Evidence of an Essential Histidine Residue in Thermolysin†

Yigal Burstein,‡ Kenneth A. Walsh, and Hans Neurath*

ABSTRACT: Thermolysin is inhibited by ethoxyformic anhydride in the pH range of pH 6–8. The inhibition is reversed by hydroxylamine and prevented by the competitive inhibitor Cbz-L-phenylalanine. Although several ethoxyformyl groups are introduced into the enzyme molecule, inactivation can be correlated with modification by a single group. Spectral analysis indicates that the removal of an ethoxyformyl group from one histidine residue per molecule is responsible for the reactivation of the enzyme.

Thermolysin is an extracellular, thermostable proteolytic enzyme from *Bacillus thermoproteolyticus* (Endo, 1962; Ohta *et al.*, 1966). The enzyme is a neutral metalloendopeptidase which catalyzes the hydrolysis of peptide bonds on the amino side of hydrophobic residues (Matsubara *et al.*, 1965, 1966; Matsubara, 1966; Morihara and Tsuzuki, 1966, 1970; Bradshaw, 1969).

The amino acid sequence of thermolysin has been described by Titani *et al.*, (1972), and the three-dimensional structure by Matthews *et al.* (1972a,b). Colman *et al.* (1972) have identified nine amino acid residues in the region of the active site of thermolysin, *i.e.*, three histidines, two aspartic acids, two glutamic acids, one tyrosine, and one arginine. Two of the three histidine residues (residues 142 and 146) are zinc ligands and the third, His-231, appears to be linked to Asp-226 by a salt bridge.

The enzyme is inhibited by metal-chelating agents, but not by inhibitors of serine proteases or sulfhydryl enzymes. Rose Bengal catalyzed photooxidation inactivates thermolysin (Abe *et al.*, 1971) and the inhibition can be correlated with the oxidation of one or two histidine residues.

The present study describes the inhibition of thermolysin by ethoxyformic anhydride and provides evidence of the importance of a histidine residue for the activity of the enzyme.

Experimental Procedures

Materials. Crystalline thermolysin was purchased from Daiwa Kasei K. K., Osaka, Japan; ethoxyformic anhydride was a product of Eastman Kodak Co.; [^{14}C]ethoxyformic anhydride (5.9×10^{-2} Ci/mol) was a gift from Dr. D. Fahrney; *N*-acetyl-*O*-ethoxyformyl-L-tyrosine ethyl ester was synthesized according to Melchior and Fahrney (1970); Cbz-L-phenylalanine, Cbz-glycine, and *N*-acetylglycine were purchased from Fox Chemical Co., Hepes,[1] Pipes and Tris from Calbiochem, and FAGLA from Cyclo Chemical Co. Buffers used were acetate (pH 5.0–6.3), Pipes (pH 6.3–7.0), Hepes (pH 7.0–8.0), and Tris (8.0–9.0). An affinity chromatography column (1.5 × 5.0 cm) was prepared from Adsorbent 1 of Pangburn *et al.* (1973). This adsorbent contains a Gly-D-Phe ligand attached to Sepharose 4B by a spacer of 23 atoms.

Analytical Procedures. Optical density measurements were performed with a Cary Model 16 spectrophotometer; difference spectra were measured with a Cary Model 15. Thermolysin concentration was calculated from absorption measurements using ϵ_M of 61,000 at 280 nm (Pangburn *et al.*, 1973). Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. An efficiency of 66% was observed with 1 ml of an aqueous sample dispersed in 10 ml of Aquasol scintillant (New England Nuclear).

Enzyme Assay. Thermolysin-catalyzed hydrolysis of FAGLA was monitored at 345 nm in a thermostated cell compartment of the Cary Model 16 spectrophotometer at 25° according to Feder (1968). The substrate (1 mM) was dissolved in 0.1 M Hepes, 5 mM $CaCl_2$, and 10^{-5} M $ZnCl_2$ (pH 7.2) containing 0.5% dimethylformamide. Caseinolytic activity was assayed according to Keay and Wildi (1970).

