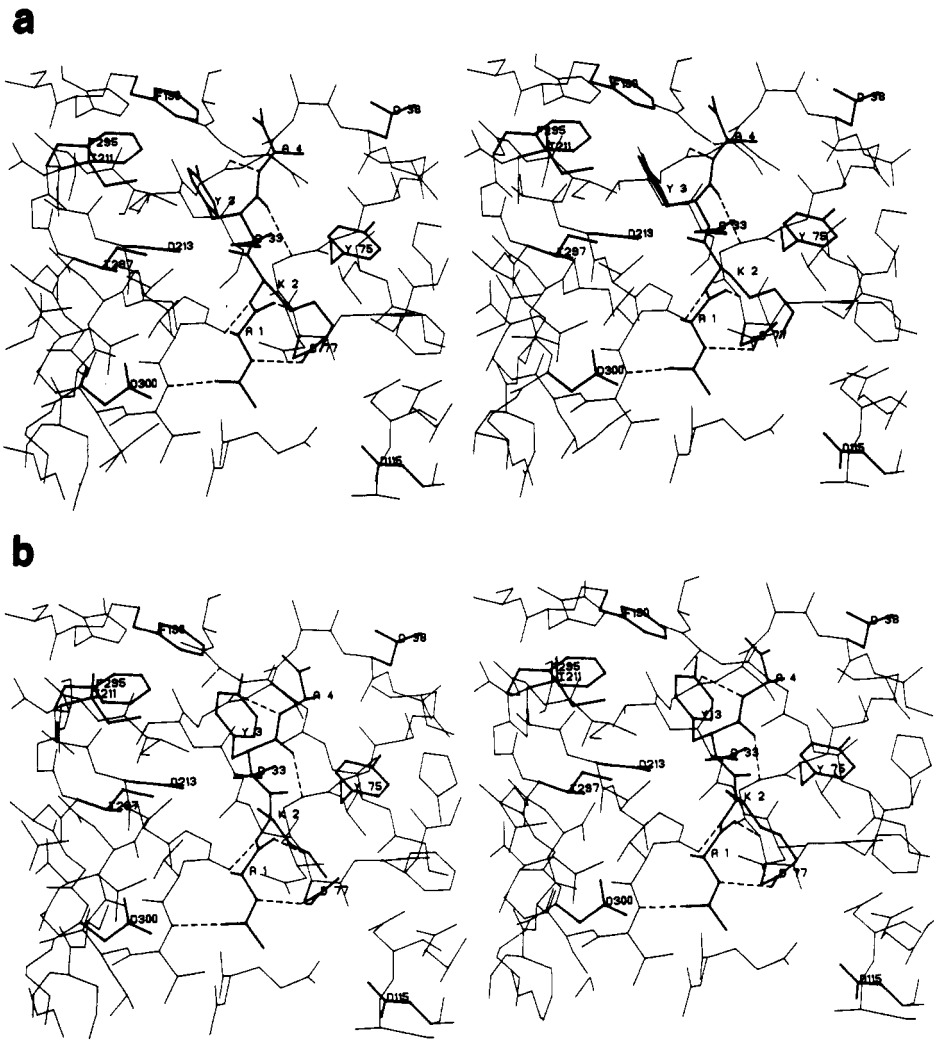

FIGURE 5: (a) Stereoview of the active site region of penicillopepsin with the substrate Ac-Ala-Lys-Tyr-Ala amide in the proposed productive binding mode. The residues of the S_1' binding site phenylalanine-190(189), isoleucine-211(213), phenylalanine-295(299), and isoleucine-297(301) are highlighted. The side chain of isoleucine-293(297) is also close. Also drawn with thick lines are the substrate residues and aspartic acid-33(32), aspartic acid-38(37), tyrosine-75(75), aspartic acid-77(77), aspartic acid-115(114), aspartic acid-213(215), and aspartic acid-300(304) of the enzyme. The side chain of aspartic acid-38(37) is shown in its native position; the flap is in the conformation observed with the bound inhibitor Iva-Val-Val-Sta-OEt. Hydrogen bonds from the tentative binding position of the substrate to penicillopepsin are shown with dashed lines. The substrate binding mode depicted here corresponds to the model of Figure 4a with the negative end of the peptide dipole of the scissile bond directed in toward the catalytic carboxyl groups. (b) Similar view of the penicillopepsin binding site with an alternative binding mode of the substrate Ac-Ala-Lys-Tyr-Ala amide shown. The conformation adopted by the polypeptide chain of the substrate results in too close contacts of the three adjacent carbonyl oxygen atoms from the P_2, P_1, and P_1' residues. In addition, an impossibly close contact results from atoms C^β and C^γ of the P_1 lysyl side chain with the carboxyl group of Asp-33(32). In spite of the favorable hydrogen-bonding interactions depicted and the favorable direction of the peptide dipole, the unfavorable nonbonded contacts rule out this binding mode as a productive one for the aspartic proteinases.

