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Superactivation of Thermolysin by Acylation with Amino Acid *N*-Hydroxysuccinimide Esters†

Shmaryahu Blumberg and Bert L. Vallee*

ABSTRACT: Synthesis of a series of active *N*-hydroxysuccinimide esters of aliphatic and aromatic amino acids has yielded a new class of reagents for the covalent modification of proteolytic enzymes such as thermolysin. The activities of aliphatic acyl amino acid thermolysins are from 1.7 to 3.6 times greater than that of the native enzyme when hydrolyzing furylacryloyl-Gly-Leu-NH_2, the substrate employed most widely. By comparison, the aromatic acylamino acid derivatives are "superactive," their activities being as much as 70-fold greater. Apparently, the aromatic character of the amino acid introduced is a critical variable in the determination of the functional response. The increased activity is completely restored to that of the native enzyme by deacylation with nucleophiles, such as hydroxylamine, and the rate of restoration of native activity is a function of the particular acyl group incorporated. Preliminary evidence regarding the chemical properties of the modified enzyme suggests that tyrosine, rather than lysine, histidine, or arginine, may be the residue modified. The functional consequences of successive modification with different reagents, moreover, indicate that each of them reacts with the same protein residue. The competitive inhibitors β-phenylpropionyl-Phe and Zn^{2+} do not prevent modification with these active esters. Hence, the site(s) of their inhibitory action differ(s) from that at which modification occurs. The structure of the substrate is also a significant variable which determines the rate at which each acyl amino acid thermolysin hydrolyzes peptides. Depending on the particular substrate, the activity of aromatic derivatives can be as much as 400-fold greater than that of the native enzyme, and the resultant activity patterns can be ordered in a series characteristic for each enzyme derivative.

Thermolysin from *Bacillus thermoproteolyticus* is a zinc metalloenzyme; like other metalloendopeptidases it is inhibited by metal chelating agents but is insensitive to inhibitors of thiol and serine proteases (Latt et al., 1969; Matsubara and Feder, 1971). Both its amino acid sequence (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972a,b; Colman et al., 1972) have been determined. Recently, reagents which characteristically affect the catalytic properties of this enzyme through covalent modification have been employed to implicate the involvement of specific amino acid residues in activity. Thus, diethyl pyrocarbonate[1] (DEP) is a reversible inactivator of thermolysin. It

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[1] Abbreviations used are: DEP, diethyl pyrocarbonate; FAGLA, furylacryloyl-Gly-Leu-NH_2; Dnp, 2,4-dinitrophenyl; Dns, 5-dimethylaminonaphthalene-1-sulfonyl; FDNB, 1-fluoro-2,4-dinitrobenzene; 4-DnpNH, 4-(2,4-dinitroanilino); DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; FA, furylacryloyl; DMF, dimethylformamide; Osu, oxysuccimide. All amino acids are of the L configuration unless otherwise indicated.

modifies a single histidyl residue, likely His-231, though its definitive identity remains to be established (Blumberg et al., 1973; Burstein et al., 1974).

Modification with DEP in the presence of β-phenylpropionyl-L-phenylalanine, an inhibitor of the enzyme, not only prevents the inactivation but instead, generates a marked and reversible increase in activity toward both peptide and ester substrates (Blumberg and Holmquist, 1973; Blumberg et al., 1974). The formation of a reactive mixed anhydride intermediate, generated in situ by interaction of DEP with β-phenylpropionyl-L-phenylalanine, was considered a likely mechanism which could lead to such increases in activity by acylating yet another enzyme residue, perhaps tyrosine.

Exploration of the nature of such an intermediate and of other carboxyl activated derivatives of the inhibitor led us to synthesize a series of active *N*-hydroxysuccinimide esters of amino acids and of substituted amino acids which we have now employed as acylating agents to modify thermolysin. They all bring about different degrees of activation, dependent on the amino acid or derivative employed. Moreover, the maximal enhancement observed with any one modification is a function of the substrate examined resulting in activity patterns characteristic of each acyl-thermolysin derivative. The extensive changes in activity result from controlled alterations of the native structure affecting areas of the enzyme involved in activity. Thus, these *N*-hydroxysuccinimide esters constitute a new class of functional amino acids or agents for the covalent modification of proteins, particularly proteolytic (and other) enzymes such as thermolysin.

Materials and Methods

Thermolysin, three times crystallized (A grade, lots 200397,201095, and 400187), was obtained from Calbiochem. The enzyme was recrystallized by dissolving (50 mg/ml) in 5*M* NaBr–0.05 *M* Tris-Cl, 0.01 *M* $CaCl_2$ (pH 7.5) followed by dialysis to low ionic strength with the same buffer but in the absence of NaBr, as described (Latt et al., 1969; Holmquist and Vallee, 1974). Other commercial reagents were obtained as follows: 2-furanacrylic acid (Eastman); Ac-Ala, Ac-D-Ala, Ac-D-Val, Ac-Phe, Ac-D-Phe, Ac-Tyr(Ac), Ala-Gly, and Gly-D-Phe (Sigma Chem. Corp.); Ac-Val and Ac-Leu (Fox Chem. Co.); Ac-Ile (Mann); Ac-Gly, Ac-Trp, Ac-D-Trp, Tyr(Bzl), Ac-Tyr, Phe(4-NO_2), Leu-Gly, Leu-Ala, and FAGLA (Cyclo Chem. Co.). All other peptide intermediates were synthesized according to standard procedures except as indicated below. Solutions of Zn^{2+} were prepared from the spectroscopically pure sulfate salt (Johnson-Matthey "Specpure" grade). All other materials were of reagent grade or the highest purity available. Adventitious metal ions were removed from all buffers and substrates by extraction with a freshly prepared solution of dithizone, 0.01%, in CCl_4 (Thiers, 1957).

Dnp-Gly-D-Phe, mp 113–115° (from ethanol–water), and Dns-Gly-D-Phe were obtained by reacting equimolar amounts of Gly-D-Phe and FDNB or dansyl chloride, respectively, in 1:1 dioxane–water at pH 8.5 for 1 hr. Ac-Tyr(Dnp), mp 175–177°, was prepared by reacting equimolar amounts of Ac-Tyr with FDNB at pH 9.5 in 1:1 dioxane–water for 1 hr. After extraction with ether, the solution was acidified with 2 *N* HCl and extracted with ethyl acetate. The organic layer was washed with water and dried over Na_2SO_4 and the solvent evaporated in vacuo. The product was recrystallized from ethyl acetate. Anal. Calcd for $C_{17}H_{15}N_3O_8$: C, 52.44; H, 3.88; N, 10.79. Found: C, 52.68; H, 4.00; N, 10.72. Ac-Phe(4-DnpNH), mp 140–142°, was obtained from Ac-Phe(4-NO_2) after reduction to Ac-Phe(4-NH_2) by catalytic hydrogenation over 10% Pd/C followed by reaction with FDNB at neutral pH in 1:1 dioxane–water. The product, isolated by acidification with 2 *N* HCl and extraction into ethyl acetate, was recrystallized from methanol–water. Anal. Calcd for $C_{17}H_{16}N_4O_7$: C, 52.58; H, 4.15; N, 14.43. Found: C, 52.47; N, 4.46; N, 14.19.

The affinity adsorbant for thermolysin, agarose-Gly-Gly-Gly-D-Phe, was prepared by coupling Gly-Gly-Gly-D-Phe, obtained by condensation of Z-Gly-Gly-Osu to Gly-D-Phe under standard conditions followed by removal of the blocking group by hydrogenolysis, to Sepharose 4B activated with cyanogen bromide (Cuatrecasas, 1970). The coupling to the adsorbant was allowed to proceed for 1 day followed by thorough washing of the affinity adsorbant with water.

