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Absence in Papaya Peptidase A Catalyzed Hydrolyses of a pK_a ~ 4 Present in Papain-Catalyzed Hydrolyses[†]

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ABSTRACT: The pH dependence below pH 6 of k_{cat}/K_m for the papaya peptidase A catalyzed and papain-catalyzed hydrolyses of the *p*-nitrophenyl esters of hippuric acid, benzyloxycarbonylglycine, and benzyloxycarbonyl-L-lysine has been determined and compared. In the case of papain, k_{cat}/K_m was observed to be modulated by two acidic ionizations, whereas for papaya peptidase A a single ionization controls the pH dependence of k_{cat}/K_m below pH 6. A comparison of the values of the pH-independent parameter k_{cat}'/K_m' for the two enzymes toward the three substrates indicates that papaya peptidase A performs catalytically as well as papain without reflecting the specificity requirements known to be essential for the catalytic efficiency of the latter enzyme.

For the hydrolysis of specific substrates catalyzed by papain (EC 3.4.22.2), the pH dependence of k_{cat}/K_m is generally observed to be bell shaped with pK_a values of about 4 and 8.5 (Glazer & Smith, 1971; Lowe, 1976). Originally, these pK_a values were ascribed to the ionizations of, respectively, a carboxylic acid group and the thiol group of the active-site cysteine (Smith & Parker, 1958). However, with the seeming acceptance of the thiolate–imidazolium ion pair formed between cysteine-25 and histidine-159 as the predominant and catalytic competent enzyme form in the pH range 4–8.5 [Drenth et al., 1975; Polgar, 1977; Brocklehurst et al. (1981) and references cited therein], a new view on the catalytic mechanism of papain and in particular on the assignment of the observed pK_a values has been established.

During the past decade evidence has accumulated for the influence of a second acidic ionizing group on the active-center characteristics and catalytic activity of papain (Brocklehurst & Little, 1970; Sluyterman & Wijdenes, 1973; Bendall & Lowe, 1976a,b; Lewis et al., 1978). Thus, the formerly controversial assignment of the pK_a of about 4 to either histidine-159 or aspartic acid-158 now seems replaced with the contention that two deprotonations resulting in the formation of the S^-–ImH^+ interactive system and the carboxylic anion of aspartic acid-159, respectively, are reflected in the acidic limb of the pH profile for papain-catalyzed hydrolyses.

In the initial investigations of papaya peptidase A, that enzyme was shown to catalyze the hydrolysis of casein and urea-denatured hemoglobin with pH dependences quite similar to those observed for papain (Schack, 1967), and the (k_{cat}/K_m)–pH profile for the papaya peptidase A catalyzed hydrolysis of *N*-benzoyl-L-arginine ethyl ester was shown to be bell shaped with apparent pK_a values of 4.3 and 8.5 (Robinson, 1975). However, recent studies of the pH dependence of the rate of irreversible inhibition by 2,2′-dipyridyl disulfide of the three monothiol proteases ficin (EC 3.4.22.3), actinidin, and papaya peptidase A have revealed that these enzymes appear to lack an ionizing group with the characteristics attributed to the aspartic acid-158 residue of papain (Malthouse & Brocklehurst, 1976; Brocklehurst et al., 1981; Baines & Brocklehurst, 1982). In order to establish whether this observation extends to the catalytic action of papaya peptidase A, we have carried out a comparative study of the acidic limbs of the (k_{cat}/K_m)–pH profiles for papain-catalyzed and papaya peptidase A catalyzed hydrolyses of specific substrates. The results presented in this work show that below pH 6 the pH dependence of k_{cat}/K_m is determined by two ionizations for papain-catalyzed hydrolyses but by only a single ionization for papaya peptidase A catalyzed hydrolyses.

Materials and Methods

Enzymes. Dried papaya latex (crude, type 1) was obtained from Sigma Chemical Co. Spray-dried papaya latex was generously supplied by Powell & Scholefield Ltd., Liverpool, U.K. From these materials, papain and papaya peptidase A were purified to the level of 1.0 mol of SH/mol of protein by procedures described elsewhere (Kaarsholm & Schack, 1983; P. Schack and N. C. Kaarsholm, unpublished results). The enzymes were stored at 4 °C as the mercury derivative until required.

