REVERSIBLE INACTIVATION AND SUPERACTIVATION BY COVALENT MODIFICATION OF THERMOLYSIN*

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Diethylpyrocarbonate (DEP) in the pH range 6.1 - 7.5 inactivates thermolysin by ethoxyformylation. Restoration of activity by hydroxylamine at pH 6.2 correlates with the regeneration of a single histidyl residue. Exposure of the enzyme to DEP together with the reversible inhibitor β-phenylpropionyl-L-phenylalanine, or acylation with the mixed anhydride of β-phenylpropionyl-L-phenylalanine and ethoxyformic acid or with β-phenylpropionyl-L-phenylalanyl-imidazole increases the activity by an order of magnitude toward both peptide and ester substrates. Treatment with NH_2OH restores the catalytic properties of this superactive derivative to that of the native enzyme while modification with DEP destroys activity completely.

Previous studies in this laboratory have shown that thermolysin, a neutral protease from B. thermoproteolyticus, contains 1 g at of catalytically essential Zn as well as 4 g at of Ca per mole of enzyme (1). Chemical modification with diethylpyrocarbonate (DEP) now demonstrates that a histidyl residue is also essential to the catalytic function of the enzyme. Further, these studies indicate the existence of another residue which, when suitably modified, results in an exceptionally large increase in activity, a "superactivation" (2).

MATERIALS AND METHODS: Thermolysin (Calbiochem, A grade, lot 200397) was recrystallized and assayed as described (1). Stock solutions, 8×10^{-4} M, were prepared by dissolving water-washed crystals in 2.5 M NaBr, 0.01 M $CaCl_2$, 0.01 M Tris, pH 7.5.

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Diethylpyrocarbonate (Sigma Chemical Corp.) was redistilled before use. N-Acetyl-O-ethoxyformyl-L-tyrosine ethyl ester and N-ethoxyformyl-imidazole were prepared as described (3). The reversible inhibitor β-phenylpropionyl-L-phenylalanine, (m.p. 162° - 163°) and the depsipeptide benzoyl-glycyl-L-phenyllactyl-L-alanine, (m.p. 167° - 168°) were prepared from commercially available intermediates using the N-hydroxysuccinimide ester method (4). The mixed anhydride of β-phenylpropionate-L-phenylalanine and ethoxyformic acid, 0.2 M, was prepared prior to use by mixing a 10% molar excess of the former with ethylchloroformate and one equivalent of triethylamine in ice-cold DMF. The synthesis of β-phenylpropionyl-L-phenylalanine imidazole (m.p. 98° - 103°) was carried out by reacting equimolar amounts of the inhibitor and carbonyldiimidazole (5) in ice-cold CH_2Cl_2 and recrystallizing from ethyl acetate.

Modifications of thermolysin were performed routinely in the pH-stat at 25°. The reaction mixture contained 2 ml of enzyme, 4×10^{-5} M, 0.20 M NaCl, 0.12 M NaBr, 0.01 M $CaCl_2$ and 5×10^{-4} M Tris. Inactivation was initiated by the addition of freshly prepared 10-20% solutions of DEP in peroxide-free dioxane; its concentrations never exceeded 1% of the total volume. Absorption spectra were obtained with a Cary 14 spectrophotometer. Modified thermolysins were separated from reagents and products by gel filtration on a Biogel P-4 column (0.9 x 20 cm) equilibrated with 0.2 M NaCl, 0.01 M $CaCl_2$, 2 mM Tris, pH 7.1.

RESULTS AND DISCUSSION: DEP has been reported to be a highly reactive acylating agent for catalytically essential histidyl residues of a number of enzymes. The products are stable and exhibit characteristic spectra (6-8). DEP inactivates thermolysin at rates and to a degree determined both by its concentration and the pH of modification. Variation in DEP concentrations from 2 to 16 mM progressively decreases activity from 55 to 3 per cent of that of the native enzyme when the reaction is carried out at pH 6.5 for 30 minutes. The initial rate and