Kinetics of Inactivation. Thermolysin (0.5–1.0 mg/ml) was dissolved in solutions containing 0.1 M NaCl, 5 mM $CaCl_2$, 2 mM buffer, and suitable concentrations of a competitive inhibitor. The enzyme solution was incubated at 25° in a pH-Stat and a solution of ethoxyformic anhydride (0.3 M) in ethanol was added to a final concentration of 3 mM to initiate the reaction. Aliquots were removed to measure remaining enzymatic activity. The pseudo-first-order rate constant for inactivation (k_{app}) was calculated from the initial rates of inhibition according to eq 1, where E_1 and E_2 are

$$\ln (E_1/E_2) = k_{app}(T_2 - T_1) \quad (1)$$

the observed activities at times T_1 and T_2. The modified enzyme was separated from excess reagent and reaction products by gel filtration on a column (1.6 × 18 cm) of Sephadex G-25 equilibrated with 20 mM acetate–5 mM $CaCl_2$ (pH 6.0).

Incorporation of Radioactivity. To a solution of thermolysin (1 mg/ml) in 0.1 M NaCl, 5 mM $CaCl_2$, 25 mM buffer, and where indicated a competitive inhibitor (5 mM), a solution of [^{14}C]-ethoxyformic anhydride (3 mM final concentration) was added at 25°. After 45 min, an aliquot (1 ml) was removed and the protein was separated from small molecules by gel filtration as described earlier. Two other aliquots were separately treated at pH 7 with 20 mM hydroxylamine (1 hr) or 1.0 M hydroxylamine (24 hr) and similarly separated from reagents. The protein fractions were pooled and the protein concentration, enzymatic activity, and radioactivity were determined. These procedures were repeated after 60 min of inactivation.

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‡ On leave from the Weizmann Institute of Science, Rehovot, Israel.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); FAGLA, furylacryloylglycyl-L-leucinamide.

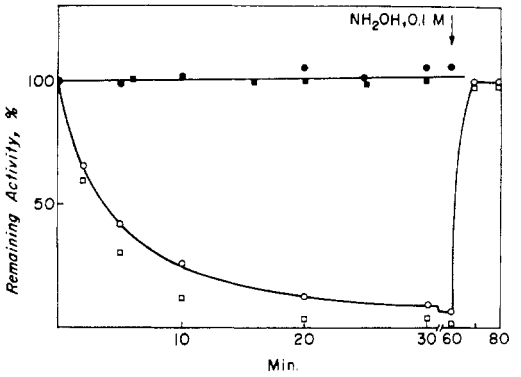

FIGURE 1: Inactivation of thermolysin (1.5×10^{-5} M) by ethoxyformic anhydride (3 mM) in 5 mM $CaCl_2$–20 mM Hepes (pH 7.2) at 25° in the presence (●, ■) and in the absence (○, □) of 5 mM Cbz-L-phenylalanine. Activity was measured towards FAGLA (●,○) and casein (■,□).

Results

Ethoxyformylation of Thermolysin. When thermolysin was incubated with 3 mM ethoxyformic anhydride at pH 7.2, over 90% of the enzymatic activity towards both FAGLA and casein was lost in 45 min (Figure 1). The remaining activity could be abolished by adding an equal aliquot of reagent. Both activities were rapidly restored by 0.1 M hydroxylamine (5 min) or by 20 mM hydroxylamine (30 min) at pH 7.2.

The enzyme was protected against inactivation by the competitive inhibitor Cbz-L-phenylalanine (5 mM), as shown in Figure 1. Acetylglycyl-L-leucine and acetyl-L-phenylalanine (in concentrations of 5–20 mM) provided only partial protection while acetylglycylglycine (20 mM) was ineffective.

Further evidence for the modification of residue(s) of the active site was obtained by the observation (Figure 2B) that *inactive* ethoxyformyl-thermolysin was not adsorbed by the affinity chromatography column.[2] In contast, the enzyme which was ethoxyformylated in the presence of Cbz-L-phenylalanine was *active*, and could be adsorbed and eluted (Figure 2C) under the same conditions as the native enzyme (Figure 2A). The chromatographic elution pattern of hydroxylamine-*reactivated* enzyme (20 mM hydroxylamine) was also identical with that of the native enzyme.

The extent of inactivation of thermolysin by ethoxyformic anhydride is dependent on pH as shown in Table I. The data can be expressed by

$$k_{app} = \frac{k_{max}}{1 + [H^+]/K_a} \quad (2)$$

which in linear form becomes

$$k_{app}[H^+] = K_a k_{max} - K_a k_{app} \quad (3)$$

where K_a is the dissociation constant of a reacting group and k_{max} is the second-order rate constant when the reacting group is unprotonated. By plotting $k_{app}[H^+]$ *vs.* k_{app} (values taken from Table I), a straight line was obtained. Values of $k_{max} = 0.91 \pm 0.01$ min^{-1} and $pK_a = 7.6 \pm 0.2$ were calculated from the ordinate intercept and the slope, respectively, using the least-squares method.

