antibodies may, therefore, serve as a tool to search for such differences in many other tissues.

Acknowledgments

We wish to thank Professor M. Sela for very helpful discussions. This work was supported, in part, by U.S. National Cancer Institute Contract No. NOICP 33220 and by Grant No. 1 RO1 AI 11405-01 from the National Institutes of Health, United States Public Health Service.

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Thermolysin and Neutral Protease: Mechanistic Considerations[†]

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ABSTRACT: The pH dependence of the catalytic activities of thermolysin and neutral protease indicates that each enzyme is dependent upon the protonation of a single residue with a pK_a of 7.5. Chemical modification with ethoxyformic anhydride indicates that a single histidyl residue, with a pK_a of 7.6–7.9, is essential for the catalytic activity of each enzyme. The only free histidyl residue in the active site of thermolysin is His-231, which, in crystallographic studies, was found to be the binding site for several heavy-atom derivatives. The present study demonstrates that these heavy metal compounds inactivate the enzymes reversibly and protect them from covalent inactivation by ethoxyformic anhydride. These findings lead to the proposal that His-231 may serve as a proton donor during catalysis. Other structural considerations indicate that Glu-143 may be in the proper orientation to serve as an attacking nucleophile. The proposed mechanism resembles certain features of the mechanism of carboxypeptidase A on the one hand and of serine proteases on the other.

Detailed descriptions of the amino acid sequence of thermolysin (Titani et al., 1972) and of its three-dimensional structure (Matthews et al., 1972a,b; Colman et al., 1972) have established that this proteolytic enzyme is unique as it bears no structural resemblance to any of the four other sets of homologous proteases, i.e. the serine proteases, the sulfhydryl proteases, the metalloexopeptidases, and the acid proteases (Hartley, 1960; Walsh, 1975).

Two neutral proteases (A and B) isolated from *Bacillus subtilis* resemble thermolysin in specificity, metal dependence, and molecular weight (Tsuru et al., 1964; Matsubara and Feder, 1971; Pangburn et al., 1973b). Neutral proteases A and B differ threefold in their specific activity (Pangburn et al., 1973a) but no differences in amino acid composition or sequence are yet evident. Preliminary se-

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quence analysis of neutral protease A[1] suggests that it is homologous with thermolysin (Pangburn et al., 1973a,b) raising the possibility that these two enzymes have similar three-dimensional structures and analogous mechanisms of action.

Besides their substrate specificity and metal requirements, little is known of the catalytic mechanism of this set of enzymes. The electron density map of thermolysin reveals nine residues near the active site which could participate in catalysis (Colman et al., 1972). Three of these bind the catalytically essential zinc atom. Chemical modification studies (Blumberg et al., 1973; Burstein et al., 1974) have implicated a histidyl residue in the catalytic mechanism, but the specific histidyl residue or the function of this residue has not been identified.

The present study provides additional measurements of pH dependence and chemical inactivation of both thermolysin and neutral protease A which allow a hypothesis of the catalytic mechanism of thermolysin to be constructed. This hypothesis is tested for consistency with the functional characteristics of the homologous enzymes neutral proteases A and B.

Experimental Section

Materials

Crystalline thermolysin was purchased from Daiwa Kasei K.K., Osaka, Japan; crude neutral protease (*Bacillus subtilis* strain NRRL B3411) was obtained from the Monsanto Company, St. Louis, Mo., through the courtesy of Dr. J. Feder. Two forms of neutral protease (A and B) were purified by affinity chromatography as described by Pangburn et al. (1973a). Furylacryloylglycyl-L-leucinamide was purchased from Cyclo Chemical Co. and Hepes,[2] Pipes, and Tris were from Calbiochem. Ethoxyformic anhydride was a product of Eastman Kodak Co. Reagent grade metal chlorides were used. Dimercury acetate was a gift from Dr. Brian Matthews. *o*-Chloromercuriphenol was obtained from K and K Laboratories, Inc. A specific adsorbent for affinity chromatography was prepared by coupling BrCN-activated Sepharose 4B through a spacer of triethylenetetraamine to chloroacetyl-D-phenylalanine. This preparation, as described by Walsh et al. (1974), provides a Gly-D-Phe ligand on a nine-atom spacer.