N-Hydroxysuccinimide Esters. N-Blocked amino acid and peptide derivatives were esterified as suggested by Anderson et al. (1964) by reacting with equimolar amounts of *N*-hydroxysuccinimide and DCC in dioxane or DMF (2–6 ml/mmol according to solubility) at 4°. The reaction mixture was kept at 4° for 16 hr, the DCU was removed by filtration, the solvent was evaporated in vacuo and the residue was twice crystallized from 2-propanol, unless otherwise stated. The following derivatives were prepared in dioxane: Ac-Ala-Osu, mp 121–122° (Anal. Calcd for $C_9H_{12}N_2O_5$: C, 47.37; H, 5.30; N, 12.28. Found: C, 47.70; H, 5.19; N, 12.56); Ac-D-Ala-Osu, mp 125–126°; Ac-Phe-Osu, mp 153–154° (Anal. Calcd for $C_{15}H_{16}N_2O_5$: C, 59.20; H, 5.30; N, 9.21. Found: C, 59.00; H, 5.24; N, 8.98); Ac-D-Phe-Osu, mp 151–152°. In DMF were prepared the following active esters: Ac-Gly-Osu, mp 113–115° (Anal. Calcd for $C_8H_{10}N_2O_5$: C, 44.86; H, 4.71; N, 13.08. Found: C, 44.55; H, 4.75; N, 12.70); Ac-Val-Osu, mp 140–141° (Anal. Calcd for $C_{11}H_{16}N_2O_5$: C, 51.55; H, 6.29; N, 10.93. Found: C, 51.44; H, 6.35; N, 10.96); Ac-D-Val-Osu, mp 143–144°; Ac-Ile-Osu, mp 124–125° (Anal. Calcd for $C_{12}H_{18}N_2O_5$: C, 53.32; H, 6.71; N, 10.37. Found: C, 52.78; H, 6.83; N, 10.60); Ac-Leu-Osu, mp 117–118°; Ac-Trp-Osu, mp 189–190° (from 2-propanol–DMF) (Anal. Calcd for $C_{17}H_{17}N_3O_5$: C, 59.47; H, 4.99; N, 12.24. Found: C, 59.24; H, 5.30; N, 12.09); Ac-D-Trp-Osu, mp 197–198°; Ac-Tyr(Ac)-Osu, mp 177–179° (from 2-propanol–DMF) (Anal. Calcd for $C_{17}H_{18}N_2O_7$: C, 56.35; H, 5.01; N, 7.73. Found: C, 55.82; H, 5.06; N, 7.69); Ac-Tyr(Bzl)-Osu, mp 184–186° (from 2-propanol–DMF) (Anal. Calcd for $C_{22}H_{22}N_2O_6$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.07; H, 5.56; N, 6.63); Ac-Tyr(Dnp)-Osu, mp 155–160° (soften) (Anal. Calcd for $C_{21}H_{18}N_4O_{10}$: C, 51.85; H, 3.73; N, 11.52. Found: C, 51.78; H, 4.34; N, 11.51); Ac-Phe(4-DnpNH)-Osu, mp 155–160° (Anal. Calcd for $C_{21}H_{14}N_5O_9$: C, 51.96; H, 3.95; N, 14.43. Found: C, 51.58; H, 4.14; N, 14.10); Dnp-Gly-D-Phe-Osu, mp 187–190° (from 2-propanol–DMF) (Anal. Calcd for $C_{21}H_{19}N_5O_9$: C, 51.96; H, 3.95; N, 14.43. Found: C, 51.48; H, 3.96; N, 14.14); Dns-Gly-D-Phe-Osu, mp 80–110° (soften) (Anal. Calcd for $C_{27}H_{28}N_4O_7S$: C, 58.68; H, 5.11; N, 10.14. Found: C, 58.28; H, 5.58; N, 9.54).

Furylacroyl Intermediates and Substrates

Fa-Osu. A solution of 2-furanacrylic acid, 0.1 mol, and *N*-hydroxysuccinimide, 0.1 mol, in 200 ml of dioxane was cooled to 4° and DCC, 0.1 mol, was added. The mixture

was stirred at 4° for 16 hr. The DCU was filtered and the dark brown solution evaporated in vacuo. The residue was recrystallized twice from 2-propanol, yield 50%, mp 119–120°. Anal. Calcd for $C_{11}H_9NO_5$: C, 56.17; H, 3.86; N, 5.96. Found: C, 56.23; H, 4.01; N, 6.06.

FA-Gly. A solution of FA-Osu, 30 mmol, in 75 ml of dioxane, dissolved by heating and cooling, was mixed with a solution of glycine, 36 mmol, and $NaHCO_3$, 72 mmol, in 75 ml of water. The mixture was left for 6 hr at room temperature, the dioxane evaporated in vacuo, and the solution acidified with 1 *N* HCl. After cooling for 16 hr, the precipitate was filtered, washed with cold water, and recrystallized from methanol–water. Yield 60%, mp 220–222° (lit. 213–215° (Feder and Schuck, 1970)).

FA-Gly-Osu. A solution of FA-Gly, 15 mmol, and *N*-hydroxysuccinimide, 15 mmol, in 60 ml of dioxane–DMF, 1:1, was cooled to 4° and DCC, 15 mmol, was added. The solution was stirred at 4° for 16 hr. The DCU was filtered, the solution evaporated in vacuo, and the residue recrystallized twice from 2-propanol, yield 65%, mp 158–159°. Anal. Calcd for $C_{13}H_{12}N_2O_6$: C, 53.42; H, 4.14; N, 9.59. Found: C, 53.19; H, 4.27; N, 9.60. Furylacryloyl tripeptides were prepared by reacting either FA-Osu or FA-Gly-Osu with the appropriate di- or tripeptides according to the following procedure. The *N*-hydroxysuccinimide ester (5 mmol) in 25 ml of dioxane was added at room temperature to a solution of the peptide, 6 mmol, and sodium bicarbonate, 12 mmol, in 25 ml of water. The mixture was kept at room temperature for 6 hr, the dioxane evaporated, and the residue acidified with 1 *N* HCl. After the mixture was cooled overnight at 4°, the precipitate was collected by filtration and dried. The yields were between 40 and 60%. The following compounds were thus prepared.

FA-Gly-Leu-Gly. This compound was prepared from FA-Gly-Osu and Leu-Gly, mp 180–181° (from 2-propanol–water). Anal. Calcd for $C_{17}H_{23}N_3O_6$: C, 55.88; H, 6.35; N, 11.50. Found: C, 55.80; H, 6.62; N, 11.57.

FA-Gly-Ala-Gly. The compound was made from FA-Gly-Osu and Ala-Gly, mp 150–155° (from water). Anal. Calcd for $C_{14}H_{17}N_3O_6$: C, 52.01; H, 5.30; N, 13.00. Found: C, 52.33; H, 5.32; N, 11.57.

FA-Gly-Leu-Ala. The compound was prepared from FA-Gly-Osu and Leu-Ala, mp 170–171° (from 2-propanol–ether). Anal. Calcd for $C_{18}H_{25}N_3O_6$: C, 56.98; H, 6.64; N, 11.08. Found: C, 57.09; H, 6.81; N, 11.14.

FA-Gly-Leu-Phe. The compound was synthesized from FA-Gly-Osu and Leu-Phe (mp 262°), mp 165–166° (from methanol–ether). Anal. Calcd for $C_{24}H_{29}N_3O_6$: C, 63.28; H, 6.42; N, 9.23. Found: C, 63.68; H, 6.36; N, 8.86.

FA-Ala-Ala-Ala. This compound was made by reaction of FA-Osu and Ala-Ala-Ala (mp 245–248°) and was crystallized from methanol–ether, mp 227–228°. Anal. Calcd for $C_{16}H_{21}N_3O_6$: C, 54.69; H, 6.02; N, 11.96. Found: C, 54.69; H, 5.95; N, 12.25.

FA-Phe-Leu-Gly. The compound was prepared from FA-Osu and Phe-Leu-Gly, mp 201–202° (from methanol–ether). Anal. Calcd for $C_{24}H_{29}N_3O_6 \cdot H_2O$: C, 60.87; H, 6.60; N, 8.88. Found: C, 60.98; H, 6.39; N, 8.73.