[2] The affinity column was prepared by coupling acetyl-D-phenylalanine to Sepharose through a spacer molecule; for details, see Pangburn *et al.* (1973).

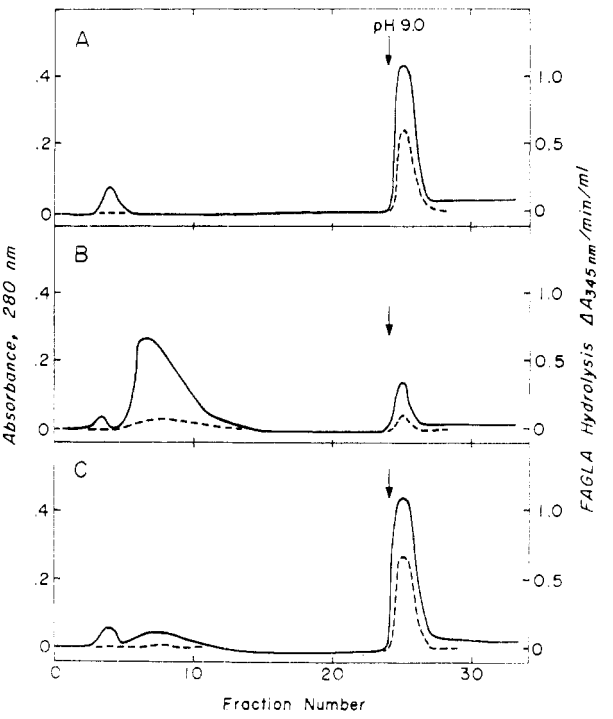

FIGURE 2: Affinity chromatography of thermolysin and ethoxyformyl-thermolysin. A column of adsorbent (see Materials) was equilibrated with 0.1 M NaCl, 5 mM $CaCl_2$, and 0.01 M Hepes (pH 7.0). The enzyme (1.5–2.0 mg) was applied in 2.0 ml of this buffer and the column was washed at a rate of 48 ml/hr for 40 min. Fractions of 1.5–2.0 ml were collected and assayed with FAGLA (broken line). Bound protein (solid line) was eluted with 0.1 M Tris–5 mM $CaCl_2$ (pH 9.0): (A) native thermolysin; (B) thermolysin, inactivated with ethoxyformic anhydride; (C) thermolysin treated with ethoxyformic anhydride in the presence of Cbz-L-phenylalanine.

TABLE I: Kinetics of Inactivation of Thermolysin by Ethoxyformic Anhydride.[a]

pH	k_{app} (min^{-1})
6.2	0.033
6.6	0.076
6.9	0.14
7.1	0.21
7.6	0.42
8.0	0.64

[a] Thermolysin (1.08×10^{-5} M) was treated with ethoxyformic anhydride (3 mM) in 0.1 M NaCl, 5 mM $CaCl_2$, and 2 mM buffer at 25°. The pH was maintained in a pH-Stat by the addition of 0.2 M NaOH.

Figure 3 illustrates the dependence on pH of the pseudo-first-order rate constant of the inhibition of thermolysin by ethoxyformic anhydride. The solid line represents the theoretical curve calculated from eq 2 assuming one ionizing group having $pK_a = 7.6$ and $k_{max} = 0.91$ min^{-1}.

Incorporation of [^{14}C]Ethoxyformic Anhydride into Thermolysin and Removal by Hydroxylamine. The incorporation of [^{14}C]ethoxyformyl groups into thermolysin was a function of the initial concentration of the reagent and of pH. At a reagent concentration of 3 mM, 3.5 groups were incorporated at pH 5.7, whereas 9.5 and 13.4 groups were incorporated at pH 6.8 and 7.2, respectively.

TABLE II: Incorporation of [^{14}C]Ethoxyformyl Groups into Thermolysin.