Methods

Measurement of Enzymatic Activity. The concentration of active enzyme was determined spectrophotometrically at 25° by following the hydrolysis of furylacryloylglycyl-L-leucinamide (ΔE_m of 317 cm^{-1} at 345 nm) using the conditions described by Pangburn et al. (1973a). Protein concentrations and specific activities were based on the extinction coefficients and kinetic constants reported by the same authors.

The dependence of enzymatic activity on pH was measured at 25° with a Cary 16 recording spectrophotometer using solutions containing 1 m*M* furylacryloylglycyl-L-leucinamide, 50 m*M* buffer, and 0.01 m*M* $ZnCl_2$ brought to an ionic strength of 0.1 with NaCl. The buffers employed were sodium acetate (pH 4.38–5.21), Pipes (pH 5.28–7.07), Hepes (pH 6.14–7.93), and Tris (pH 7.01–9.03).

Inhibition by Ethoxyformic Anhydride and by Heavy Metals. The modification of neutral protease A by ethoxyformic anhydride followed the procedures described by Burstein et al. (1974) for the reaction of thermolysin with this reagent. Inhibition by compounds containing heavy metals was determined in 0.9 m*M* furylacryloylglycyl-L-leucinamide, 0.09 *M* Hepes (adjusted to pH 7.2 with NaOH), 0.45% dimethylformamide, and various concentrations of the metal ions.

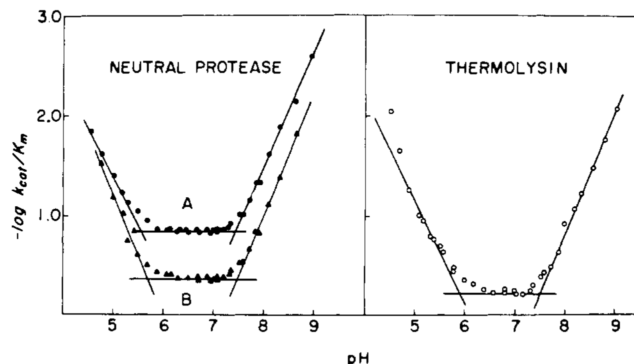


FIGURE 1: pH dependence of neutral proteases and thermolysin. The apparent pK_a values in the acidic region are 5.6 and 5.7 for neutral proteases A and B, respectively, and 5.9 for thermolysin. In the alkaline region the pK_a is 7.5 for each enzyme. The slopes for the acid, neutral, and alkaline regions are, respectively, −1.0, 0, and 1.2 for neutral protease A, −1.2, 0, and 1.2 for neutral protease B, and −1.0, 0, and 1.4 for thermolysin. The units of k_{cat}/K_m are sec^{-1} (mg/ml)$^{-1}$.

Results

The Dependence of Catalysis on pH. Theoretically, the pK_a of an ionizable group which affects enzymatic activity can be determined from the pH dependence of k_{cat} or K_m. However, even the best substrate for thermolysin has a large K_m and a low solubility, precluding explicit measurements of k_{cat} and K_m over a sufficiently wide range of pH. However, the ratio k_{cat}/K_m can be obtained at nonsaturating substrate concentrations as described by Feder (1968). A plot of $-\log k_{cat}/K_m$ vs. pH for thermolysin (Figure 1) shows two inflection points, one at pH 7.5 and another at pH 5.9. Similar determinations for neutral proteases A and B show identical inflection points at pH 7.5, whereas in the acidic region the inflection points occur at pH 5.6 and 5.7 for neutral proteases A and B, respectively. These data are in accord with earlier measurements of pH optima by Feder and Schuck (1970).