preserved. Nevertheless, as a result of reversing the bond dipole there are three serious steric contacts that preclude this conformation as an alternative for the productive binding mode of the scissile peptide.

The three contiguous carbonyl oxygen atoms of residues P_2, P_1, and P_1' are all in much too close contact (<2.04 Å) when the peptide backbone has the conformation shown in Figure 5b. This conformation has ϕ and ψ equal to −44° and −97° for P_1 lysine and 42° and 119° for P_1' tyrosine which are both in energetically forbidden regions on a Ramachandran diagram. In addition to these sterically impossible contacts of the substrate carbonyl oxygen atoms, the side-chain atoms C^β and C^γ of P_1 lysine make too close contacts with the carboxyl group of Asp-33(32). Therefore, it is impossible to maintain the binding interactions for P_1 and P_1' groups, with the attendant hydrogen-bonding interactions from P_3 to P_1, and have simultaneously the scissile peptide approach the active site with the positive end of its dipole moment directed toward the net negative charge on Asp-33(32) and Asp-213(215).

The main chain of the substrate in the proposed productive binding mode as shown in Figure 5a adopts an extended conformation in accord with proposals by Fruton (1976) and Hofmann (1974). The hydrogen-bonding interactions for substrate main-chain atoms from P_2 to P_2' are consistent with those observed with a bound inhibitor and penicillopepsin (James et al., 1982).

pH Dependence of k_{cat}/K_m for Penicillopepsin and Rhizopus Pepsin. The pH dependences of k_{cat} for the hydrolysis of Ac-Ala-Ala-Lys-Nph-Ala-Ala amide by penicillopepsin and *Rhizopus* pepsin are shown in Figures 1 and 2, respectively. They show maximal values at pH 4.5 and 4.2, respectively. The pH dependences of the specificity constants k_{cat}/K_m for two substrates—a dipeptide and a hexapeptide—with penicillopepsin and for the hexapeptide only with *Rhizopus* pepsin are shown in Figure 6. The maxima range from pH 4.5 for the latter to about 6 for the hydrolysis of the dipeptide by penicillopepsin. No attempt has been made to extract pK_a values for ionizing groups in the catalytic site from the curves.

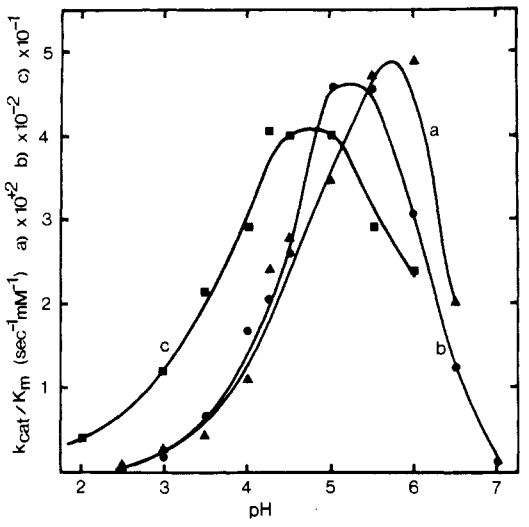

FIGURE 6: pH dependence of k_{cat}/K_m for penicillopepsin and *Rhizopus* pepsin. The values of k_{cat}/K_m with the substrate Ac-Ala-Ala-Lys-Nph-Ala-Ala amide were obtained from the k_{cat} and K_m values given in Figure 1 [penicillopepsin (●)] and Figure 2 [*Rhizopus* pepsin (■)]. The values for k_{cat}/K_m for the substrate Ac-Lys-Nph amide with penicillopepsin (▲) were obtained from separately determined k_{cat} and K_m values for pH 4.5–6.5 and were determined directly for pH 2.0–4.25 (where the K_m values are too high to be measurable). Note that the scales of the ordinate for the three curves differ by several orders of magnitude, as indicated.