Condns of Incorpn[a]	Act. Remaining[b] (%)	Ethoxyformyl Groups Incorpd[c]	Treatment with 20 mM NH_2OH for 1 hr: Act. Regained (%)	Treatment with 20 mM NH_2OH for 1 hr: ^{14}C Groups/ Molecule Removed	Treatment with 1 M NH_2OH for 24 hr: ^{14}C Groups/ Molecule Removed	Treatment with 1 M NH_2OH for 24 hr: ^{14}C Groups/ Molecule Remaining
pH 5.7						
45 min	90	3.5	97	0.7	1.2	2.3
60 min	92	3.5	98	0.7	1.3	2.2
pH 7.2						
45 min	<10	13.3	100	2.1	5.3	8.0
60 min	<10	13.4	98	2.1	5.3	8.1
pH 7.2 + Cbz-L-Phe[d]						
45 min	94	12.5	101	1.3	4.3	8.2
60 min	95	12.4	99	1.1	4.3	8.1

[a] Enzyme (1 mg/ml) in 0.1 M NaCl, 5 mM $CaCl_2$, 25 mM buffer, and 3 mM ethoxyformic anhydride at 25°. [b] Activity toward FAGLA. [c] Residues per molecule. For reaction conditions and isolation procedures, see Experimental Procedures. [b] Cbz-L-phenylalanine, 5 mM.

At pH 5.7 ethoxyformylation abolished less than 10% of the activity of thermolysin. Of the 3.5 ethoxyformyl groups incorporated, 0.7 group could be removed by mild hydroxylamine treatment (20 mM for 60 min) and about 0.5 additional group could be removed by the more drastic treatment (1 M for 24 hr). The remaining 2.3 groups appeared to be stable in hydroxylamine.

At pH 7.2 over 90% of the activity of the native enzyme was abolished upon ethoxyformylation (Table II). The competitive inhibitor Cbz-L-phenylalanine protected the enzyme from inhibition and *prevented the incorporation of 0.9 ethoxyformyl group* into the enzyme (13.4 groups were incorporated in the absence of Cbz-L-phenylalanine and 12.5 groups were incorporated in its presence).

Mild hydroxylamine treatment fully restored the activity of the inhibited enzyme and removed 2.1 ethoxyformyl groups from the inhibited enzyme. The same treatment removed only 1.2 ethoxyformyl groups from *active* ethoxyformyl-thermolysin (which had been modified in the presence of the competitive inhibitor). Thus a single ethoxyformyl group appears to be responsible for the inactivation of thermolysin and reactivation results from removal of this group by mild hydroxylamine treatment. More drastic treatment removed about 3.2 additional ethoxyformyl groups from both ethoxyformyl thermolysin derivatives (pH 7.2), leaving each modified enzyme molecule with about 8.1 stable ethoxyformyl groups.

Although inactivation and reactivation of the enzyme were ascribed to a single ethoxyformyl group, this correlation rested upon small differences (0.9–1.2 groups) between large numbers (11–13 groups). The correlation was confirmed by examining the [^{14}C]ethoxyformylation of thermolysin which had previously been treated with *nonradioactive* ethoxyformic anhydride in the presence of Cbz-L-phenylalanine. In this experiment, 5.4 [^{14}C]ethoxyformyl groups were introduced and only 1.0 of these was removed during reactivation with 20 mM NH_2OH (Table III). A control of inactive thermolysin (ethoxyformylated in the absence of Cbz-L-phenylalanine) incorporated 4.5 groups but no radioactivity was released during treatment with 20 mM NH_2OH (Table III). In another control, inactive thermolysin was first reactivated with NH_2OH and then inactivated with [^{14}C]ethoxyformic anhydride. As in the control experiment in Table II, more than one group (1.7) was released by hydroxylamine, indicating additional sites of acylation unrelated to inactivation.

Spectral Changes Accompanying Inactivation and Reactivation. Figure 4A illustrates the changes in the absorption spectrum of thermolysin during ethoxyformylation at pH 7.2. The absorption at 278 nm decreased while that at 242 nm increased markedly. The increase at 242 nm is indicative of histidine modification (Ovádi *et al.*, 1967; Mühlrád *et al.*,