While measurements of k_{cat}/K_m at high substrate concentrations yield values of pK_a for the enzyme–substrate complex, measurements at substrate concentrations smaller than K_m yield the pK_a of free enzyme or free substrate (Dixon and Webb, 1964). Since the present substrate is uncharged, the data can only reflect the ionization of groups in the free enzyme which influence enzymatic activity.

The slopes of the lines in Figure 1 are indicative of the number of ionizing species causing the inflection. The data suggest that the acidic and alkaline limbs each result from the ionization of a single catalytically important residue. The slopes are not precisely integers, however, suggesting some denaturation, pH-dependent structural fluctuations, or solvent effects.

Modification with Ethoxyformic Anhydride. Studies of

[1] Sequence analysis of neutral protease A has placed 55% of the residues. Portions of this structure are clearly homologous with the active site of thermolysin. A description of these findings is in preparation (Pangburn et al., 1975; Levy et al., 1975).

[2] The following abbreviations are used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

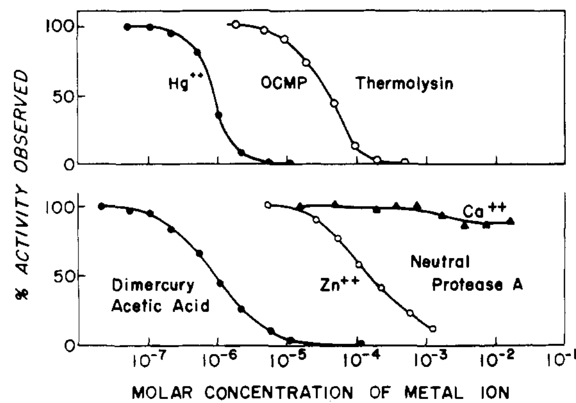


FIGURE 2: Inhibition of thermolysin and neutral protease A by mercury (Hg^{2+}), *o*-chloromercuriphenol (OCMP), dimercury acetate (DMA), zinc, and calcium. The percent inhibition was determined by assaying the enzymes (25 µl) with 3 ml of 0.9 m*M* furylacryloylglycyl-L-leucinamide–0.09 *M* Hepes (pH 7.2) containing the metal ions at the concentrations indicated. Controls lacking only the metal ions were taken as 100%.

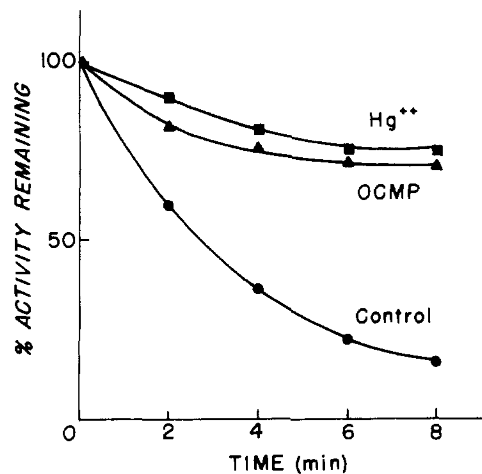


FIGURE 3: Time course of inhibition of thermolysin by ethoxyformic anhydride. Thermolysin (0.2 mg/ml) in 10 m*M* Hepes–2 m*M* $Ca(OAc)_2$ was treated with 10 m*M* ethoxyformic anhydride at pH 7 (the pH was maintained in a pH-Stat at 25°) in the absence (●) and in the presence of 1 m*M* *o*-chloromercuriphenol (▲) or 0.2 m*M* $HgCl_2$ (■). Samples (50 µl) were removed, diluted with 200 µl of 0.2 *M* NaOAc–2 m*M* $Ca(OAc_2)$ (pH 5), and kept in ice until assayed with furylacryloylglycyl-L-leucinamide. Dilution into the substrate solution reduced the metal inhibition. Measurements at zero time (before adding ethoxyformic anhydride) were taken as 100%.

inactivation of thermolysin by ethoxyformic anhydride by Blumberg et al. (1973, 1974) and by Burstein et al. (1974) have established the importance of a single histidyl residue to the catalytic function of this enzyme. The pH dependence of the rate of inactivation of thermolysin correlated with a single site of modification having a pK_a of 7.6.

