LH-RH and its analogues in $^{13}C$ should allow investigation of the binding properties of these peptides, if not to membrane receptors due to low receptor populations, to model receptors.

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# Superactivation of Neutral Proteases: Acylation with *N*-Hydroxysuccinimide Esters†

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ABSTRACT: A series of *N*-hydroxysuccinimide esters of acylamino acids previously shown to acylate and thereby increase the activity of thermolysin by several orders of magnitude (Blumberg, S., and Vallee, B. L. (1975), *Biochemistry 14,* 2410) has been used to modify the related neutral proteases from *Bacillus subtilis, Bacillus megaterium,* and *Aeromonas proteolytica.* Each of these enzymes is activated to a level characteristic of the particular protein and the particular acyl group incorporated when monitored with the substrate furylacryloyl-Gly-Leu-$NH_2$. Thus, for the modification of *B. megaterium, B. subtilis,* and *A. proteolytica* proteases with Ac-Trp-ONSu, $k_{cat}/K_m$ increases 11-, 2.5-, and 18-fold whereas those of the Ac-Phe(4-DnpNH)-ONSu derivatives increase 23-, 22-, and 3.4-fold, respectively. Absorption spectra of the Ac-Phe(4-DnpNH)-ONSu modified enzymes before and after deacylation with hydroxylamine indicate that from 1 to 2 residues are modified. The rate of removal of the Ac-Phe(4-DnpNH) label by 0.1 M hydroxylamine correlates directly with that of the return of native enzymatic activity, at a rate comparable with the rate of deacylation of *O*-acyltyrosine models. The competitive inhibitors $Zn^{2+}$ and $\beta$-phenylpropionyl-Phe do not prevent activation indicating that modification occurs at a site(s) distinct from that at which inhibitors bind. The degree of activation depends also on the substrate employed, generally being greater for substrates which the native enzymes hydrolyze slowly. These data are interpreted to indicate the modification of a residue near the active site, but which serves as a subsite for substrate interaction.

Complementary interactions of enzyme active centers with substrates and inhibitors are fundamental to the mode of action of enzymes (cf. Jencks, 1975). Synthetic approaches designed to further the understanding of enzyme mechanisms, however, have been restricted largely to systematic variations of substrate and inhibitor structure. Chemical modification resulting in incorporation of organic moieties into the active center of enzymes augments this approach when modulating enzymatic function. We have recently demonstrated that *N*-hydroxysuccinimide esters of acylamino acids or peptides covalently link amino acids or peptides to a chemically reactive side chain of thermolysin, thereby markedly increasing its activity (Blumberg et al., 1973, 1974; Blumberg and Holmquist, 1973; Blumberg and Vallee, 1975). The structure of these reagents

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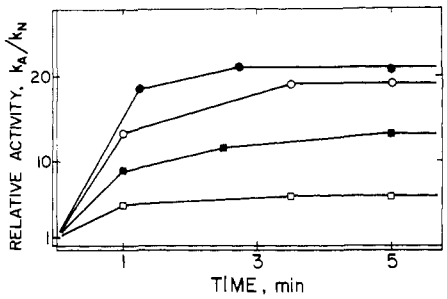


FIGURE 1: Activity of *Bacillus megaterium* protease (●), thermolysin (○), *Aeromonas proteolytica* protease (■), and *Bacillus subtilis* protease (□) when acylated with 1 mM Ac-Tyr(OAc)-ONSu. Reactions were performed at pH 8.0. Activities, $k_N$ of the native enzyme, and $k_A$ of the activated enzyme, were measured by diluting aliquots, 15 μl, at the times indicated into the assay mixture containing FAGLA and buffer.

can be varied systematically to alter enzyme structure and function in turn, providing a versatile class of reagents for the modification of proteins. We have now examined the consequences of modifying three additional bacterial neutral proteases, the enzymes from *Bacillus subtilis* var. *amylosaccharitiicus, Bacillus megaterium,* and *Aeromonas proteolytica,* with these reagents and compared the results with those obtained earlier with thermolysin.