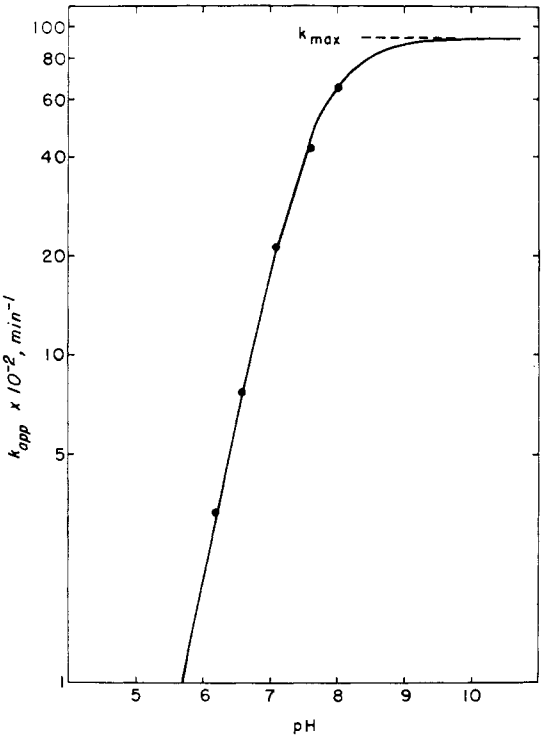

FIGURE 3: Dependence on pH of the pseudo-first-order rate constant for the inhibition of thermolysin (1.5×10^{-5} M) by ethoxyformic anhydride (3 mM) at 25°. Values of k_{app} were taken from Table I. The line is a theoretical curve calculated from eq 2, assuming a single ionizing group of $pK_a = 7.6$ and $k_{max} = 0.91$ min^{-1}.

TABLE III: Incorporation of [^{14}C]Ethoxyformic Anhydride into Ethoxyformyl-thermolysin

Ethoxyformyl-thermolysin	Initial Enzymatic Act. (%)	Incorpn of [^{14}C]-Ethoxyformic Anhydride[a]		Treatment with 20 mM NH_2OH for 1 hr (pH 6.0)		Treatment with 1 M NH_2OH for 24 hr (pH 6.0)	
		Act. Remaining (%)	^{14}C Groups/ Molecule Incorpd	Act. Regained (%)	^{14}C Groups/ Molecule Removed	^{14}C Groups/ Molecule Removed	^{14}C Groups/ Molecule Remaining
Active[b]	105	9	5.4	107	1.0	2.9	2.5
Inactive[c]	9	7	4.5	93	<0.1	1.9	2.6
Reactivated[d]	95	10	5.9	106	1.7	3.5	2.4

[a] Treatment with 5 mM [^{14}C]ethoxyformic anhydride (pH 7.2) at 25°. [b] Previously treated with cold ethoxyformic anhydride (pH 7.2) in the presence of 5 mM Cbz-L-Phe as in Table II. [c] Inactivated with cold ethoxyformic anhydride (pH 7.2) as in Table II. [d] Treated with cold ethoxyformic anhydride (pH 7.2), then reactivated with 20 mM NH_2OH as in Table II.

1969; Melchior and Fahrney, 1970). The decrease in the absorption at 278 nm is similar to the changes observed upon O-acetylation or O-alkylation of tyrosine residues (Shaltiel and Patchornik, 1963; Simpson *et al.*, 1963). O-Ethoxyformylation of the model compound *N*-acetyl-L-tyrosine ethyl ester resulted in a difference spectrum which showed a minimum at 278 nm ($\Delta\epsilon_{278}$ = 1310 l mol^{-1} cm^{-1}), and a major decrease at wavelength below 240 nm (Figure 4B).

The time course of modification of the chromophores was investigated by spectral analysis at pH 7.0 (Figure 5). Within 30 min 85% of the activity was lost during treatment with 3 mM ethoxyformic anhydride, but the activity was restored by hydroxylamine (20 mM).

Modification of two tyrosine residues was followed by a *decrease* in absorption at 278 nm. These tyrosine residues were not deacylated during reactivation by the mild hydroxylamine treatment. Thus, a tyrosine residue cannot be directly involved in the inhibition of thermolysin by ethoxyformic anhydride.