Similar experiments were undertaken with neutral protease A using the methods described by Burstein et al. (1974). The results were similar in all respects. That is, the enzyme was protected from inactivation by the competitive inhibitor Cbz-L-Phe (50 m*M*) and could be partially reactivated by treatment with hydroxylamine (25 m*M*). The inactivated protein failed to bind to an affinity chromatography column, while the reactivated enzyme was adsorbed and eluted in a manner similar to the native enzyme. The reactivation of the modified enzyme correlated with the deethoxyformylation (followed spectrally) of 1.1 histidyl residues. Finally, the pH dependence of the pseudo-first-order rate constant of inactivation indicated that inactivation results from the acylation of the unprotonated form of a single site with an apparent pK_a of 7.9 ± 0.2.

Inhibition of Catalytic Activity by Metal Ions. Thermolysin and neutral protease A were inhibited by low concentrations of mercuric ions and mercury compounds and by somewhat higher concentrations of Zn as shown in Figure 2. All of these compounds were in fact tested on both enzymes and the concentrations of each compound causing 50% inhibition were identical for the two enzymes.

Affinity Chromatography of Thermolysin in the Presence of $HgCl_2$. Since mercuric ions inhibit thermolysin, their effect on the binding of thermolysin to the affinity adsorbent was examined. In control experiments, thermolysin (4 mg) in 5 ml of 10 m*M* Hepes, 2 m*M* $Ca(OAc)_2$, and 100 m*M* NaOAc (pH 7) was applied to a column (0.9 × 27 cm) of the specific adsorbent (see Materials) which had been equilibrated with the same buffer. The enzyme was bound to the column as previously described (Pangburn et al., 1973a) and eluted normally with 20 m*M* $Ca(OAc)_2$–100 m*M* NaOAc (pH 9). When 2×10^{-4} *M* $HgCl_2$ was included in all buffers, and the column preequilibrated with Hg-containing buffer, the chromatographic behavior of thermolysin (preincubated for 15 min in the Hg-containing buffer, pH 7.0) was not changed. The concentration of mercuric chloride was sufficient to inhibit the enzymatic activity. This is in contrast to the behavior of the enzyme inactivated by ethoxyformic anhydride, which was not bound (vide supra). Thus, thermolysin apparently bound -Gly-D-Phe ligands at pH 7 even in the presence of inhibiting concentrations of $HgCl_2$.

Protection by Metal Ions against Inactivation by Ethoxyformic Anhydride. The interference of one inhibitor with the effect of another suggests that similar sites of action are involved. Figure 3 illustrates the results of an experiment to test this hypothesis and shows that the rapid inactivation of thermolysin by ethoxyformic anhydride was indeed largely prevented by the presence of 0.2 m*M* mercury(II) or 1 m*M* *o*-chloromercuriphenol.

Discussion

The 2.3-Å electron density map and the amino acid sequence of thermolysin have provided a detailed description of the molecular structure of this enzyme (Matthews et al., 1972a,b, 1974; Titani et al., 1972). Colman et al. (1972) have identified nine amino acid residues which are sufficiently close to bound competitive inhibitors to assign these groups a catalytic role (Figure 4). The present observations on thermolysin and neutral protease are consistent with a model for the mechanism of action of these enzymes in which His-231 acts as a proton donor in the catalytic process.