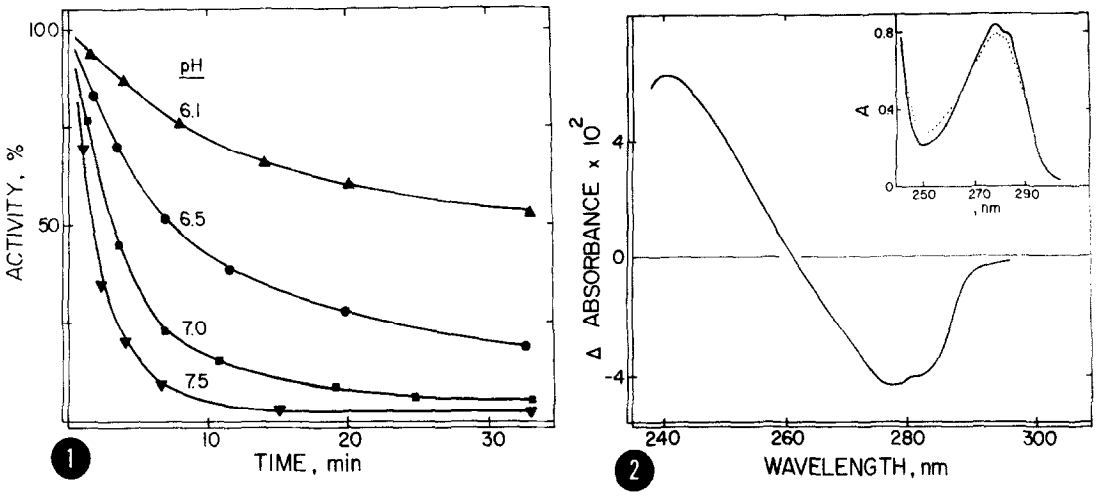


Fig. 1. Inactivation of thermolysin by DEP, 4 mM, as a function of pH. Conditions as described under Materials and Methods. Assays were performed with furylacryloyl-glycyl-L-leucineamide (FAGLA) (1).

Fig. 2. Difference absorption spectrum of ethoxyformylthermolysin versus thermolysin, both 1.3 x 10^{-5} M. Ethoxyformylthermolysin, exhibiting 10% of the activity of the native enzyme, was obtained by treating the enzyme with DEP, 8 mM, pH 6.5 for 30 min. INSERT: Absorption spectra of ethoxyformylthermolysin (···) and thermolysin (——).

extent of inactivation increase on raising the pH from 6.1 to 7.5 (Fig. 1).

Significant spectral alterations accompany these changes in activity. Ethoxyformylation of histidyl residues of proteins increases absorbance at 242 nm, $\Delta\varepsilon_{242}$ = 3200 (9), and such changes were observed with thermolysin (Fig. 2). In addition, the absorbance at 278 nm decreases suggesting tyrosine modification. Indeed, similar to O-acetylation, O-ethoxyformylation markedly decreases the absorbance of N-acetyl-L-tyrosine ethyl ester at 278 nm, $\Delta\varepsilon_{278}$ = 1380. Under the conditions here employed to inactivate thermolysin DEP modifies both histidyl and tyrosyl residues of the enzyme.

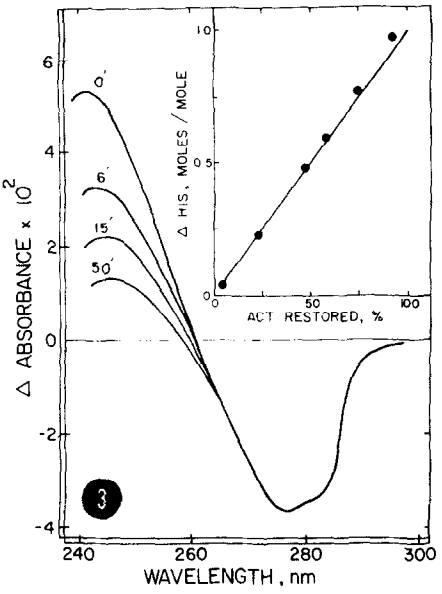
As a step in differentiating their functional roles, the enzyme, 4 x 10^{-5} M, was treated with N-acetylimidazole, 20 mM, pH 7.5, 25° for 30 minutes resulting in acetylation of 9 tyrosyl residues but without significantly affecting activity. However, subsequent exposure to DEP

completely abolishes the activity of O-acetylthermolysin, a first indication that this reagent inactivates the enzyme by modifying a residue other than tyrosine.

The inactivation of thermolysin with DEP is completely reversible, and the kinetics and spectral changes accompanying the reaction identify the critical amino acid residue. Thermolysin, 4×10^{-5} M, was exposed to DEP, 8 mM, pH 6.5 for 30 minutes. The product exhibits 10 per cent of the activity of the native enzyme and contains 1 g at of Zn, subsequent to purification by gel filtration. Incubation with Tris, 2 mM, pH 7.1 slowly restores activity to that of the native control with a T 1/2 $\sim$ 1 week. Other nucleophiles can substitute for Tris, and hydroxylamine is particularly effective in this regard.