The modification of histidine was followed by monitoring the *increase* of absorption at 242 nm. The change in the absorption corresponded to ethoxyformylation of 1.2 histidine residues. Hydroxylamine (20 mM) treatment removed about 0.85 ethoxyformyl group from this residue. At this pH, the initial rate of inactivation appeared to be slower than the initial rate of histidine modification, but the rate of reactivation of the enzyme was similar to the rate of removal of ethoxyformyl groups from the histidine. The discrepancy between the number of histidine residues ethoxyformylated and the number de-ethoxyformylated indicated that spectral changes during *inactivation* were not a reliable measure of the involvement of histidine. The measurements during inactivation are complicated by the fortuitous fact that the positive peak in the difference spectrum arising during ethoxyformylation of imidazole (232 nm) is very close to a negative peak arising during ethoxyformylation of *N*-acetyl-L-tyrosine ethyl ester (234 nm). Thus, when histidyl and tyrosyl residues are simul-

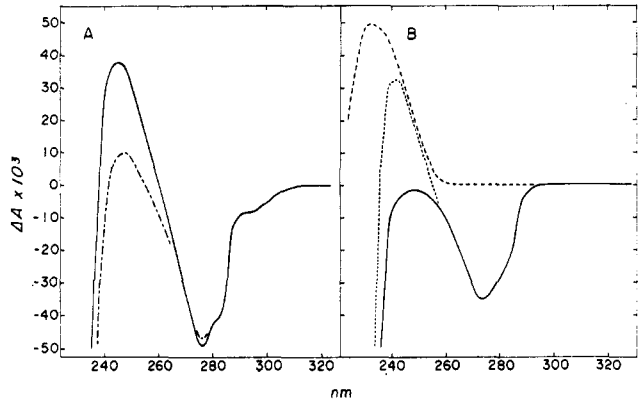

FIGURE 4: (A) Difference spectra after inactivation and reactivation of thermolysin. Solutions of the enzyme (1.5×10^{-5} M) in 2.5 ml of mM $CaCl_2$, 0.1 M NaCl, and 20 mM Hepes (pH 7.2) were placed in two spectrophotometer cells. Ethoxyformic anhydride (25 μl of a 0.3 M solution in ethanol) was added to one cuvette (and ethanol to the reference cuvette) and the difference spectrum was measured after 40 min (solid line). Hydroxylamine (25 μl of a solution of 2 M, pH 7.0) was then added to both cells and the difference spectrum was measured 40 min later (broken line). (B) Spectral changes of model compounds after treatment with ethoxyformic anhydride (1.5×10^{-5} M) in 25 mM Hepes (pH 7.0). Solid line: *N*-acetyl-L-tyrosine ethyl ester (5×10^{-5} M); broken line: imidazole (2 mM); dotted line: the algebraic sum of the two difference spectra.

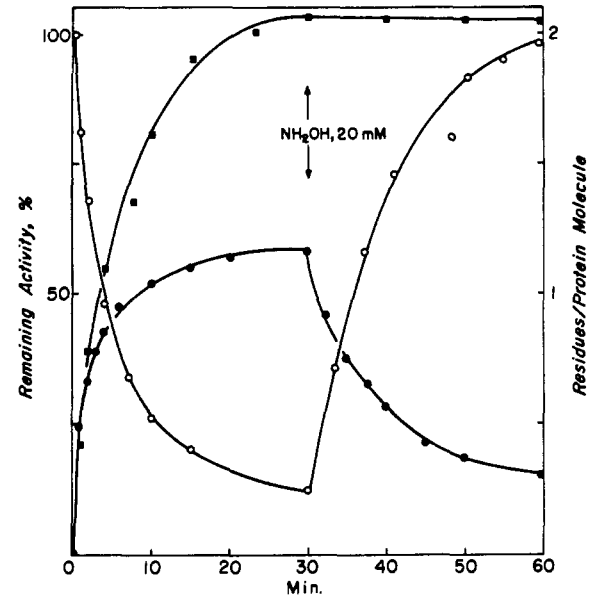

FIGURE 5: Modification of chromophores during reaction of thermolysin (1.5×10^{-5} M) with ethoxyformic anhydride (3 mM) in 25 mM Hepes (pH 7.2) at 25° followed by reactivation with hydroxylamine (20 mM). Enzymatic activity was measured with FAGLA (○). Modification of tyrosine residues was followed by the decrease in the absorption at 278 nm (■) using $\Delta\epsilon_{278}$ = 1310 l. mol^{-1} cm^{-1}. Modification of histidine residues was followed by the increase in absorption at 242 nm (●) using $\Delta\epsilon_{242}$ = 3200. The course of histidine Modification during *reactivation* with NH_2OH is more reliable than that during *inactivation* (see text).

taneously modified, the combined difference spectrum shows an anomalously small change in the apparent histidine modification (Figure 4B). It is possible that as many as two histidyl residues are modified and that only one is deacylated by 20 mM hydroxylamine.