## Experimental Section

*Enzymes.* Thermolysin (A grade, lots 200397, 201095, and 400187, Calbiochem) was recrystallized twice by dissolving in 5 M NaBr, 0.01 M $CaCl_2$, 0.01 M Tris,[1] pH 7.5, and dialyzing to low ionic strength (0.01 M $CaCl_2$, 0.01 M Tris, pH 7.5), as described previously (Latt et al., 1969). *Bacillus subtilis* var. *amylosaccharitiicus* neutral protease (once crystallized, lot 8 × 24) was obtained from Miles Lab. Inc. Dr. J. M. Prescott kindly provided *Aeromonas proteolytica* neutral protease (Griffin and Prescott, 1970). Crude extract of *Bacillus megaterium* strain MA neutral protease (Feder et al., 1971) was a gift of Dr. J. Feder, Monsanto Co., St. Louis, Mo., and was purified by affinity chromatography on an agarose-Gly-Gly-Gly-D-Phe column (Blumberg and Vallee, 1975). All of the neutral proteases were dissolved in 0.2 M NaCl, 0.01 M $CaCl_2$, 2 mM Tris, pH 7.1, followed by gel filtration on a Bio-Gel P-4 column (0.9 × 20 cm) equilibrated and eluted with the same buffer. The enzymes contained 1.0 ± 0.1 g-atom of zinc per mol of protein, as determined by atomic absorption spectrophotometry (Fuwa and Vallee, 1963).

*Reagents and Substrates.* Amino acids and peptides were obtained from Cyclo Chem. Co., Sigma Chem. Corp., Mann Res. Lab, or Fox Chem. Co. or were synthesized according to standard procedures. Substrates and the *N*-hydroxysuccinimide esters of N-blocked amino acids or peptides were prepared as suggested by Anderson et al. (1964). The details of these syntheses and the properties of the derivatives have been described (Blumberg and Vallee, 1975).

*Assay and Kinetic Measurements.* Activities were determined from the decrease in absorbance at 345 nm on hydrolysis of 1 mM *N*-3-(2-furyl)acryloylglycyl-L-leucylamide (FAGLA) in 0.1 M NaCl, 0.05 M Tris, 0.01 M $CaCl_2$, pH 7.5, 25 ° C (Latt et al., 1969). Under these conditions, hydrolysis of this substrate by each of these neutral proteases was first-order. Rate constants were determined from the observed first-order rates or from the half-lives of substrate hydrolysis. Hydrolyses of *N*-3-(2-furyl)acryloyl blocked tripeptides were examined in a similar manner by monitoring the change in absorption at 330–345 nm using 0.1 to 0.2 mM substrate under the same conditions as those used for the FAGLA assay. The strict first-order behavior at these low substrate concentrations and the absence of product inhibition indicate that the observed first-order rates are true $k_{cat}/K_m$ values.

*Chemical Modifications.* Unless otherwise indicated chemical modifications were performed at constant pH using a Radiometer ABU-12 and a TTT-1a autotitrator with 1–2 ml of 0.01 to 0.1 mM enzyme solutions, containing 0.2 M NaCl, 0.01 M $CaCl_2$, 1 mM Tris at pH 8.0. Reactions were initiated by addition of a 0.05 to 0.1 M solution of the *N*-hydroxysuccinimide ester dissolved in dimethylformamide. Protein concentrations were determined by absorbance based on the following molar absorptivities; 66 000 $cm^{-1}$ $M^{-1}$ at 280 nm for thermolysin (Ohta et al., 1966; Titani et al., 1972), 55 750 $cm^{-1}$ $M^{-1}$ at 278.5 nm for *Aeromonas proteolytica* protease (J. M. Prescott, personal communication), and 54 200 $cm^{-1}$ $M^{-1}$ at 280 nm for *Bacillus subtilis* and *Bacillus megaterium* proteases (Keay, 1969; Keay et al., 1971). All other procedures, equipment, and methods employed have been described (Blumberg and Vallee, 1975).

## Results

For the *N*-hydroxysuccinimide esters of *N*-acylamino acids which modify thermolysin and increase its activity (Blumberg and Vallee, 1975), maximal activation is attained at pH 8.0 using 0.1 to 5.0 mM acylating agent and from 0.02 to 1 mM enzyme. These conditions now have been employed to modify the neutral proteases from *Bacillus subtilis, Bacillus megaterium,* and *Aeromonas proteolytica.* Figure 1 illustrates the time course for the changes of activity on acylation of each of these enzymes with 1 mM Ac-Tyr(OAc)-ONSu. Activity becomes maximal in approximately 5 min and remains unchanged thereafter.

Similar experiments were performed under identical conditions using a large number of *N*-hydroxysuccinimide esters of aliphatic and aromatic *N*-acylamino acids of varying structures (Table I). For each agent, the effect on the rate of hydrolysis of FAGLA was monitored as a function of time. In all instances maximum activity was reached within 5 min, and addition of a second aliquot of acylating agent thereafter did not further increase activity significantly indicating virtually complete modification by the first aliquot. The degree of activation of each particular enzyme is a function of the structure of the acylating agent (Table I).