The rate of restoration of the activity with NH_2OH increases sharply as the pH decreases from pH 7.6 to 6.0. In the presence of NH_2OH, 0.014 M, at pH 7.6, the T 1/2 is about 50 minutes while it is about 5 minutes at pH 6.2, 25°. Importantly, acid catalyzes the NH_2OH promoted deacylation of ethoxyformylimidazole but not that of N-acetyl-O-ethoxyformyl tyrosine ethyl ester, suggesting that the inactivation of thermolysin by DEP is due to modification of a histidyl residue. Indeed, exposure of ethoxyformyl-thermolysin to NH_2OH, 0.02 M, pH 6.2, concurrently regenerates 1 histidyl residue per mole of enzyme and full activity (Fig. 3). Thus, ethoxyformylation and subsequent deacylation of a single histidine would appear to account for the reversible inactivation and reactivation of the enzyme.

Few instances have presented the opportunity to inspect the molecular basis of significant reversible <u>increases</u> in activity of enzymes subsequent to covalent modification. Remarkably, thermolysin has also proven to be an exceptionally suitable system for this purpose since we have found a number of reagents which covalently modify thermolysin to increase its activity by an order of magnitude, a veritable "superactivation" toward both the peptide and the ester substrate here employed.



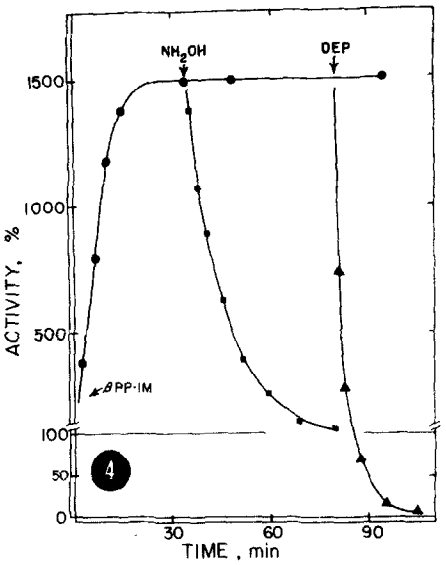


Fig. 3. Difference spectra of ethoxyformylthermolysin versus thermolysin, both 1.3 x 10^{-5} M, on deacylation with NH_2OH, 0.02 M, pH 6.2. Activity of the modified enzyme was 10% of that of the native enzyme.
INSERT: Activity, restored by treatment with NH_2OH, correlated with the number of histidines regenerated based on $A_{242} = 3200$ (9).

Fig. 4. Superactivation of thermolysin by β-phenylpropionyl-L-phenylalanylimidazole (β-PP-IM) and its reversal by NH_2OH. Modification was performed with 1 mM β-PP-IM, pH 7.5 in 2% DMF. Other conditions as under Materials and Methods. The enzyme was gel filtered after 35 min of reaction and then treated with NH_2OH, 0.1 M, pH 7.5, or with DEP, 8 mM, pH 6.5. Assays were performed with FAGLA.

The activity of the enzyme increases 8-fold after 80 minutes when exposed simultaneously both to the reversible inhibitor β-phenyl-L-phenylalanine .06 M and DEP, 16 mM at pH 6.5. This remarkable activation, thought to be the consequence of acylation by a reactive mixed anhydride formed between the inhibitor and DEP, is enhanced when different reactive derivatives of the inhibitor are employed. Thus, the mixed anhydride of β-phenylpropionyl-L-phenylalanine and ethoxy formic acid or β-phenylpropionyl-L-phenylalanylimidazole acylate the enzyme and superactivate it 15-fold (Fig. 4). NH_2OH, 0.1 M, pH 7.5, 25°, restores the activities of such superactive thermolysins to that of the native enzyme. This process follows pseudo-first order kinetics with a T 1/2 $\simeq$ 8 min,

accompanied by an increase in absorbance at 278 nm. DEP completely inactivates such superactive enzymes. Further, prior O-acetylation of tyrosyl residues with N-acetylimidazole suppresses superactivation.

The data suggest that the residue(s) involved in superactivation differ from that involved in inactivation. Lysines would seem to be excluded since hydroxylamine does not ordinarily cleave amide bonds. The data available point to tyrosine and we are attempting currently to differentiate between this and other alternatives. Studies are underway also to determine the kinetic basis for the activity changes and to locate the residues modified in the primary sequence and in the three-dimensional structure of the protein (10-12). While the present data are clearly insufficient to permit conclusions with respect to any particular residues modified, it is intriguing to note that both Tyr 157 and His 231 are in close proximity to the active site zinc atom (11,12).

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