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The Comparative Specificity of Acid Proteinases

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ABSTRACT **Examination of the kinetic parameters for the hydrolysis, by acid proteinases, of a single peptide bond (between *p*-nitro-L-phenylalanyl and L-phenylalanyl) in a series of oligopeptides has shown that secondary interactions are important factors in determining the catalytic efficiency. Comparison of the action of highly purified pepsinlike enzymes (*Rhizopus* proteinase, *Mucor* proteinase, rennin) with that of swine pepsin A indicates significant differences among them, either in the binding of the substrate (as estimated by K_m), or in the catalytic efficiency (as measured by k_{cat}), or both. It may be concluded from these data that, in their action on oligopeptide substrates, the specificity of proteinases operating by a similar catalytic mechanism cannot be explained solely in terms of the amino acid residues flanking the sensitive peptide bond; in addition, the specificity includes significant contributions from secondary interactions arising from complementary relations between parts of the substrate and of the enzyme at a distance from the catalytic site. Data are also presented for the effect of urea (about 1 M) on the kinetic parameters of several acid proteinases; under the conditions of these studies, the binding of the substrate is affected to a much lesser degree than is the catalytic efficiency.**

Many of the known proteinases have been classified into distinct types, according to the nature of the enzymic groups identified with the catalytic process (1). The so-called serine proteinases, which include chymotrypsin, trypsin, elastase, and subtilisin, are characterized by the presence of a reactive seryl residue and a histidyl residue at the catalytic site. In the cysteine proteinases (papain, ficin, streptococcal proteinase, etc.), a cysteinyl residue and a histidyl residue are involved. These enzymes act optimally on proteins and on synthetic peptide substrates at pH values in the range 5–9, and the available evidence is consistent with the intermediate formation of an acyl-enzyme. In addition, there is a sizeable group of enzymes whose optimal action on protein substrates is in the pH range 2–5, and that have been termed "acid proteinases." The best-known member of this group is swine pepsin A (2); others are rennin (3), cathepsin D (4), and the acid proteinases of several molds, including *Rhizopus chinensis* (5), *Penicillium janthinellum* (penicillopepsin (6)), and *Mucor miehei* (7). Besides pepsin, several of these enzymes have been prepared in crystalline form (3, 5, 6).

A common feature of the acid proteinases appears to be their inhibition by diazo compounds (8, 9); in the case of pepsin, the carboxyl group of an aspartyl residue in the sequence Ile-Val-Asp-Thr-Gly-Thr-Ser has been identified as the site of attack by such a reagent (10). A reactive aspartyl residue has also been identified as part of the active site of penicillopepsin (6). Although further studies are needed, the available information suggests that in the action of pepsin (and possibly of other acid proteinases) an imino-enzyme intermediate is involved in the catalytic mechanism; it has been suggested that the active site contains a carboxylate group acting as a nucleophile and another carboxyl group (in its protonated form) acting as a proton donor (11, 12).

Whereas the serine proteinases exhibit significant differences in specificity with respect to the amino acid residue, in the substrate, which provides the carbonyl group of the sensitive peptide bond, the known acid proteinases appear to have similar (although not identical) preference for hydrophobic amino acid units flanking the sensitive bond (13). Thus, the available data on the cleavage of the oxidized B chain of insulin by pepsin (14), rennin (3), and the acid proteinases of *Rhizopus* (15) and *Mucor* (16) show, in all cases, a preferential attack at Leu-Tyr (*15-16*), Tyr-Leu (*16-17*), Phe-Phe (*24-25*), and Phe-Tyr (*25-26*), although differences have been noted in the apparent susceptibility of other peptide bonds.