Ac-Gly-Leu-Gly. This compound was obtained by reacting 1 equiv of Ac-Gly-Osu, 1.1 equiv of Leu-Gly, and 2.2 equiv of $NaHCO_3$ in 1:1 dioxane–water. After 6 hr at room temperature the solution was acidified with HCl and evaporated to dryness. The residue was extracted with dioxane, the solvent was removed in vacuo, and the residue recrystallized from 2-propanol–petroleum ether (40–60°), mp 180–181°. Anal. Calcd for $C_{12}H_{21}N_3O_5$: C, 50.16; H, 7.37; N, 14.63. Found: C, 50.65; H, 6.63; N, 14.17.

Unless otherwise stated, chemical modifications were performed at pH 8.0 and 25° using the Radiometer ABU-12, TTT-1a pH-Stat system. The reaction mixture contained 2 ml of 0.2 *M* NaCl, 0.01 *M* $CaCl_2$, and 0.001 *M* Tris. Enzyme concentrations were 20–50 μM. Reaction was initiated by the addition of 40 μl of reagent in DMF. Modified thermolysins were separated from reagents and products by gel filtration on a Bio-Gel P-4 column (0.9 × 20 cm), equilibrated with 0.2 *M* NaCl, 0.01 *M* $CaCl_2$, 2 m*M* Tris (pH 7.1) at 4°. Concentration of thermolysin was determined by measuring absorbance at 280 nm based on $A_{1\%}(280) = 17.65$ (Ohta et al., 1966), mol wt 34,600 (Titani et al., 1972). The concentration of thermolysin modified with Ac-Phe(4-DnpNH)-Osu was determined by measuring the absorbance at both 280 nm and 370 nm using ϵ_{280} 7000 and ϵ_{370} 18,500 for the chromophore and that of thermolysin modified with Ac-Tyr(Dnp)-Osu by measuring absorbance at 280 and 310 nm using ϵ_{280} 9000 and ϵ_{310} 9750 for this chromophore. Both native thermolysin and these chromophoric derivatives of thermolysin contain 1.0 ± 0.1 g-atoms of zinc/mol of enzyme, as determined by atomic absorption spectrophotometry (Fuwa and Vallee, 1963). Concentrations of other thermolysin derivatives were estimated by measuring the absorbance at 280 and the zinc content. Since modification of aromatic residues decreases molar absorptivity at 280 nm, zinc analyses were high by 10–15% when enzyme concentration was based on the molar absorptivity of the native enzyme.

The conditions under which acylamino acid *N*-hydroxysuccinimide esters undergo racemization are much more drastic than those employed here for modification; yet the occurrence of partial racemization remains to be ruled out. Further, the possible formation of oxazolones to serve as active acylating species is under investigation.

Peptidase activity was assayed with the chromophoric substrate, FAGLA, by monitoring the decrease in absorbance at 345 nm due to hydrolysis of the Gly–Leu bond (Feder, 1968). Assays were performed with a Gilford 200 instrument, using a Beckman DU monochromator, equipped with double thermospacers through which 25° water was circulated from a Haake constant temperature bath. Standard assay conditions were: 1 m*M* FAGLA, 0.1 *M* NaCl, 0.05 *M* Tris, and 0.01 *M* $CaCl_2$, pH 7.5, 25°. Under these conditions, hydrolysis of substrate followed pseudo-first-order kinetics, unless otherwise indicated. Values of k_{obsd}, the observed first-order rate constant, were calculated from plots of log $[(A_0 - A_\infty)/(A_0 - A_t)]$ vs. time, where A_0 is the initial absorbance, A_∞ is the absorbance after complete hydrolysis, and A_t is the absorbance at time t or, alternatively, from the half-life of the hydrolysis.

Hydrolysis of the additional substrates was followed in the same manner by monitoring the change in absorbance at 335–345 nm for furylacryloyl tripeptides and at 230 nm for Ac-Gly-Leu-Gly. Values of k_{cat}/K_M for the native enzyme, designated k_N, and for the activated enzyme, k_A, were obtained by dividing the observed first-order rate constants by enzyme concentration under conditions where $[S] \ll K_M$. To establish these conditions for the native enzyme and for each particular enzyme derivative, substrate concentration was decreased progressively until the observed rate constant was independent of [S].

Inhibition constants for Zn^{2+} and β-phenylpropionyl-L-Phe were determined by following the hydrolysis of 0.4 m*M*

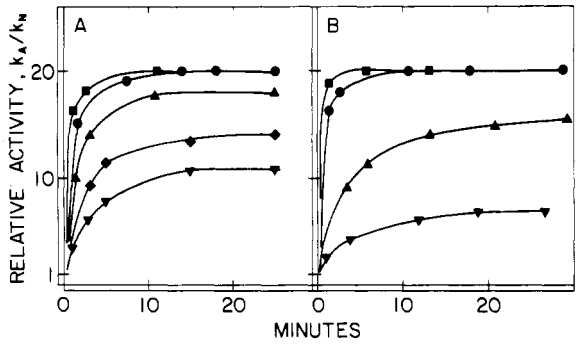

FIGURE 1: Modification of thermolysin with Ac-Phe-Osu. (A) Effect of variation of the concentration of the reagent on modification of thermolysin, 30 μM, pH 8.0. Peptidase activity was measured as a function of time after the enzyme was exposed to reagent: 0.25 mM (▼), 0.5 mM (♦), 1 mM (▲), 2 mM (●), and 5 mM (■). (B) Effect of pH on the modification of thermolysin, 30 μM, with 5 mM reagent at pH 6.5 (▼), 7.0 (▲), 8.0 (●), and 8.5 (■).

FAGLA, in 0.1 M NaCl–0.05 M Tris–0.01 M $CaCl_2$ (pH 7.5). Plots of k_0/k_i vs. inhibitor concentration where k_0 and k_i are the observed rate constants in the absence and presence of inhibitor, respectively, are linear, yielding K_I values according to the relation $K_I = [I]/(k_0/k_i - 1)$. Under these conditions competitive inhibition is indistinguishable from noncompetitive inhibition.

Results

Modification of Thermolysin with Ac-Phe-Osu. General conditions for modification of thermolysin with *N*-hydroxysuccinimide esters were established using Ac-Phe-Osu. Such active esters hydrolyze readily and, hence, their effective concentration decreases significantly during the course of the modification reaction. Accordingly, the consequences of varying reagent concentration were examined. On increasing Ac-Phe-Osu from 0.25 to 2.0 mM, with thermolysin held constant at 30 μM, both the rate at which activity increases and the maximal value attained after 20 min increase progressively (Figure 1A). There is a further increase in the rate of activation when the reagent concentration is increased to 5 mM, but the maximal degree of activation stays the same, i.e., 20-fold.

The modification is pH dependent (Figure 1B). Raising the pH from 6.5 to 8.0 increases both the rate of activation by 5 mM Ac-Phe-Osu and the maximal value attained after 20 min. At even higher pH values the maximal activity remains the same, i.e., 20-fold, but is reached more rapidly. A concentration of 2 mM reagent and pH 8 proved optimal for modification. However, even with 0.25 mM reagent, more than 50% of the maximal change in activity is achieved.

The same increase in activity occurs in the presence of the inhibitor, β-phenylpropionyl-Phe. Further, zinc which is a catalytically essential constituent of the enzyme but a potent inhibitor when added in excess (Holmquist and Vallee, 1974), like β-phenylpropionyl-Phe, fails to prevent the changes in activity due to acylation. Apparently acylation does not occur at the site where these agents exert their effects.

Modification with Aliphatic Active Esters. A series of active esters of acetyl amino acids in which aliphatic amino acids replace phenylalanine were also used as acylating agents under the conditions found optimal for acylation with Ac-Phe-Osu. The peptidase activities of these aliphatic acyl-thermolysins are only 1.7–3.6 times that of the native enzyme (Table I). Lowering the reagent concentration to 1 mM results in a somewhat smaller increase in activities, but raising it to 5 mM does not increase them further. Again, neither β-phenylpropionyl-Phe nor Zn^{2+} prevents modification or alters significantly the maximal value attained by acylation with Ac-Ala-Osu.