Spectral changes during *reactivation* of the inhibited enzyme by mild hydroxylamine treatment are much easier to interpret because *O*-ethoxyformyltyrosyl residues were not hydrolyzed by this treatment, and ΔA_{238} can be related to the removal of ethoxyformyl groups from histidyl residues. We chose, therefore, to follow the changes in the difference spectrum during reactivation of the modified enzyme. The results are presented in Table IV and Figure 6. Thermolysin modified

TABLE IV: Extent of Tyrosyl and Histidyl Modification During Ethoxyformylation of Thermolysin and Its Reversal with Hydroxylamine.

Ethoxyformylation[a]			Hydroxylamine Treatment[b]		
				Residues Deethoxyformylated	
Conditions[c]	Act. Remaining[d] (%)	Tyr Modified[e]	Act. Restored (%)	Tyr[e]	His[f]
pH 5.5	99	0.2	100	0	0
pH 7.2 + Cbz-L-Phe[g]	98	2.1	101	0	0
pH 7.2	9	2.1	98	0	0.9

[a] Thermolysin (1 mg/ml) was treated with 3 mM ethoxyformic anhydride for 60 min in 5 mM $CaCl_2$ and 20 mM buffered solutions. The modified protein was isolated by gel filtration on Sephadex G-25 columns equilibrated with 20 mM NaOAc–5 mM $CaCl_2$ (pH 5.5). [b] 20 mM, at pH 5.5 for 1 hr. [c] Buffers were acetate (pH 5.5) and Hepes (pH 7.2). [d] Activity toward FAGLA. [e] Residues per molecule, determined from the changes in absorption at 278 nm. [f] Residues per molecule, determined from the decrease in absorption at 238 nm (Figure 6). [g] Cbz-L-phenylalanine, 5 mM.

at pH 5.5 had not lost enzymatic activity and showed no spectral change when treated with 20 mM hydroxylamine. Ethoxyformyl-thermolysin, prepared at pH 7.2 in the presence of Cbz-L-phenylalanine, was fully active and again no spectral changes were observed upon treatment with 20 mM hydroxylamine (1 hr). Ethoxyformyl-thermolysin, prepared at pH 7.2 in the absence of the competitive inhibitor, was inactive and treatment with 20 mM hydroxylamine reactivated the enzyme. The accompanying spectral changes indicate loss of one ethoxyformyl group from a histidine residue (Figure 6).

Inactive ethoxyformyl-thermolysin was relatively stable at neutral pH ($\tau_{1/2}$ = 2.5 days in 10 mM Hepes, pH 7.0), but treatment with hydroxylamine (20 mM) rapidly regenerated enzyme activity. At pH 7.0 full activity toward FAGLA or casein was regained within 30 min; at pH 5.5 activity was regained in 12 min.

Discussion

Ethoxyformic anhydride reacts in proteins with several amino acid side chains such as phenolates, imidazoles, carboxylates, sulfhydryls, α- and ϵ-amines and guanidino groups

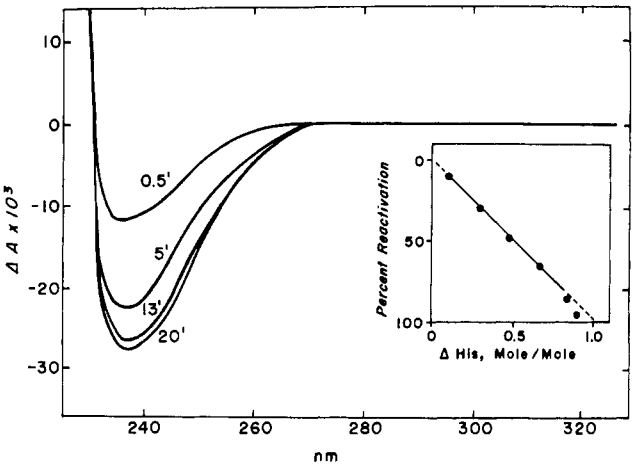

FIGURE 6: Difference spectra of ethoxyformyl-thermolysin during reactivation with hydroxylamine. Ethoxyformyl-thermolysin (10^{-6} M, 90% inactive) was dissolved in 5 mM $CaCl_2$–25 mM acetate (pH 5.7). At zero time 20 mM hydroxylamine (pH 5.7) was added to one cuvette and an equal volume of buffer to the reference cuvette. Insert: regeneration of activity during treatment with hydroxylamine plotted against the release of histidyl residues as measured by the difference spectra at 238 nm (ϵ_M 3200 cm^{-1}).