Evidence of Histidine Involvement in Catalysis. The inhibition of thermolysin (Burstein et al., 1974) and of neutral protease A by ethoxyformic anhydride indicates the importance of a single histidyl residue for the catalytic activity of each enzyme. In each case, inactivation can be correlated with modification of a single site having an apparent pK_a of 7.6 in thermolysin and 7.9 in neutral protease A. The dependence of activity on pH indicates that the deprotonation of a single group with a similar pK_a (7.5) inactivates each enzyme (Figure 1). It seems reasonable to suggest that the same residue is responsible for these observations and that the *protonated* form of this histidyl residue is necessary for

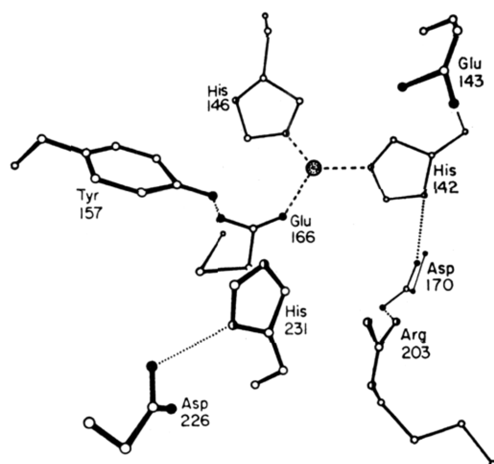


FIGURE 4: Sketch illustrating the position of some of the residues in the active-site cleft of thermolysin. The direction of viewing is down into the active site along the fourth zinc ligand axis (Colman et al., 1972).

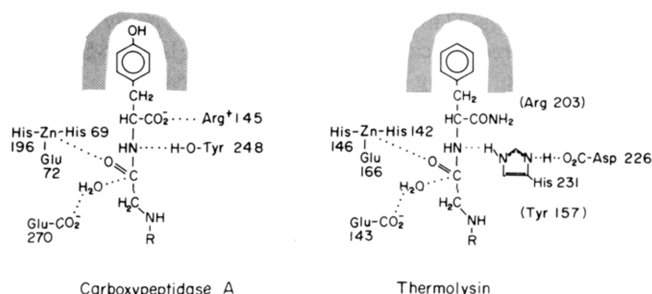


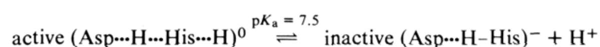
FIGURE 5: Comparison of the hypothetical active site of thermolysin with that proposed by Lipscomb et al. (1969) for the active site of carboxypeptidase A. In each case a substrate is illustrated in a productive binding mode. A general base mechanism is shown involving Glu-143 in thermolysin (or Glu-270 in carboxypeptidase) and a proton donor, His-231 in thermolysin (or Tyr-248 in carboxypeptidase).

catalysis whereas the *unprotonated* form reacts with ethoxyformic anhydride.

Of the three histidyl residues in the active site of thermolysin, two residues (142 and 146) serve as zinc ligands; only the third (His-231) is free to participate directly in catalysis. Although the ethoxyformylated peptide is too unstable to be isolated and identified (Burstein et al., 1974), the protection of the critical site by competitive inhibitors lends credence to the proposal that His-231 is the modified residue responsible for inactivation. More convincing support can be derived from the studies of inhibition by metals. Colman et al. (1972) found that mercury compounds ($HgCl_2$, dimercury acetic acid, and mercury succinimide) all bind to His-231 in the crystals. They also showed that silver ions bind to His-231 in the crystals and Endo (1962) reported that silver ions inhibit thermolysin in solution. Furthermore, Holmquist and Vallee (1974) have reported that the addition of zinc, in excess of that needed to restore activity to the apoenzyme, inhibits thermolysin, seemingly by binding to a specific inhibitory site. The present observation that in low concentrations, zinc, mercuric chloride, dimercury acetic acid, and *o*-chloromercuriphenol each inhibit thermolysin, combined with the crystallographic data, implicate His-231 as the site of heavy metal inactivation. The protection provided by these heavy metal compounds against inhibition by ethoxyformic anhydride (Figure 3) supports the hypothesis that the site of metal binding is also the histidine residue which upon ethoxyformylation inhibits the enzyme.