Table I: Acylation of Thermolysin by Aliphatic *N*-Acetyl Amino Acid *N*-Hydroxysuccinimide Esters.[a]

Ester	Activity Ratio[b] (k_A/k_N)
Ac-Ala-Osu	1.7
Ac-Val-Osu	2.2
Ac-Ile-Osu	2.4
Ac-Leu-Osu	3.6

[a] 50 μM thermolysin, modified with 2 mM reagent, pH 8.0, 25°. [b] Maximal activities measured after 15 min of reaction using FAGLA as substrate.

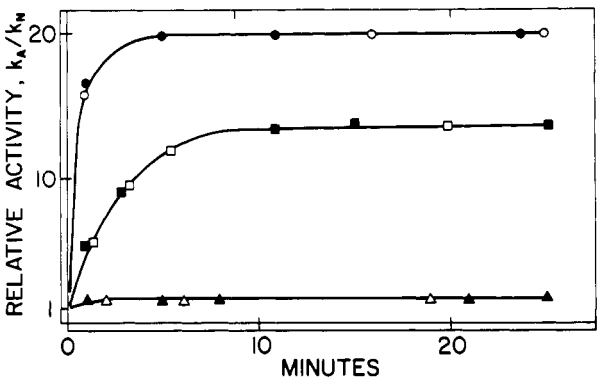

FIGURE 2: Effect of the optical configuration of the reagent on the modification of thermolysin. Reagents of L configuration, solid symbols; of D configuration, open symbols. Modification with 5 mM Ac-Phe-Osu, (●,○), 0.5 mM Ac-Phe-Osu (■,□), or 5 mM Ac-Ala-Osu (▲,△).

Effect of Optical Configuration of the Reagent. To probe other features of reagent structure potentially essential to changes in activity, thermolysin was reacted at pH 8.0 with two different concentrations of Ac-D-Phe-Osu, 0.5 mM and 5 mM (Figure 2). The rates of reaction of the D isomer and the degree of maximal activation are very different at these two concentrations, but they are identical with those observed with the L isomer. Quite analogous effects are found on modification with Ac-D-Ala-Osu and Ac-D-Val-Osu, 5 mM.

Modification with Aromatic Active Esters. Comparison of the maximal activity of aliphatic acyl-thermolysins with that of Ac-Phe-thermolysin suggests that the aromatic character of the phenyl ring in Ac-Phe-Osu might be a critical variable in determining the magnitude of the functional response. Indeed, substitution of Trp, or derivatives of Tyr and their analogs, for Phe give products whose activities are substantially greater than those of any of the aliphatic acyl-thermolysins examined. The activities are in the order of Trp < Phe = Tyr(Ac) ≃ Tyr(Bzl) < Tyr(Dnp) < Phe(4-DnpNH). The Trp derivative is 12 times and the Phe(4-DnpNH) derivative is 70 times as active as the native enzyme (Table II).

Modification of thermolysin with 1 mM Ac-Trp-Osu or Ac-D-Trp-Osu causes a 12-fold increase in activity in 10 min. Higher concentrations of the reagents do not enhance

Table II: Acylation of Thermolysin by Aromatic *N*-Acetyl Amino Acid *N*-Hydroxysuccinimide Esters.[a]

Ester	Reagent Concn (m*M*)	Activity Ratio[c] (k_A/k_N)
Ac-Phe-Osu	2	20
Ac-Trp-Osu	1	12
Ac-Tyr(Ac)-Osu	1	20
Ac-Tyr(Bzl)-Osu	1	20
Ac Tyr(Dnp)-Osu[b]	0.5	40
Ac-Phe(4-DnpNH)-Osu[b]	0.5	70
Dnp-Gly-D-Phe-Osu	0.5	15
Dns Gly-D-Phe-Osu	0.5	15

[a] All modifications carried out at pH 8.0, 25°. Enzyme was 20–30 μM and activity measured with 1 m*M* FAGLA. [b] Enzyme was 100 μM and activity measured with 200 μM FAGLA. [c] The activities are the maximal ones observed.

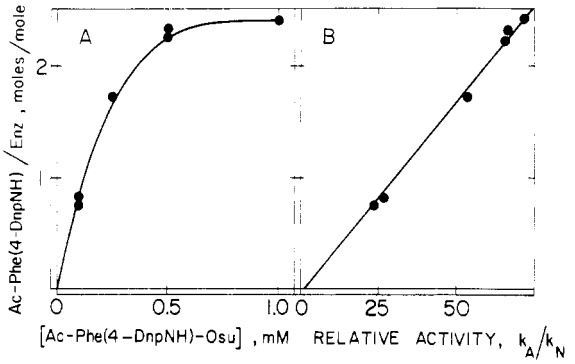

FIGURE 3: Modification of thermolysin with Ac-Phe(4-DnpNH)-Osu. (A) The number of groups incorporated as a function of the concentration of the reagent. Thermolysin, 0.1 m*M*, in 0.2 *M* NaCl–0.36 *M* NaBr–2 m*M* Tris (pH 8.0) was modified with various concentrations of the reagent in 2% DMF, pH 8.0, 25° for 10 min, and the product gel-filtered. Residues incorporated were measured spectrophotometrically as described in the text. (B) Correlation of the activity of modified thermolysin with the degree of modification. Activity was measured with 0.2 m*M* FAGLA.

activity further, but eventually inactivate by precipitating the enzyme. Similarly, modification with Ac-Tyr(Ac)-Osu or Ac-Tyr(Bzl)-Osu yields products with maximal activities close to that observed for Ac-Phe-Osu (Table II) while Ac-Tyr(Dnp)-Osu and Ac-Phe(4-DnpNH)-Osu yield maximal activities 40 and 70 times greater, respectively, than that of the native enzyme (Table II). Thus, the induction of high activity is not limited to the incorporation of only D or L amino acids. Indeed, for aromatic acyl-thermolysins substitution in the phenyl ring, e.g., DnpO or DnpNH, magnifies the functional response.

The introduction of such chromophoric para substituents provides potential environmental probes of the sites of modification. Chromophoric amino blocking groups such as Dns-Gly or Dnp-Gly, replacing Ac, can also contribute probe properties. Dnp-Gly-D-Phe-Osu and Dns-Gly-D-Phe-Osu increase activity 15-fold.

Thermolysin modified with Ac-Phe(4-DnpNH)-Osu was studied in detail as the prototype of a chromophoric derivative. To avoid large molar excesses of reagent during modification, enzyme concentration was increased to 0.1 and/or 0.3 m*M* while the reagent concentration was varied from 0.1 to 1.0 m*M*. The reaction was allowed to proceed for 10 min at 25° followed by gel filtration and measurement of spectra and activity. Figure 3A shows the incorporation of

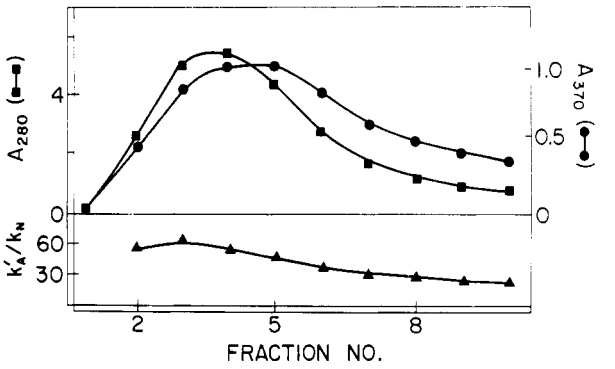

FIGURE 4: Fractionation of thermolysin, partially modified with Ac-Phe(4-DnpNH)-Osu on an agarose-Gly-Gly-Gly-D-Phe column. Thermolysin, 0.33 m*M*, was modified with Ac-Phe(4-DnpNH)-Osu, 0.48 m*M*, in 2.5 *M* NaBr–0.1 *M* NaCl–0.01 *M* $CaCl_2$–10 m*M* Tris (pH 8.0), 25°, for 10 min, and the product was gel-filtered on a Bio-Gel P-4 column equilibrated and eluted with 0.2 *M* NaCl–0.01 *M* $CaCl_2$–2 m*M* Tris (pH 7.1); 40 ml of the gel-filtered enzyme product, 0.2 m*M* containing 0.97 group of chromophore/mol of enzyme, was passed through the affinity column (0.9 × 18 cm) and 20-ml fractions were collected and absorption at 280 and 370 nm and activity were measured. Absorption at 280 nm after correction for absorption of the chromophore (■); absorption at 370 nm (●); the activity per mole of chromophore incorporated, k'_A, relative to the activity of the native enzyme, k_N (▲).

reagent as a function of its concentration. The extent of modification increases with reagent concentration until, at 0.5 m*M*, 2.3 mol of chromophore are incorporated. With 1.0 m*M* reagent, much of the enzyme precipitates and the soluble, gel-filtered fraction contains 2.4 groups/mol of enzyme. The activities of the modified enzymes increase linearly in direct proportion to the number of groups incorporated (Figure 3B).