Despite their similarity of action on the B chain of insulin, however, several of the known acid proteinases have been reported to be relatively inactive toward simple synthetic substrates for crystalline swine pepsin A. Recent work has provided a large number of new pepsin substrates, some of which are cleaved at a rapid rate, and conclusions have been drawn concerning the specificity of the action of this enzyme at peptide bonds (13). In particular, it has been found that with small synthetic substrates of the type A-X-Y-B, where X and Y are L-amino acid residues flanking a sensitive peptide bond, pepsin prefers a phenylalanyl residue in the X position and a tryptophyl, tyrosyl, or phenylalanyl residue in the Y position; other hydrophobic amino acid residues also promote pepsin action, but to a much lesser degree (17). Moreover, it has been found that the rate of pepsin action at the Phe-Phe [or Phe(NO_2)-Phe] bond of substrates of the type A-Phe-Phe-B [or A-Phe(NO_2)-Phe-B] is greatly influenced by the nature of the A and B groups on either side of the sensitive dipeptidyl unit (18, 19). These effects have been interpreted as providing evidence for the view that the "secondary" interactions of the A and B groups of the substrate with complementary enzymic groups relatively distant from the catalytic site may alter the conformation of the enzyme in a manner that affects the efficiency of catalysis. It appeared likely, therefore, that the apparent discrepancy between the similarity of the action of the acid proteinases on the B chain of insulin and the wide differences among them in the cleavage of small synthetic substrates might be related to the effect of

Abbreviations: In addition to the usual abbreviations for L-amino acid residues, the following have been used: Phe(NO_2), *p*-nitro-L-phenylalany ; Z, benzyloxycarbonyl; OMe, methoxy.

such secondary interactions. The present experiments were undertaken to examine this possibility.

MATERIALS AND METHODS

Swine pepsin (twice crystallized, lot PM693-7) was obtained from the Worthington Biochemical Corp. When assayed in the usual manner (20), with hemoglobin as the substrate, the proteinase activity of this enzyme preparation was 2700 units/mg of protein. The acid proteinase of *Rhizopus chinensis* (3 times crystallized) was obtained from the Miles Laboratories, and had a specific activity of 870 units/mg of protein in the above assay (at pH 3.8, a similar activity was noted). The acid proteinase of *Mucor meihei* (7) was generously provided by Dr. M. Ottesen. Crystalline rennin was prepared from commercial rennet powder according to the method of Berridge (21); material generously provided by Dr. B. Foltmann and Dr. J. Garnier was used for seeding, and greatly accelerated the preparation. The specific activity (milk-clotting assay (22)) was 60 units/mg of protein.

The synthesis of the oligopeptides used as substrates in the present study has been described previously, as has the spectrophotometric method employed for the determination of the initial rates of cleavage of the Phe(NO_2)-Phe bond in such substrates (19). In all cases, the data were obtained at pH 4.0 (0.04 M formate buffer) and 37°C, and accorded with Michaelis-Menten kinetics over the range of substrate concentration (0.02–0.4 mM) employed; 5–8 runs were performed for each determination of K_m and of k_{cat} (V_m per molar equivalent of enzyme); the enzyme concentration (0.003–5.4 μM) was chosen to give reliable data for the initial rate of hydrolysis. In the calculation of k_{cat}, the molecular weights of the four enzymes were assumed to be: pepsin, 34,200; *Rhizopus* proteinase, 35,000; *Mucor* proteinase, 38,000; and rennin, 34,000. In all cases, the position of cleavage was shown by thin-layer chromatography (19) to be restricted to the Phe(NO_2)-Phe bond under the conditions of these studies. The precision (95% confidence limits) of the kinetic parameters was estimated by computer analysis (18).