Substrates. *N*-Benzyloxycarbonylglyine *p*-nitrophenyl ester (Z-Gly-ONp)[1] was obtained from Sigma Chemical Co., and *N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (Z-Lys-

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[1] Abbreviations: pHNP, hippuric acid *p*-nitrophenyl ester; Z-Gly-ONp, benzyloxycarbonylglycine *p*-nitrophenyl ester; Z-Lys-ONp, benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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ONp) was the product of Bachem, Bubendorf, Switzerland. The *p*-nitrophenyl ester of hippuric acid was synthesized by the method of Bodanszky & DuVigneaud (1962) for the preparation of Z-Gly-ONp. Three recrystallizations from hot ethanol [containing 1% (v/v) glacial acetic acid] yielded products with constant melting point: *p*-nitrophenyl hippurate (pHNP) mp 170–171 °C [lit. mp values 167–168 °C (McDonald & Balls, 1957) and 170–171 °C (Lowe & Williams, 1965; Hollaway, 1968)]. Upon treatment with strong base as well as upon treatment with papain or papaya peptidase A, the esters gave the theoretical amount of nitrophenol.

Kinetic Measurements. Prior to a kinetic experiment, a sample of mercury enzyme was activated by treatment with a 50-fold molar excess of DL-dithiothreitol (DTT) in 1 mM EDTA at pH 6. After 20 min, the enzyme was assayed toward Z-Gly-ONp as described by Asboth & Polgar (1977). The sample thus activated was used for kinetic experiments within the next 2.5 h. This is well within the range of time in which both enzymes were shown to maintain constant specific activity. All preparations used in the present work contained at least 0.9 mol of SH group/mol of protein.

All reactions were followed at 340 nm in 1-cm cuvettes at 25 °C in a Cary 16 spectrophotometer. To assure that the reported (k_{cat}/K_m)–pH profiles were not perturbed by the buffers used, 0.1 M acetate buffer containing NaCl to produce an ionic strength of 0.3 M was employed over the entire pH range of 3–5.5.

A typical kinetic experiment was performed as follows: The pH of the acetate buffer was measured with a PHM 84 pH meter of Radiometer, Copenhagen, and the spectrophotometer was balanced at 340 nm with 2500 μL of buffer in both cuvettes. A 100-μL aliquot of substrate in acetonitrile was added to the sample cuvette, and the base line was recorded for about 20 s. The reaction was started by the addition of 100 μL of enzyme solution to the sample cuvette, and the complete progress curve of the reaction was recorded. Subsequently, the pH of the sample cell content was checked. The initial concentration of reactants varied depending on the enzyme and the substrate. For papaya peptidase A, the following conditions were employed: Z-Gly-ONp, $[S]_0$ 50–100 μM, [E] (12–24) × 10^{-8} M; Z-Lys-ONp, $[S]_0$ 50–100 μM, [E] (5–10) × 10^{-8} M; pHNP, $[S]_0$ 25–60 μM, [E] (15–30) × 10^{-8} M. For papain, the reaction conditions were as follows: Z-Gly-ONp, $[S]_0$ 50–100 μM, [E] 20–60 × 10^{-8} M; Z-Lys-ONp, $[S]_0$ 50–100 μM, [E] (1–10) × 10^{-8} M; pHNP, $[S]_0$ 25–80 μM, [E] (40–80) × 10^{-8} M. For both enzymes and each substrate, the values of k_{cat}/K_m were independent of the concentration of enzyme in the above given ranges, indicating that the observed pH dependence of k_{cat}/K_m was not influenced by possible self-association of the enzymes.

Since both enzymes are known to be irreversible denatured at low pH values (Baines & Brocklehurst, 1982), special precautions were taken to ensure that (k_{cat}/K_m)–pH profiles did not reflect irreversible denaturation. Both enzymes were found to be stable under all assay conditions for the times used in the assays. For each (k_{cat}/K_m)–pH profile, control experiments were run, wherein the enzyme was incubated at the lowest pH values for the time of the kinetic run, whereupon the reaction was started by addition of substrate. The (k_{cat}/K_m)–pH profiles were also independent of the presence of DTT and EDTA during the assays. Thus, identical results were obtained regardless of whether or not the EDTA and activator were removed from the enzyme solutions by gel filtration prior to the kinetic experiments. This fact indicates that oxidation of the active-site thiol group was not a complication. Finally, under the conditions used here, a typical reaction was complete within 1–3 min, and spontaneous hydrolysis of the substrates was found to be negligible.