Although activity is maximal on incorporation of 2.4 mol of reagent, it is not clear if all of the modified residues are critical to the observed changes in activity. This question was examined by partially modifying the enzyme and fractionating the product on an agarose-Gly-Gly-Gly-D-Phe affinity column. The elution pattern of the enzyme modified to contain 0.95 mol of chromophore/mol of enzyme is shown in Figure 4, monitoring the absorption at both 370 and 280 nm. The absorptions of the chromophore and of the protein do not coincide completely. Partially modified enzyme elutes first, followed by enzyme which is modified more extensively. The activity per mole of chromophore incorporated is higher for the early fractions and lower for the retarded fractions (Figure 4). Since the activity per mole of chromophore incorporated exceeds 80% of the maximal activation achievable (Figure 3), it appears that modification of but one residue may account for the activation observed.

Stability and Reversibility of Modification. The acyl-enzyme derivatives (Tables I and II) are stable and can be gel-filtered without losing their characteristic activities. The products of the reaction of thermolysin with Ac-Ala-Osu, Ac-Leu-Osu, Ac-Trp-Osu, or Ac-Phe-Osu (Figure 5A) after gel filtration in 0.2 *M* NaCl–0.01 *M* $CaCl_2$–2 m*M* Tris, pH 7.1, 4°, lose no more than 3% of their activity per day, when stored for 10 days at 4°. However, rapid deacylation, accompanied by concomitant restoration of native activity, can be achieved by exposure to nucleophiles (Figure 5B). The rate of return to native activity depends on the nature of the acyl group introduced (Table III). Using 0.1 *M* NH_2OH, the half-lives of deacylation for the Ac-Phe, Ac-Trp, Ac-Ala, and the Ac-Leu derivatives are 8, 9, 10,

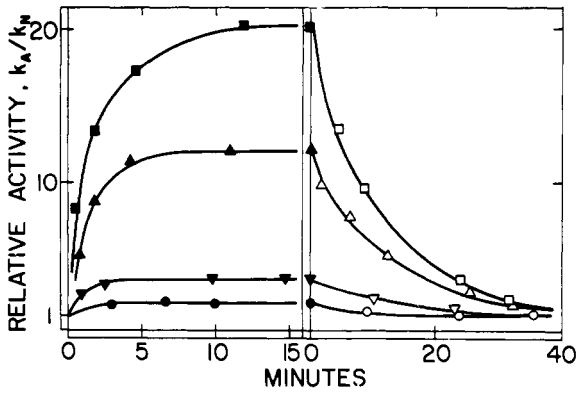

FIGURE 5: Activation of thermolysin by covalent modification with *N*-acetylamino acid *N*-hydroxysuccinimide esters and restoration of native activity by deacylation with hydroxylamine. (A) Thermolysin, 50 μM, in 0.2 *M* NaCl, 0.24 *M* NaBr, 0.01 *M* $CaCl_2$, 1 m*M* Tris, was acylated at pH 8.0, 25° for 15 min with 1.5 m*M* Ac-Ala-Osu (●), 2 m*M* Ac-Leu-Osu (▼), 1 m*M* Ac-Trp-Osu (▲), or 1.5 m*M* Ac-Phe-Osu (■). Activities were assayed with FAGLA as described under Materials and Methods. (B) The Ac-Ala, Ac-Trp, and Ac-Phe thermolysin derivatives (20 μM) after gel filtration were treated with 0.1 *M* NH_2OH–0.015 *M* Tris, pH 7.5, 25° and the Ac-Leu derivative with 0.2 *M* NH_2OH–0.03 *M* Tris (○,▽,△,□). Activities assayed with FAGLA.

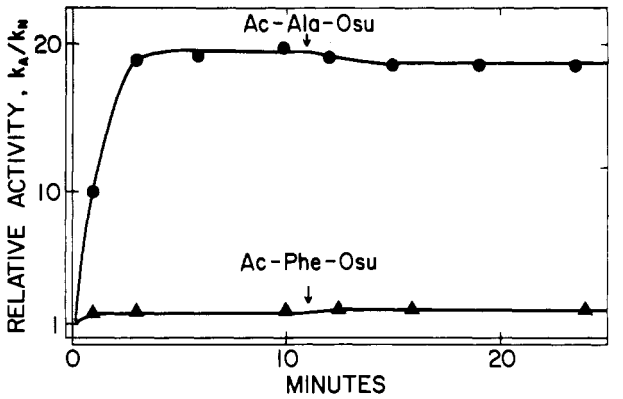

FIGURE 6: Effect of sequential modifications with *N*-hydroxysuccinimide esters of *N*-acetyl amino acids on the activity of thermolysin. Thermolysin, 20 μM, in 0.2 *M* NaCl–0.12 *M* NaBr–0.1 *M* $CaCl_2$–0.5 m*M* Tris was acylated at pH 8.0 with 1.5 m*M* Ac-Phe-Osu for 11 min (●), followed by 1.5 m*M* Ac-Ala-Osu added at the point indicated by the arrow. Similarly, thermolysin was acylated with 1.5 m*M* Ac-Ala-Osu for 11 min (▲) followed by 1.5 m*M* Ac-Phe-Osu as indicated by the arrow.

Table III: Deacylation of Thermolysins with Hydroxylamine and Restoration of Activity Characteristic of the Native Enzyme.[a]

Acyl-thermolysin	NH_2OH (*M*)	$t_{1/2}$ (min)
Ac-Phe	0.1	8
Ac-Trp	0.1	9
Ac-Ala	0.1	10
Ac-Leu	0.1	24
Ac-Leu	0.2	11
Ac-Val	0.5	22
Ac-Ile	0.5	38
Ac-Phe(4-DnpNH)	0.1	4

[a]Deacylation was carried out by adding a stock solution of 2 *M* NH_2OH–0.3 *M* Tris (pH 7.5) to the enzyme in 0.2 *M* NaCl–10 m*M* $CaCl_2$–2 m*M* Tris (pH 7.1) incubated at 25°. Activity was assayed with FAGLA as described under Materials and Methods.

Table IV: Inhibition of Acyl-thermolysins by Zn^{2+} and β-Phenylpropionyl-Phe.[a]

Enzyme	Zn^{2+} $K_I \times 10^5$ (*M*)	β-Phenylpropionyl-Phe $K_I \times 10^3$ (*M*)
Thermolysin	4.2	1.6
Ac-Ala-thermolysin	3.9	1.8
Ac-Phe-thermolysin	3.4	1.2
Ac-Phe(4-DnpNH)-thermolysin	5.8	1.0

[a]Inhibition of hydrolysis of 0.4 m*M* FAGLA was determined in 0.1 *M* NaCl–50 m*M* Tris–10 m*M* $CaCl_2$ (pH 7.5). Zn^{2+} concentration ranged from 2 to 20 × 10^{-5} *M*; β-phenylpropionyl-Phe concentration ranged from 1.8 to 7.2 m*M*. Enzyme concentrations were 6 × 10^{-7} *M*, 6 × 10^{-7} *M*, 5 × 10^{-8} *M*, and 3 × 10^{-8} *M* for the native, Ac-Ala, Ac-Phe, and Ac-Phe(4-DnpNH) enzymes, respectively.

and 24 min, respectively. The rates of deacylation for the branched amino acids Ac-Val and Ac-Ile are much lower. Even with 0.5 *M* NH_2OH the half-lives are 22 and 38 min, respectively. Significantly, in 0.1 *M* NH_2OH the half-life for deacylation of the highly active, chromophoric derivative Ac-Phe(4-DnpNH)-thermolysin is only 4 min.