(Larrouquère, 1964; Mühlrád *et al.*, 1967; Melchior and Fahrney, 1970). It was not surprising, therefore, to find that many (13.5) ethoxyformyl groups per molecule were incorporated into thermolysin upon treatment with ethoxyformic anhydride at pH 7.2. Control experiments, however, indicated that only one of these groups seems to be responsible for the loss of activity. Spectral changes during reactivation with hydroxylamine verified the observation that modification of a single residue had caused the inactivation.

The competitive inhibitor Cbz-L-phenylalanine protects the enzyme from inhibition by [^{14}C]ethoxyformic anhydride and prevents the incorporation of a single ethoxyformyl group into the modified enzyme. Since Matthews and coworkers[3] have shown that Cbz-L-phenylalanine binds to the active site of thermolysin, it follows that the single residue modified lies within the active site of thermolysin. The pH dependence of the inactivation confirmed that loss of activity was correlated with only one site of modification having a pK_a of 7.6.

The spectral changes of thermolysin during ethoxyformylation are characteristic of the modification of both histidine and tyrosine residues. Since the spectral changes relating to tyrosine modification (278 nm) are not prevented by the competitive inhibitor (Cbz-L-phenylalanine) ethoxyformylation of a tyrosine residue does not appear to be the cause of enzyme inactivation.

The effect of Cbz-L-phenylalanine on the ethoxyformylation of histidine residues could not be measured accurately due to the absorption of Cbz-L-phenylalanine at that particular wavelength.

No spectral changes were observed at 278 nm during reactivation with hydroxylamine, thus confirming the conclusion that tyrosine residues were not deacylated by this treatment and that their acylation could not have been the cause of inactivation. The reversibility of inactivation appears to rule out lysyl and arginyl residues as functional sites of modification, since the corresponding derivatives should be resistant to nucleophilic attack (Mülrád *et al.*, 1967; Melchior and Fahrney, 1970). Furthermore, Cbz-L-phenylalanine did not prevent

[3] Private communication.

the incorporation of "stable"[4] ethoxyformyl groups into thermolysin (8.1 groups, Table II), suggesting that none of the ethoxyformylated lysine or arginine residues is located at the active site of the enzyme.

Only deacylation of histidine could be correlated with the reactivation reaction. Spectral changes in the 235–250-nm range indicated that the extent of release of the reagent from a single histidyl residue was proportional to the extent of reactivation by dilute hydroxylamine (Figure 6). The spectral changes that occur during reactivation, unlike those that accompany inactivation, were free of interference by concurrent tyrosine deacylation. Moreover, controls of *active* ethoxyformyl-thermolysin (modified at pH 5.7 or in the presence of Cbz-L-Phe) revealed no spectral changes at 238 nm when treated with 20 mM hydroxylamine. Radioactivity data indicate that two ethoxyformyl groups were released during reactivation and one of these must be from histidine. The location of the other is unknown, but control experiments which included Cbz-L-Phe provide evidence that this second group can be both introduced and released without altering the enzymatic activity.

Because of the limited stability of ethoxyformylhistidine residues it did not seem to be possible to isolate the modified histidine peptide and to identify the specific histidyl residue whose modification is responsible for loss of activity. The indirect proof discussed above does indicate that the integrity of a single histidyl residue in the active site of thermolysin is essential for enzymatic activity. The same conclusions have been independently reached by Blumberg *et al.* (1973) in a concurrent investigation of this problem. Matthews and coworkers (Colman *et al.*, 1972) have identified three histidine residues in the active site of thermolysin. Two of these bind zinc, and the third, His-231, can bind a silver ion which inhibits the enzyme. His-231 is likely to be the site of interaction with the present reagent, especially since the reactivity of this histidine residue may be accentuated by a neighboring aspartyl residue (Asp-226).

Recent investigations (Walsh *et al.*, 1973) have shown that two neutral proteases of *Bacillus subtilis* are similarly inactivated by ethoxyformic anhydride and reactivated by hydroxylamine. Since thermolysin and these neutral proteases contain homologous amino acid sequences, it appears that their active sites are structurally and functionally similar as well.

Acknowledgment

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[4] Stable during treatment with 1 M hydroxylamine (pH 5.5) for 24 hr.

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