Data Treatment. For the *p*-nitrophenyl ester substrates, a value of $\Delta\epsilon_{340} = 6600\ M^{-1}\ cm^{-1}$ was employed. This value was found to be essentially independent of pH in the range studied, i.e., pH about 3–6.

The kinetic parameters were determined from the complete progress curves of the reactions. For both enzymes, the absence of product inhibition was assured in that, at several pH values, different initial substrate concentrations gave the same K_m within experimental error at each pH. All calculations were performed on an ABC 80 computer (Scandia Metric AB, Solna, Sweden). For a give progress curve, the polynomial of the third order that best fitted the experimental data was calculated by a least-squares program. From a knowledge of the enzyme concentration and the extinction coefficient of the appropriate substrate, the Michaelis–Menten parameters were determined by a least-squares fit to the [S] vs. [S]/v data. Thus, each progress curve provided a single determination of k_{cat}/K_m.

If one assumes that below pH 6 ionizations involving either one proton (eq 1) or two protons (eq 2) control the activity of an enzyme and provided that the enzyme is active in only one ionization state, the following equations apply (Cleland, 1979; Lewis et al., 1978):

$$\log \frac{k_{cat}}{K_m} = \log \frac{k_{cat}'/K_m'}{1 + [H^+]/K_I} \tag{1}$$

$$\log \frac{k_{cat}}{K_m} = \log \frac{k_{cat}'/K_m'}{1 + [H^+]/K_{Ib} + [H^+]^2/(K_{Ia}K_{Ib})} \tag{2}$$

The superscript slant prime denotes pH-independent kinetic parameters, and K_I, K_{Ia}, and K_{Ib} represent macroscopic ionization constants. The fact that k_{cat}/K_m decreases at alkaline pH values in parallel with an ionization of pK about 8.5 (Whitaker & Bender, 1965; Sluyterman & De Graaf, 1972; Mole & Horton, 1973) was ignored in the derivation of eq 1 and 2, since below pH 6 the effect of this ionization on the value of k_{cat}/K_m is negligible (Lewis et al., 1978). The observed log (k_{cat}/K_m)–pH profiles were evaluated on the basis of eq 1 and 2 by a nonlinear least-squares curve-fitting program.

Results

Figure 1, upper panel, illustrates the pH dependence of log (k_{cat}/K_m) for the papain-catalyzed hydrolyses below pH 6 of pHNP, Z-Gly-ONp, and Z-Lys-ONp, respectively. The dashed and solid lines in the figure represent the best fit of the experimental data to eq 1 and 2, respectively. The standard deviations of the calculated values of log (k_{cat}/K_m) from the experimentally observed values were 3–6 times smaller when eq 2 rather than eq 1 was used to fit the experimental data. Accordingly, inspection of Figure 1, upper panel, reveals that eq 2 fits the data much better than eq 1. The most striking difference in the fit of the data to eq 1 and 2 is seen at low pH values where slopes of the plots of log (k_{cat}/K_m) vs. pH approach 2 and not 1. Thus for the papain-catalyzed hydrolyses of the *p*-nitrophenyl ester substrates, two ionizations control the pH dependence of k_{cat}/K_m below pH 6.

The acidic limb of the pH dependence of log (k_{cat}/K_m) for the papaya peptidase A catalyzed hydrolyses of pHNP, Z-Gly-ONp, and Z-Lys-ONp is shown in the lower panel of Figure 1. The solid lines in the figure represent the best fit of the experimental data to eq 1. When eq 2 rather than eq