Successive Modification of Thermolysin. The changes in activity correlate so well with the structure of the modifying reagent as to suggest that each of them interacts with the same protein residue. This supposition was examined by measuring the functional consequences of successive modifications with different reagents. Thermolysin was acylated first with Ac-Ala-Osu, 1.5 m*M*, and the product, 1.7 times more active than the native enzyme, was then treated with Ac-Phe-Osu, 1.5 m*M*. No further increase in activity was found (Figure 6, lower curve). A 20-fold activity increase would have been expected had reaction with Ac-Phe-Osu occurred (Table II). The same experiment was carried out in the reverse order. The enzyme was modified first with Ac-Phe-Osu, 1.5 m*M*, yielding a product 20 times more active than the native enzyme (Figure 6, upper curve). Subsequent modification with 1.5 m*M* Ac-Ala-Osu failed to affect activity. Apparently, the same residue is modified by both Ac-Ala and Ac-Phe. Once reaction with one agent has occurred, the site no longer reacts with the other. The smaller change in activity resulting from modification with Ac-Ala-Osu, relative to Ac-Phe-Osu, is more likely a consequence of differences in reagent structure than of modification of different residues. Similar conclusions can be drawn from sequential modifications of thermolysin with Ac-Trp-Osu and Ac-Val-Osu.

Inhibition of the Modified Enzymes by β-Phenylpropionyl-Phe and Zn^{2+}. As noted, the competitive inhibitors, β-phenylpropionyl-Phe and Zn^{2+}, do not prevent modification of the native enzyme by these active ester reagents, suggesting that modification and inhibition occur at different sites on the enzyme. It follows that Zn^{2+} and β-phenylpropionyl-Phe should still inhibit the activated enzyme. Indeed, not only are they inhibited but the inhibition constants for all the acyl-enzymes are closely similar to those for the native enzyme varying only twofold (Table IV).

Effect of Substrate Structure on Observed Activity of Native Thermolysin. The above activities and activity ratios have all been determined using FAGLA, the standard substrate for thermolysin (Feder, 1968). A series of N-blocked tripeptides were synthesized which differ systematically both in amino acid composition and sequence to investigate whether or not these changes in activity are charac-

Table V: Thermolysin-Catalyzed Hydrolysis of Tripeptide Substrates[a]

Substrate	[S] (mM)	$k_{cat}/K_M \times 10^{-4}$ (sec^{-1} M^{-1})	Relative[b] k_{cat}/K_M
FA-Gly-Ala-Gly	0.4	0.013	1
FA-Gly-Leu-NH_2	0.2	2.2	170
FA-Ala-Ala-Ala	0.4	5.5	420
FA-Gly-Leu-Gly	0.2	8.3	640
FA-Gly-Leu-Phe	0.05	30	2,300
FA-Gly-Leu-Ala	0.1	87	6,700
FA-Phe-Leu-Gly	0.1	230	18,000
Ac-Gly-Leu-Gly	1.0	0.064	5

[a] All reactions carried out in 0.1 M NaCl–0.05 M Tris–0.01 M $CaCl_2$ (pH 7.5), 25°. [b] k_{cat}/K_M for FA-Gly-Ala-Gly is taken as one.

Table VI: Comparison of Acyl-thermolysins and Native Thermolysin Catalyzed Hydrolysis of Tripeptide Substrates.[a]

	$k_{cat}/K_M \times 10^{-4}$ (sec^{-1} M^{-1})				
Substrate	Native	Ac-Ala	Ac-Val	Ac-Phe	Ac-Phe(4-DnpNH)
FA-Gly-Ala-Gly	0.013	0.039[b]	0.052[b]	1.3	5.2
FA-Gly-Leu-NH_2	2.2	3.8	4.8	45	160
FA-Ala-Ala-Ala	5.5	8.5	5.8	18	12
FA-Gly-Leu-Gly	8.3	17	22	170	180
FA-Gly-Leu-Phe	30	74	79	450	300[c]
FA-Gly-Leu-Ala	87	130	130	300	130
Ac-Gly-Leu-Gly	0.06	0.12	0.18	2.7	0.23

[a] All reactions carried out in 0.1 M NaCl–0.05 M Tris–0.01 M $CaCl_2$ (pH 7.5) at 25°. [b] Values derived from initial rates. [c] Substrate concentration 0.02 mM; other concentrations are as given in Table V.

teristic of the enzyme, pertain solely to FAGLA, or extend to other substrates. Values of k_{cat}/K_M for native thermolysin, spanning four orders of magnitude (Table V), reflect some important elements of specificity of the enzyme (Matsubara et al., 1965; Morihara and Tsuzuki, 1966, 1970; Feder and Schuck, 1970). Substrates with a hydrophobic residue contributing the amino group to the susceptible bond are hydrolyzed at higher rates as is apparent from a comparison of, e.g., FA-Gly-Leu-Gly with FA-Gly-Ala-Gly, the former being hydrolyzed 640 times more rapidly than the latter. Comparison of the four FA-blocked tripeptide substrates with Leu as the penultimate amino acid reveals that hydrophobicity of the other two amino acids is also an important specificity factor. Relative to FA-Gly-Leu-Gly, replacement of the C-terminal Gly by Ala or Phe increases activity ten- and fourfold, respectively. The most pronounced effect occurs on substituting Phe for the Gly residue which contributes the carboxyl group to the susceptible bond. This enhances activity 28-fold. Moreover, when Ala replaces both Gly residues this obviates the requirement for a hydrophobic residue in the penultimate position; the activity toward FA-Ala-Ala-Ala is almost the same as that toward FA-Gly-Leu-Gly. Interestingly, the blocking group, FA, also participates significantly in the interaction of these tripeptides with the enzyme; the k_{cat}/K_M value for FA-Gly-Leu-Gly is 130 times higher than that for Ac-Gly-Leu-Gly.

Effect of Substrate Structure on Observed Activity of Modified Thermolysins. Assays using the above substrates provide a more subtle discrimination of the effects of enzyme modification. The relevant k_{cat}/K_M values, presented in Table VI, can be analyzed by comparing either one substrate being acted upon by different enzymes or one enzyme acting on different substrates; each approach reveals different details. For example, Ac-Phe-thermolysin acting on FA-Gly-Leu-Phe reveals the highest value of k_{cat}/K_M, 4.5×10^6 sec^{-1} M^{-1}, a value among the highest ever observed for the hydrolysis of peptides by *any* proteolytic enzyme. Similarly high values are observed for both Ac-Phe-thermolysin and Ac-Phe(4-DnpNH)-thermolysin acting on the leucyl containing peptides FA-Gly-Leu-Gly, FA-Gly-Leu-Phe, and FA-Gly-Leu-Ala, all being higher than 1×10^6 sec^{-1} M^{-1}.