RESULTS AND DISCUSSION

It will be noted from Table 1 that the rate of cleavage, by pepsin, of the Phe(NO_2)-Phe bond in substrates of the type A-Phe(NO_2)-Phe-B is greatly influenced by changes in the nature of the B group and, as reported previously (19), the effect on the magnitude of k_{cat} is greater than on that of K_m. Examination of the action of the *Rhizopus* proteinase on these substrates indicated that when A = Z-His, the values of k_{cat}/K_m for the three substrates with B = OMe, Ala-OMe, and Ala-Ala-OMe were roughly similar to those obtained with pepsin, both k_{cat} and K_m being lower. One of the possible explanations for this difference in the kinetic parameters observed with pepsin and the *Rhizopus* proteinase is that, in the latter case, nonproductive interaction makes a relatively larger contribution to the binding of the substrates (19, 23). That such nonproductive interaction may involve the benzyloxycarbonyl group is suggested by the finding that when A = Phe-Gly-His, the K_m values for the two substrates tested with both pepsin and the *Rhizopus* proteinase are the same, although k_{cat} is much lower in the latter case. In contrast to the *Rhizopus* proteinase, the enzyme from *Mucor* cleaves the substrates with A = Z-His much more slowly than does pepsin, the K_m values being much higher; the effect of the replacement of B = OMe by Ala-OMe on the value of k_{cat}/K_m is similar, however, to the results for pepsin and the *Rhizopus* proteinase. With the *Mucor* proteinase, the replacement of A = Z-His by Phe-Gly-His exerts a markedly unfavorable effect on k_{cat}; thus the substrate with B = OMe was not cleaved at a measurable rate at the highest enzyme concentration (13 μM) tested, and the substrate with B = Ala-Phe-OMe was cleaved at about 0.01% of the rate found with pepsin.

The data in Table 1 for the action of rennin on pepsin substrates show that, of those tested, only those with B = Ala-OMe, Ala-Ala-OMe, and Ala-Phe-OMe were cleaved at the Phe(NO_2)-Phe bond to a measurable extent under the conditions of these studies. With B = Ala-OMe, the value of K_m was too high to permit an estimate of k_{cat} to be made, and the value of k_{cat}/K_m was calculated from the first-order rate constant for the hydrolysis of this substrate (19). In those cases in which an estimate of K_m could be made, it was clear that the substrate in question was bound much more weakly by rennin than by pepsin. In view of the similarity in the action of rennin and pepsin on the B chain of insulin, it may be surmised that the two enzymes exhibit considerable difference in affinity toward oligopeptide substrates. In this connection, it may be noted that, in the action of rennin on casein in the milk-clotting process, a Phe-Met bond is preferentially cleaved (24); that this cleavage is not a consequence of a specificity of rennin for the Phe-Met unit, as against the Phe-Phe unit, is indicated by our finding that Z-His-Phe(NO_2)-Met-OMe (prepared in a manner analogous to that described (17) for Z-His-Phe-Met-OMe) is completely resistant to rennin action at the highest enzyme concentration

TABLE 1. *Kinetics of cleavage of the Phe(NO_2)-Phe bond of A-Phe(NO_2)-Phe-B*

A-Phe(NO_2)-Phe-B		Pepsin			*Rhizopus*			*Mucor*			Rennin		
A	B	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m
Z-His	OMe	0.26 ± 0.05	0.43 ± 0.06	0.6	0.06 ± 0.01	0.12 ± 0.04	0.5	0.024 ± 0.005	1.0 ± 0.2	0.024	—	—	<0.005
Z-His	Ala-OMe	3.3 ± 0.3	0.4 ± 0.1	8.3	0.77 ± 0.11	0.08 ± 0.01	9.6	0.7 ± 0.2	1.5 + 0.3	0.47	—	—	0.01
Z-His	Ala-Ala-OMe	28 ± 2	0.13 ± 0.01	215	7.5 ± 1.2	0.06 ± 0.01	125						
Phe-Gly-His	OMe	0.12 ± 0.04	0.4 ± 0.1	0.3	0.01 ± 0.005	0.4 ± 0.1	0.025	—	—	<0.005	—	—	<0.005
Phe-Gly-His	Ala-Ala-OMe	28 ± 1	0.16 ± 0.02	175							0.07 ± 0.02	3.6 ± 1.0	0.02
Phe-Gly-His	Ala-Phe-OMe	20 ± 2	0.04 ± 0.01	500	0.45 ± 0.008	0.04 ± 0.01	10.1	0.01 ± 0.005	0.14 ± 0.02	0.07	0.02 ± 0.008	1.0 ± 0.02	0.02