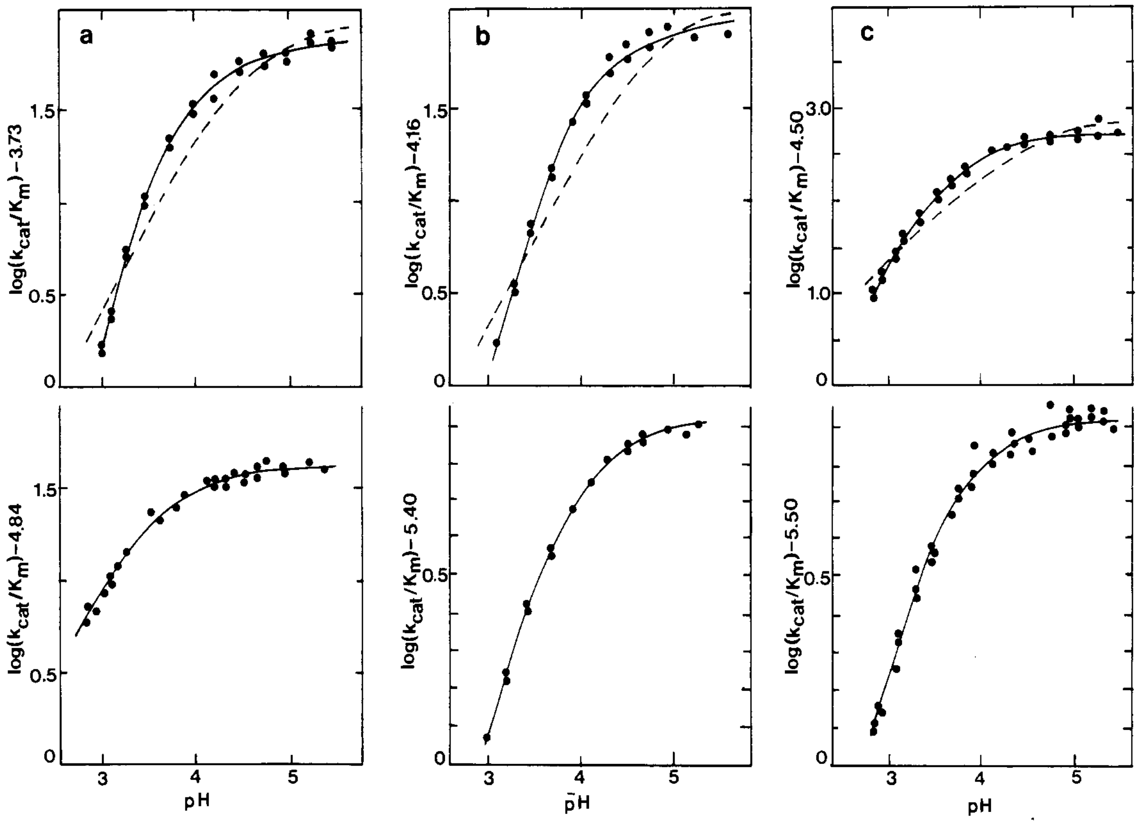

FIGURE 1: pH dependence of log (k_{cat}/K_m) for papain-catalyzed (upper panels) and papaya peptidase A catalyzed (lower panels) hydrolyses of *p*-nitrophenyl esters at 25 °C and an ionic strength of 0.3 M. The substrates were *p*-nitrophenyl esters of (a) hippuric acid, (b) benzyloxycarbonylglycine, and (c) benzyloxycarbonyl-L-lysine. (Upper panels) Solid lines represent theoretical curves on the basis of eq 2 and the parameters of Table I. Dashed lines are calculated on the basis of eq 1. (Lower panels) Solid lines are theoretical curves on the basis of eq 1 and the data of Table I.

Table I: Values of Ionization Constants and k_{cat}'/K_m' for Papaya Peptidase A Catalyzed (Eq 1) and Papain-Catalyzed (Eq 2) Hydrolyses at 25 °C and an Ionic Strength of 0.3 M

substrate	$pK_{a(I)}$[a] for papaya peptidase A	$pK_{a(Ia)}$[a] for papain	$pK_{a(Ib)}$[a] for papain	k_{cat}'/K_m' ($\times 10^{-6}$ M^{-1} s^{-1})[a] papaya peptidase A	k_{cat}'/K_m' ($\times 10^{-6}$ M^{-1} s^{-1})[a] papain
pHNP	3.56 ± 0.16	3.53 ± 0.08	3.96 ± 0.13	2.97 ± 0.27	0.41 ± 0.04
Z-Gly-ONp	3.81 ± 0.16	3.87 ± 0.05	4.03 ± 0.10	2.05 ± 0.08	1.27 ± 0.11
Z-Lys-ONp	3.57 ± 0.22	3.35 ± 0.17	4.00 ± 0.15	2.68 ± 0.18	16.63 ± 0.35

[a] Plus or minus standard deviation.