Overall, several features stand out. First, aliphatic acyl-thermolysins exhibit only a 2–4-fold activity enhancement toward all substrates, the largest being the fourfold increase for the Ac-Val enzyme acting on FA-Gly-Ala-Gly. In contrast, with aromatic acyl-thermolysins the maximal increase is 400-fold for the Ac-Phe(4-DnpNH) enzyme acting on FA-Gly-Ala-Gly. Interestingly, the major effect of enzyme modification manifests itself in activity toward the poorer substrates. The Ac-Phe(4-DnpNH) enzyme exhibits a 400-fold activity increase toward FA-Gly-Ala-Gly, the poorest substrate among those examined for the native enzyme, but only a 1.5-fold increase toward FA-Gly-Leu-Ala, an excellent substrate. FA-Gly-Leu-Phe is the best substrate for the aromatic acyl-enzymes. Substrates which the native enzyme hydrolyzes at almost equal rates, e.g., FA-Gly-Leu-Gly and FA-Ala-Ala-Ala, are highly differentiated when hydrolyzed by, e.g., Ac-Phe(4-DnpNH) thermolysin, the leucyl-containing peptide being hydrolyzed 15 times more rapidly than the alanine containing peptide. Moreover, with the standard substrate, FAGLA, modification with Ac-Phe(4-DnpNH)-Osu yields the largest activity changes. This does not pertain to the majority of other substrates; indeed, with Ac-Gly-Leu-Gly, the Ac-Phe enzyme is 12 times more effective than the Ac-Phe(4-DnpNH) derivative. These comparisons reveal that the modifications do not only change activity but also modify specificity requirements of the enzyme.

Discussion

Protection of enzymes by substrates, substrate analogs, or reversible enzyme inhibitors is now used almost routinely to selectively prevent the chemical modification of active center residues. Usually this is followed by a second modification—after removal of substrates or the related agents—to identify these residues, a procedure generally referred to as "differential labeling". If enzymatic activity is preserved subsequent to modification in the presence but not in the absence of substrates, it is usually assumed that the substrate has protected an active center group. In the course of such differential labeling experiments with thermolysin, we encountered surprising results. Their resolution led to the present experiments. In addition, the observations point to an unexpected complication of the differential labeling procedure, apparently not previously reported.

Inactivation of thermolysin with DEP results in the formation of ethoxyformylhistidine. However, modification with DEP in the presence of the reversible inhibitor, β-phenylpropionyl-Phe, not only prevents the inactivation but actually increases activity remarkably. This result was quite unexpected as compared with experiments with other, anal-

ogous systems using similar approaches (Singer, 1967).[2] The increased activity was attributed to the interaction of the inhibitor with DEP to form a reactive intermediate, which was thought to modify another residue (Blumberg et al., 1974). This hypothesis was examined by the synthesis of the possible intermediate, the mixed anhydride of β-phenylpropionyl-Phe and ethoxyformic acid. The product indeed induced entirely analogous increases in the activity of thermolysin. Similar functional changes were also demonstrated using an imidazole analog. These results suggested the existence in the enzyme of an additional, critical, functional site which could be modified by a series of acyl activated amino acid derivatives. A series of *N*-hydroxysuccinimide esters of a number of *N*-acyl amino acids were synthesized and their effects on the activity of thermolysin compared with those of phenylalanine derivatives.

The present study demonstrates that this class of labile amino acid esters constitutes a versatile group of reagents for the systematic modification of thermolysin and its catalytic properties. The resultant covalent incorporation of *N*-acyl amino acids increases the activity of the enzyme as a function of their structures when FAGLA is the substrate. In general, incorporations of aliphatic amino acids induce small and aromatic amino acids large increases in activity of the resultant acyl-thermolysins, as exemplified by the Ac-Ala and Ac-Phe derivatives which increase activity 1.7- and 20-fold, respectively (Figure 1, Tables I and II).

Introduction of substituents on the side chains of the acylating agent or the replacement of the acetyl moiety of the active ester with other N-blocking groups provides potential means to modulate the functional properties of the derivatives. Indeed, Ac-Phe(4-DnpNH)-Osu induces a 70-fold increase in activity, significantly greater than that achieved with the parent compound, Ac-Phe-Osu (Table II). Both the concentration of the reagent and the pH at which modification is performed are critical (Figures 1 and 4). Hence, variation of these parameters allows control of the degree of modification and its consequences which can serve to characterize these reactions (vide infra).

A number of observations suggest that the residue(s) which are acylated may be remote from the active site. Thus, the reversible inhibitor β-phenylpropionyl-Phe which has been shown to bind at the active site of the enzyme (B. Matthews, personal communication; Bigbee and Dalquist, 1974) does not prevent acylation and the accompanying increase in activity. Further Zn^{2+}—like Ag^{+}—(Colman et al., 1972) may inhibit the enzyme by binding to His-231. Both these agents inhibit the modified and native enzymes to the same degree (Table IV). This further implies that the functional group(s) modified are not essential to binding.

This view is supported by the fact that neither the rate nor the extent of activity reflects the optical configuration of the reagent (Figure 2). Yet, all of the *N*-hydroxysuccinimide esters seem to react selectively, perhaps with a hyperreactive residue, suggesting that the modification, like its functional consequences, expresses elements of enzyme specificity.

While all the acyl-thermolysins examined so far are stable during gel filtration, they undergo deacylation in the presence of strong nucleophiles, concomitant with a return of their enhanced activities to that of the native enzyme (Figure 5). Since the reaction is reversible, the changes in activity must be attributed to the characteristics of the amino acid introduced by modification rather than to irreversible changes that might accompany modification.

The rates of restoration of native activity are a function of the structure of the group incorporated. They are similar for the Ac-Phe, Ac-Trp, and Ac-Ala thermolysin derivatives, more rapid for the Ac-Phe(4-DnpNH), and slower for the Ac-Leu derivative and much slower for the derivatives of the branched amino acids Ac-Val and Ac-Ile (Table III). The very slow rates for the last two enzymes are presumably due to steric hindrances by the side chains (cf. Koltun et al., 1963). Increases in activity induced by aliphatic and aromatic pairs of reagents are mutually exclusive (Figure 6). Hence, it seems likely that the same critical enzyme residues are modified to increase activity in either one of these derivatives, independent of their structures. Variations in the rates of deacylation by nucleophiles may thus be ascribed primarily to the structural differences of the acyl moieties, although the protein environment may also be important in affecting the stability of the acyl-enzyme linkages.

The spectral characteristics of the amino acid reagents containing substituents such as Dnp can be used to quantitate modification. Further, these reagents can also affect other properties, e.g., selectivity, activity, or solubility of the products. Thus, maximal changes in activity are achieved with incorporation of up to 2.3 mol of Ac-Phe(4-DnpNH). Beyond this the enzyme precipitates. On the other hand, when partially modified enzyme is fractionated on an agarose-Gly-Gly-Gly-D-Phe column (Figure 4), the fractions which emerge from the column early exhibit high specific activity relative to the number of chromophoric groups incorporated. Extrapolation to maximal activity gives a value of 1.1–1.3 groups/mol of enzyme, suggesting that, while the reagent modifies several residues, in fact, only one of them is critical to the changes in activity. The chemical properties of the modified enzyme examined so far, i.e., reversal of the modification by NH_2OH (Table III) and stability of the acyl-enzyme linkage to acid denaturation, suggest that tyrosine most likely is the residue modified. This deduction is consistent with preliminary evidence from enzymatic digestion resulting in Dnp-labeled peptides containing tyrosine but not lysine, histidine, or arginine (Blumberg, unpublished observations).

Substrate structure also significantly determines the catalytic rate observed for each of the acyl-thermolysins. Thus, the activities of the aliphatic Ac-Ala and Ac-Val derivatives are enhanced fourfold at best when acting on any of the substrates in Table VI (columns 2 and 3). In contrast, those of the aromatic derivatives can reach very high values, also depending markedly on the structure of particular substrates. For each type of derivative the data can be ordered in a characteristic series (Table VII) depending on effectiveness of substrate hydrolysis, suggesting that an aromatic amino acid residue at the C-terminus of the substrate is optimal for hydrolysis by aromatic acyl-thermolysins.[3]

[2] Yet another though totally unexpected result of differential labeling has been observed in the syncatalytic modification of aspartate aminotransferase with tetranitromethane, a novel phenomenon where a residue becomes accessible to modification only in the presence of both substrates while catalysis is actually proceeding (Christen and Riordan, 1970), allowing recognition of conformational changes undergone by the enzyme at the moment of catalysis.