Inhibiting concentrations of $HgCl_2$ do not prevent adsorption of the enzyme to the affinity adsorbent even though the concentration of mercury was apparently sufficient for both zinc replacement and binding to His-231 (Colman et al., 1972; Holmquist and Vallee, 1974). Inhibition by ethoxyformylation, however, does prevent adsorption. These observations suggest that the site of binding of the -Gly-D-Phe ligand is sufficiently removed from His-231 to allow simultaneous binding of mercury to His-231 but not the larger ethoxyformyl group. These conclusions are consistent with spacings found in the active site of a model of thermolysin and the relative bulkiness of the two inhibitors.

These results support the proposition that His-231 is a part of the catalytic apparatus of thermolysin. Although we have not measured the pK_a of the binding site responsible for metal inhibition, the experiments with ethoxyformic anhydride indicate a pK_a of inhibition of 7.6, whereas catalysis by native enzyme depends upon a pK_a of 7.5. These values are higher than expected for a normal histidyl residue, but the electron density map of thermolysin reveals that His-231 is in a salt linkage with Asp-226 and that these two residues may ionize in an interdependent manner, e.g.:



$$\text{active (Asp}\cdots\text{H}\cdots\text{His}\cdots\text{H)}^0 \overset{pK_a = 7.5}{\rightleftharpoons} \text{inactive (Asp}\cdots\text{H–His)}^- + \text{H}^+$$

Matthews et al. (1972a) have already pointed out that this interaction may resemble that between Asp-102 and His-57 in the Asp···His···Ser "charge relay system" of chymotrypsin for which Robillard and Shulman (1972) reported a pK_a of 7.5, whereas Hunkapillar et al. (1973) reported a pK_a of 6.75 for a similar structure in α-lytic protease. Proposals of the mechanism of action of chymotrypsin have ascribed to His-57 the role of donating a proton to the departing amine (Henderson et al., 1972).

A Hypothesis for the Catalytic Mechanism. The above considerations suggest that His-231 of thermolysin is directly involved in catalysis, and that it is functional only in the protonated state, i.e. that it acts as a proton donor during catalysis. At this point it is fruitful to compare aspects of the active site of thermolysin with the active site of carboxypeptidase A (Figure 5). In both enzymes a zinc atom is coordinated in a tridentate structure of identical residues (His, His, Glu). Both enzymes have a hydrophobic pocket identified by its affinity for competitive inhibitors, and both enzymes possess a glutamyl residue in the active site. Although these enzymes bear no overall structural resemblance, this glutamyl residue (Glu-143) is located and oriented in thermolysin in precisely the same spatial relation to the zinc atom as is Glu-270 in carboxypeptidase A (Colman et al., 1972). In carboxypeptidase the peptide bond probably undergoes either a direct nucleophilic attack by Glu-270 (Lipscomb et al., 1969), or a general base attack through a water molecule as shown in Figure 5. A similar role can be proposed for Glu-143 in thermolysin, but direct chemical evidence is lacking. Both enzymes depend upon the ionization of a single residue (pK_a = 6.2 for carboxypeptidase A [Auld and Vallee, 1970] and pK_a = 5.9 for thermolysin) which may be the respective glutamyl residue in each. The clearest difference between the active sites of the two enzymes is that a tyrosine residue is proposed as the proton donor in carboxypeptidase and no corresponding tyrosine residue is similarly oriented in thermolysin. However, the

location of His-231 and the evidence that its *protonated* state is required for catalysis indicate that His-231 could be a proton donor during catalysis by thermolysin.

This proposal is illustrated on the right in Figure 5. The susceptible peptide bond, which has been polarized by coordination of the carbonyl oxygen to the zinc, is attacked by Glu-143. This attack may be mediated by a water molecule with Glu-143 acting as a general base. A concerted attack is depicted in which a proton transferred from His-231 to the amino nitrogen completes the scission of the peptide bond. (If Glu-143 attacks the carbonyl carbon directly, scission would result in an anhydride intermediate which would be hydrolyzed by water.)

Acknowledgments

The authors are grateful to Dr. Yigal Burstein for discussions of the chemical data, to Dr. Brian Matthews (University of Oregon) for discussions of the three-dimensional structure of thermolysin, and to Dr. Hans Neurath for support, advice, and helpful discussions.

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