TABLE 2. *Effect of urea on cleavage of oligopeptides by acid proteinases*

Substrate	Urea (M)	Pepsin k_{cat} (sec^{-1})	Pepsin K_m (mM)	Pepsin k_{cat}/K_m	*Rhizopus* k_{cat} (sec^{-1})	*Rhizopus* K_m (mM)	*Rhizopus* k_{cat}/K_m
Z-His-Phe(NO_2)-Phe-OMe	0.5	0.15 ± 0.04	0.37 ± 0.04	0.4			
	1.0	0.11 ± 0.03	0.47 ± 0.05	0.23	0.035 ± 0.005	0.16 ± 0.03	0.22
	2.0	0.09 ± 0.02	0.5 ± 0.1	0.18			
Z-His-Phe(NO_2)-Phe-Ala-OMe	1.0	1.8 ± 0.3	0.5 ± 0.1	3.6	0.55 ± 0.12	0.16 ± 0.04	4.1
Z-His-Phe(NO_2)-Phe-Ala-Ala-OMe	1.0	10 ± 2	0.10 ± 0.05	100			
Phe-Gly-His-Phe(NO_2)-Phe-OMe	1.0	0.06 ± 0.02	0.4 ± 0.1	0.15	0.006 ± 0.002	0.5 ± 0.1	0.012

tested (5.2 μM), under the conditions of these studies. It appears more likely that the preferential cleavage of the Phe-Met bond in casein is a consequence of the accessibility of this bond to proteolytic attack, as well as to secondary enzyme–substrate interactions. That such secondary interactions can play a role in rennin action is evident from the data in Table 1 for the effect of the replacement of B = OMe by Ala-Ala-OMe or Ala-Phe-OMe, although the rate of cleavage of the most sensitive synthetic substrates for rennin is only about 0.001% of that for the action of pepsin on Phe-Gly-His-Phe(NO_2)-Phe-Ala-Phe-OMe.

During the course of these studies, the question of the relation of conformational change to the catalytic efficiency of the acid proteinases was examined by estimation of the effect of urea on the kinetic parameters k_{cat} and K_m for several synthetic oligopeptide substrates. Earlier studies have shown that although pepsin does not exhibit large conformational changes in the presence of 6 M urea, as judged by changes in optical rotation, small changes are observed by circular dichroism measurements (25). It was of interest to find, therefore, that relatively low concentrations of urea (about 1 M) cause a significant decrease in the value of k_{cat}, with little change in the value of K_m (Table 2). This conclusion applies to all the substrates of pepsin and of the *Rhizopus* proteinase tested; in addition, the action of the *Mucor* proteinase on Z-His-Phe(NO_2)-Phe-Ala-OMe was characterized by k_{cat} = 0.29 ± 0.05 sec^{-1} and K_m = 1.5 ± 0.2 mM. It seems, therefore, that these enzymes respond to 1 M urea in a manner that does not affect greatly their ability to bind the oligopeptide substrates tested, but significantly alters the catalytic efficiency with which these substrates are cleaved. At the present stage of our knowledge about the nature of the action of urea on the conformation of proteins, and about the three-dimensional structure of the active site of pepsin, speculations regarding the significance of the data presented in Table 2 are premature. Although the results appear to be consistent with increased flexibility of the enzymic structure, too small to be detected by optical measurements, but sufficiently large to alter the catalytic efficiency, other explanations are possible. Further study of the action of relatively low concentrations of urea (and of other denaturing agents) on the kinetic parameters of the action of proteinases on oligopeptide substrates may clarify the problem.

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