1 was used to evaluate the experimental data for papaya peptidase A, a poorer fit resulted for all three substrates, as evidenced by an increased or unaltered standard deviation of the fit and by the fact that $pK_{a(Ia)}$ values below 2.5 were assigned. Furthermore, at low pH values the slopes of the plots of log (k_{cat}/K_m) vs. pH for the papaya peptidase A catalyzed hydrolyses approach 1. This is in contrast to the slopes of 2 obtained in the corresponding plots for the papain-catalyzed hydrolyses of the same substrates. Therefore, it seems that below pH 6 a single ionization controls the pH dependence of k_{cat}/K_m for the papaya peptidase A catalyzed hydrolyses of the *p*-nitrophenyl esters, while for papain two ionizations are involved.

Table I summarizes the pK_a values and the values of the pH-independent kinetic parameters, k_{cat}'/K_m', for both enzymes and for the three substrates studied. At 25 °C and an ionic strength of 0.3 M, the $pK_{a(I)}$ for papaya peptidase A has an average value of 3.65 ± 0.14, whereas $pK_{a(Ia)}$ and $pK_{a(Ib)}$ for the corresponding papain-catalyzed reactions have average values of 3.58 ± 0.26 and 4.00 ± 0.04, respectively.

The values of k_{cat}'/K_m' reflect the specificity of the enzymes toward the *p*-nitrophenyl ester substrates employed; i.e., a higher degree of specificity is reflected in a higher value of k_{cat}'/K_m' (Fersht, 1977). For papain, the specificity is very sensitive to the nature of the acyl moiety of the substrate. Thus, replacement of the benzoyl group by the carbobenzoxy group results in an increase of k_{cat}'/K_m' by a factor of about 4, and the replacement of glycine by lysine in the Z-amino acid ester substrate results in a further increase of k_{cat}'/K_m' by more than 1 order of magnitude. In contrast, the specificity of papaya peptidase A seems largely insensitive to the variations in the acyl part of the substrates employed.

Discussion

The observation that the acidic limb of the (k_{cat}/K_m)–pH profile for papain-catalyzed hydrolyses is modulated by two ionizations has been made by Sluyterman & Wijdenes (1973) and by Lewis et al. (1978). In the latter work, the pH dependence of k_{cat}/K_m for the papain-catalyzed hydrolyses of ethyl hippurate, N^{α}-benzoyl-L-citrulline ethyl ester, and the *p*-nitroanilide, amide, and ethyl ester derivatives of N^{α}-benzoyl-L-arginine was determined below pH 6.4. Lewis et al. (1978) considered that since their substrates had leaving groups of widely different basicity and yet yielded similar p*K* values, the ionization constants determined from the (k_{cat}/K_m)–pH profiles were close to the true macroscopic ionization constants of groups on the free enzyme. Curve-fitting based on eq 2 yielded values of p$K_{a(Ia)}$ = 3.78 ± 0.2 and p$K_{a(Ib)}$ = 3.95 ± 0.1. The fact that the pK values found here [i.e., p$K_{a(Ia)}$ = 3.58 ± 0.26 and p$K_{a(Ib)}$ = 4.00 ± 0.04] are in good agreement with those reported by Lewis et al. (1978) suggests that also in the case of papain-catalyzed hydrolyses of *p*-nitrophenyl ester substrates the (k_{cat}/K_m)–pH profiles are modulated by two acidic ionization constants that are close in value to the true macroscopic ionization constants of groups on the free enzyme. If, however, different steps of the catalytic pathway depend on different protonic states of the enzyme and there is a change in rate-determining step with pH, the ionization constants obtained from the (k_{cat}/K_m)–pH profile might contain ratios of rate constants (Schmidt & Westheimer, 1971; Renard & Fersht, 1973). Although we do not consider it likely, we cannot exclude the possibility that this situation rather than the experimental error is responsible for the small difference (<0.6 pK_a unit) seen with different *p*-nitrophenyl ester substrates in Table I, as well as the slight difference between the average p$K_{a(Ia)}$ value reported here (i.e., 3.58 ± 0.26) and the average p$K_{a(Ia)}$ value (i.e., 3.78 ± 0.2) given by Lewis et al. (1978) for the papain-catalyzed hydrolyses of a different set of substrates under the same conditions.

It should be noted that Jarvis & Brocklehurst (1982) reported that they have found it necessary to include a third acidic ionization constant to adequately describe the pH dependence of k_{cat}/K_m for the papain-catalyzed hydrolyses of Z-Lys-ONp and N^{α}-benzoyl-L-arginine *p*-nitroanilide below pH 6. As it may be seen already from the scatter of the experimental points in Figure 1c, upper panel, our data for the papain-catalyzed hydrolysis of Z-Lys-ONp does not make it reasonable to exploit the curve-fitting procedure in order to obtain additional parameters.