As Knowles (1976) has pointed out, the determination of intrinsic pK_a's from "pH–activity" curves is only valid under two limiting conditions, namely, "if there is only one state of ionization of the active site that is capable of catalyzing the reaction and if all prototropic equilibria involving the ionizing groups are fast with respect to all conversion steps in the reaction" [see also Peller & Alberty (1959)]. The implications of the recent binding studies of pepstatin (Rich & Sun, 1980) or other inhibitor peptides (Dunn et al., 1981) with pig pepsin and penicillopepsin (D. H. Rich and T. Hofmann, unpublished work) suggest that inhibitor as well as substrate binding involves conformational changes that are relatively slow and raise the possibility that pH-dependent conformational changes affect the pH–rate profile for k_{cat}. In any case, even in the absence of ionic side-chain interactions of substrate with enzyme, it does not seem possible to ascribe meaningful pK_a values to the catalytic groups of pepsin and presumably related enzymes. Experimentally determined values for the pK_a's of the aspartyl groups of the free enzyme (as opposed to the ES complex) range from 0.7 to 3.7 for pK_{a1} and from 2.7 to >5 for pK_{a2} (Clement, 1973). This clearly shows that the assumption that the pH dependence of k_{cat}/K_m yields the pK_a's of the free enzyme (Dixon & Webb, 1964) is not valid for pepsin and, because of the similarity in structure and enzymic properties, also not for other aspartic proteinases. We feel, therefore, that we cannot meaningfully ascribe pK_a values to the catalytic groups of these enzymes.

pH Dependence of Aspartic Proteinases. Aspartic proteinases, whose catalytic residues are two aspartic acids, are unique in that they function physiologically over a wide pH range, from pepsin at pH around 2.0 to renin, which is required to cleave angiotensinogen at pH 7.4. This appears to put unusual requirements on the catalytic apparatus. However, the physiological pH optima are misleading when the enzyme action is viewed in strictly kinetic and molecular terms. The low pH optima observed for pepsin [see review by Clement (1973)] are only seen when small peptides, which are very poor substrates, or when proteins are used. When good substrates, such as Z-His-Phe-Phe-OEt (Hollands & Fruton, 1968) or Z-Gly-Ala-Phe-Phe 3-(4-pyridyl)propyl ester (Sachdev & Fruton, 1970), are used, neither k_{cat} nor K_m is much affected by pH between 1 and 4.5, the range over which these substrates could be examined. On the other hand, renin, which acts physiologically on angiotensinogen at pH 7.4, shows pH optima from 3.5 to 6.5 when it hydrolyzes a specific octapeptide derived from angiotensinogen (Inagami et al., 1983). In this paper we show that the pH optima of k_{cat} with penicillopepsin and *Rhizopus* pepsin are between 4 and 4.5 and those for k_{cat}/K_m between 4.5 and 6. Thus, it appears that when aspartic proteinases act on good peptide substrates, their pH optima are in a much narrower range than those suggested by their physiological actions and suggests that the pH dependence of the catalytic apparatus of the different aspartic proteinases is quite similar.

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Registry No. Ac-Ala-Ala-Lys-Nph-Ala-Ala amide, 82867-31-0; Leu-Ser-Nph-Nle-Ala-Leu-OMe, 37589-79-0; Ac-Lys-Nph amide, 88412-23-1; Ac-Ala-Ala-Lys, 88412-25-3; Ac-Ala-Ala-Lys amide, 88412-24-2; Nph-Ala-Ala amide, 88412-26-4; Fo-Nph-Ala-Ala amide, 88412-27-5; Z-His-Phe-Trp-OEt, 10119-01-4; penicillopepsin, 9074-08-2; *Rhizopus* pepsin, 9074-09-3.

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