[3] The acyl-thermolysins exhibit increased activity toward all substrates examined thus far with the single exception of FA-Phe-Leu-Gly, toward which activity is actually decreased.

Table VII. Relative Order of Activities[a] of Native and Aliphatic and Aromatic Acyl-thermolysins toward Furylacryloyl-Gly-X-Y[b] Substrates.

Native	-Leu-Ala > -Leu-Phe > -Leu-Gly > -Ala-Ala > -Leu-NH_2 > -Ala-Gly
Ac-Ala-	-Leu-Ala > -Leu-Phe > -Leu-Gly > -Ala-Ala > -Leu-NH_2 > -Ala-Gly
Ac-Val-	-Leu-Ala > -Leu-Phe > -Leu-Gly > -Ala-Ala > -Leu-NH_2 > - Ala-Gly
Ac-Phe-	-Leu-Phe > -Leu-Ala > -Leu-Gly > -Leu-NH_2 > -Ala-Ala > -Ala-Gly
Ac-Phe(4-DnpNH)-	-Leu-Phe > -Leu-Gly > -Leu-NH_2 > -Leu-Ala > -Ala-Ala > -Ala-Gly

[a] For numerical values see Table VI. [b] Where X = Leu, Ala, Y = Gly, Ala, Leu, Phe, NH_2.

The question arises as to the source of the enhanced efficiency of the modified enzyme, be it an effect on the catalytic step, substrate binding, or both. There is no evidence that release of substrate or product inhibition could account for the activation. Thus far we have only determined the ratio k_{cat}/K_M and a more detailed kinetic analysis is required to elucidate the alternatives. Such an analysis for β-phenylpropionyl-Phe-thermolysin acting on Bz-Gly-Phe-Ala did not prove decisive, however. In that instance, k_{cat} increases while K_M decreases (Blumberg et al., 1974). Since this is the only system for which extensive kinetics are available, further study of other systems is mandatory.

The most remarkable characteristic of these acyl-thermolysins is their marked enhancement of activity. It makes them unusual systems for the examination of the presumable mechanism(s) underlying these changes. Interpretation of chemical modifications in terms of the specific residue(s) involved and of their mechanistic roles is complex, owing to the participation of multiple numbers of residues in catalysis and binding. Moreover, present views on mobility of enzymes, their conformational changes when binding inhibitors or substrates, and other factors essential to catalysis could imply that the functional consequences of modification might result from structural changes in areas of the molecule topologically remote from the active center.

The most frequent consequence of chemical modification procedures is enzyme inactivation. Optimally, the *loss* of activity correlates directly with the mole fraction of a given active center residue modified. This *loss* of activity is thought to signify direct or indirect participation of the residue in catalysis or substrate binding. This view has been subject to much criticism, however. Confidence in specific cause and effect relationships between chemical modification of a residue and alteration of activity is increased when modification *enhances* activity, as in the present instance. It can then justly be assumed that features of enzyme structure which are indispensable to activity have remained intact. This, in turn, allows kinetic investigations of the modulated activity of the product, an approach which is not feasible when it is catalytically inactive.

Thus, variously substituted, active acyl-thermolysins can be matched with substrates in which the residues that form either the susceptible peptide bond or the C-terminal residue are replaced. This yields experimental permutations for kinetic and thermodynamic examination of mechanistic detail. For a given mechanistic proposition, the system permits examination of alternatives. Substrate series (as in Table VII) which vary in the order and magnitudes of effectiveness of hydrolysis or of the kinetic constants which characterize it might reveal complementarities between the acyl amino acid modifying the enzyme and those of the substrate, critical to enhanced activity.

Such studies can further utilize inhibitor interactions to explore the molecular basis of inhibition. It is apparent already that the acylation with amino acids apparently does not affect the interaction of the inhibitors studied so far and a search for others which differentially affect the native and various acyl-thermolysins could prove rewarding and give leads regarding a residue which might be common to both interactions.

The catalytically active zinc and the structural calcium atoms of thermolysin offer yet additional opportunities. Zinc can be replaced by other, functional chromophoric transition metals (Holmquist and Vallee, 1974) and calcium by lanthanide magnetophores (Colman et al., 1972; Holmquist and Horrocks, 1975). Physical chemical techniques encompassing the electromagnetic spectrum, polarized light, isotope labeling, and exchange, as well as thermodynamic and kinetic properties of metal–ligand interactions, can then probe the nature and environment of metal binding sites and their proximity to the site(s) of acylation.

Whatever the ultimate results, the magnitudes of increased activity here observed are exceptionally large compared with, e.g., increases in activity by activation or the rates seen for nonenzymatic reactions. This is readily apparent from inspection of the increases in the hydrolysis of "poor" substrates when acted upon by aromatic acyl-thermolysins (Table VI). Studies of acyl-thermolysins may well uncover as yet unknown features essential to catalytic mechanisms.

Recent studies (Blumberg et al., in preparation) have revealed a variety of chemical and functional characteristics which thermolysin shares with metalloendopeptidases from *Bacillus subtilis, Bacillus megaterium,* and *Aeromonas proteolytica.* In addition to the essential zinc atoms and the features of specificity (Feder et al., 1971; Griffin and Prescott, 1970; Matsubara and Feder, 1971), all are inactivated reversibly by DEP. Like thermolysin, they are also inhibited by excess Zn^{2+} and moreover, all these enzymes undergo analogous, though not identical, increases in activity. Clearly, then, the features here reported reflect properties of a *class* of enzymes, not of a particular one, emphasizing the predictive value of these studies which thereby gain general importance for the study of enzyme mechanisms.

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Modification of Pig Heart Lipoamide Dehydrogenase by Cupric Ions†

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ABSTRACT: The insertion of a second disulfide bridge into native pig heart lipoamide dehydrogenase, requires two Cu^{2+} ions for each catalytic center inactivated under anaerobic conditions. During inactivation, both metal atoms become reducible by their juxtaposition to the two participating cysteine residues and may be removed as the Cu^{+}-chelates of neocuproine and bathocuproinesulfonate, leaving an additional disulfide bridge on the protein. Inactivation does not require the presence of oxygen, but when substoichiometric levels of copper are used under aerobic conditions the slow regeneration of Cu^{2+} becomes rate-limiting. The course of aerobic inactivation is markedly biphasic at 0° using 2 Cu^{2+}/FAD, with 30% of the total change completed rapidly, followed by a much slower phase. Both the extent of the fast phase and the rate of the second phase are enhanced by increasing levels of Cu^{2+}, but are relatively unaffected when the Cu^{2+}/FAD ratio is maintained at 2 and the protein concentration is varied. The enzyme affords several binding sites for Cu^{2+} at pH 7.8, and it is suggested that competition between these sites during the initial statistical distribution of metal ions may explain this biphasic behavior.

Pig heart lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3), a component of both the pyruvate and α-ketoglutarate dehydrogenase multienzyme complexes, catalyzes the following reaction (Massey, 1963):[1]

$$NAD^{+} + Lip(SH)_2 \rightleftharpoons NADH + Lip(S\text{–}S)$$

The enzyme, as isolated, is a dimer with one FAD (Massey et al., 1962), a single disulfide bond, and seven cysteine residues titratable with 5,5′-dithiobis(2-nitrobenzoic acid) per polypeptide (Matthews et al., 1974). The FAD and disulfide moieties comprise part of the active site and interact electronically during catalysis (Massey and Veeger, 1961). Veeger and Massey (1962) demonstrated that lipoamide dehydrogenase is readily inactivated by low levels of Cu^{2+} yielding a protein with a much lower activity in the NADH/Lip(S–S) assay but an enhanced DCI reductase activity (approximately 3 and 2000% that of the native protein, respectively). It was subsequently demonstrated that copper treatment introduces one or more additional disulfide bonds

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[1] Abbreviations used are: BCS, bathocuproinesulfonate; DCI, 2,6-dichloroindophenol; $Lip(SH)_2$ and Lip(S–S), reduced and oxidized lipoamide, respectively.