For the hydrolysis of *p*-nitrophenyl ester substrates, it is now apparent that the acidic limb of the (k_{cat}/K_m)–pH profile for papaya peptidase A differs from that of papain in that only a single ionization of pK_a = 3.65 ± 0.14 is involved in the case of papaya peptidase A. This result is consistent with the recent report of Baines & Brocklehurst (1982). On the basis of studies of the pH dependence of the rate of inhibition of papaya peptidase A by 2,2′-dipyridyl disulfide, they found that the active-center thiolate ion of this enzyme is maintained in acidic media by association of the thiol group with another acid–base system common to all of the cysteine proteinases studied so far [p$K_{a(I)}$ = 3.9; p$K_{a(II)}$ = 7.9]. It was also noted that papaya peptidase A appears to lack an ionizing group with characteristics analogous to those of the aspartic acid-158 residue of papain. Thus, for hydrolyses catalyzed by papaya peptidase A it seems that if the enzyme does possess a residue analogous to that of the aspartic acid-158 residue of papain, its pK value is either extremely low or its presence is irrelevant at least for the hydrolysis of the substrates used here.

For the papaya peptidase A catalyzed hydrolyses of the three *p*-nitrophenyl ester substrates, closely similar values of the single acidic ionization constant in the (k_{cat}/K_m)–pH profile were obtained (cf. Table I). This fact together with the observation of an ionization constant of pK = 3.9 in the pH dependence of the second-order rate constant for the reaction of the thiol group of papaya peptidase A with 2,2′-dipyridyl disulfide (Baines & Brocklehurst, 1982) suggest that the ionization constant reflected in the (k_{cat}/K_m)–pH profiles for this enzyme is close in value to a true macroscopic ionization constant of a group on the free enzyme.

The values of k_{cat}'/K_m' for the papain-catalyzed hydrolyses are in reasonable agreement with those previously reported [pHNP, Lowe & Williams (1965); Z-Gly-ONp, Kirsch & Igelström (1966), Williams & Whitaker (1967), Mole & Horton (1973), and Bendall et al. (1977); Z-Lys-ONp, Bender & Brubacher (1966) and Jarvis & Brocklehurst (1982)]. The relative values of k_{cat}'/K_m' for pHNP, Z-Gly-ONp, and Z-Lys-ONp reflect the essential specificity requirements of the enzyme. Papain is capable of accomodating in its active site up to seven amino acid residues of a peptide substrate, four residues on the N-terminal side of the point of cleavage (subsites S_1–S_4) and three residues on the C-terminal side (subsites S_1'–S_3'). The subsite S_2 has a strong preference for hydrophobic residues such as L-phenylalanine, and among *N*-acyl amino acid derivatives the most effective substrates have arginine or lysine as the amino acid moiety, indicating a preference of subsite S_1 for these amino acids (Berger & Schechter, 1970). Accordingly, the difference in specificity between pHNP and Z-Gly-ONp results from the greater structural resemblance of phenylalanine to the benzyloxycarbonyl moiety than to the benzoyl moiety, and the difference in specificity between Z-Gly-ONp and Z-Lys-ONp reflects the preference of the S_1 subsite for lysine over glycine.

In marked contrast, the k_{cat}'/K_m' values for the papaya peptidase A catalyzed hydrolyses of the same substrates reveal that the specificity of this enzyme is largely insensitive to the variations in the acyl part of the substrates employed. Thus, papaya peptidase A does not appear to possess the essential specificity requirements known for papain. Although papaya peptidase A is at least as catalytically competent as papain in the hydrolyses of pHNP and Z-Gly-ONp, the "perfect" substrate for papaya peptidase A has yet to be discovered.

The difference between papaya peptidase A and papain with respect to the number of essential acidic ionizations and apparent specificity requirements accentuates the conclusion most recently reached by Brocklehurst et al. (1983) that papain may not necessarily be regarded as archetypal of cysteine proteinases in general.

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Registry No. pHNP, 3101-11-9; Z-Gly-ONp, 1738-86-9; Z-Lys-ONp, 4272-71-3; papaya peptidase A, 39307-22-7; papain, 9001-73-4.

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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 acetylalanylalanyllysyl-*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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