The activation resulting from incorporation of aliphatic residues is generally less than that from aromatic or substituted aromatic groups. Further, the activities of the various acyl-enzyme derivatives depend on the particular enzyme employed. For example, Ac-Phe-ONSu activates FAGLA hydrolysis by thermolysin and the *Bacillus megaterium, Bacillus subtilis,* and *Aeromonas proteolytica* enzymes, 20-, 14-, 2.7-, and 11-fold, respectively (Table I). The optical configuration of the reagent does not affect the degree of activation nor the time course of the activation process. Hence the extent (Table I) as well as rate of activation of thermolysin and of the *Bacillus subtilis* protease with Ac-D-Phe-ONSu is the same as with its L-antipod.

Previous studies of the analogous activation of thermolysin indicated that acylation of the enzyme occurs (Blumberg and Vallee, 1975). In that system treatment with nucleophiles, e.g.,

[1]Abbreviations used are: FA, 3-(2-furyl)acryloyl-; FAGLA, *N*-3-(2-furyl)acryloylglycyl-L-leucylamide; ONSu, succinimidooxy; Tris, tris(hydroxymethyl)aminomethane; Bzl, benzyl; Ac, acetyl; Bz, benzoyl.

TABLE I: The Effect of Acylation by *N*-Hydroxysuccinimide Esters on the Fagla Activity of Neutral Proteases.[a]

| *N*-Hydroxysuccinimide Ester | *n*-Fold Activation[b] *B. thermoproteolyticus*[c] | *B. megaterium* | *B. subtilis* | *A. proteolytica* |
|---|---|---|---|---|
| Ac-Ala-ONSu | 1.7 | 1.6 | 1.1 | 1.8 |
| Ac-Val-ONSu | 2.2 | 1.7 | 1.7 | 1.6 |
| Ac-Ile-ONSu | 2.4 | 2.1 | 1.9 | 1.8 |
| Ac-Phe-ONSu | 20 | 14 | 2.7 | 11 |
| Ac-D-Phe-ONSu | 20 | | 2.7 | |
| Ac-Trp-ONSu | 12 | 11 | 2.5 | 18 |
| Ac-Tyr(OAc)-ONSu | 20 | 21 | 6 | 12 |
| Ac-Tyr(OBzl)-ONSu | 20 | 20 | 16 | 8 |
| Ac-Tyr(Dnp)-ONSu | 40 | 23 | 14 | 2.4 |
| Ac-Phe(4-DnpNH)-ONSu | 70 | 23 | 22 | 3.4 |
| Dnp-Gly-D-Phe-ONSu | 15 | 14 | 7 | 5 |
| Dns-Gly-D-Phe-ONSu | 15 | 8 | 17 | 2.7 |

[a] Activities were assayed with FAGLA as described in the Experimental Section, under conditions where hydrolysis followed first-order kinetics. Acylation reactions were carried out at 25 °C, pH 8, at enzyme concentrations between 0.01 and 0.05 mM and a reagent concentration of 0.1–5 mM. [b] Expressed as the ratio $k_{cat}/K_m$ for the acylated enzyme to $k_{cat}/K_m$ of the native enzyme. The $k_{cat}/K_m$ values for FAGLA hydrolysis are $2.2 \times 10^4$, $1.0 \times 10^5$, $3.8 \times 10^3$, and $3.3 \times 10^4$ $M^{-1}$ $s^{-1}$ for the native *Bacillus thermoproteolyticus, Bacillus megaterium, Bacillus subtilis,* and *Aeromonas proteolytica,* respectively. [c] Data from Blumberg and Vallee (1975).

TABLE II: Deacylation of Acyl Compounds in 0.1 M Hydroxylamine.

| Compound | Half-Life[a] (min) |
|---|---|
| *N,O*$^4$-Diacetyltyrosine | 5.7 |
| *N*-Acetyl-*O*$^4$(Ac-Phe)-tyrosine | 2.3 |
| *N*-Acetyl-*O*$^4$(Bz-Gly)-tyrosine | 1.2 |
| Acetylimidazole | 0.004[b] |
| BzGly-OEt | 6000[c] |
| Ac-Phe(4-DnpNH)-thermolysin | 4 |
| Ac-Phe(4-DnpNH) *A. proteolytica* protease | 8 |
| Ac-Phe(4-DnpNH) *B. subtilis* protease | 5 |
| Ac-Phe-thermolysin | 8 |

[a] Measured at pH 7.5, 0.03 M Tris, 0.01 M $Ca^{2+}$ in 0.1 M hydroxylamine. [b] Calculated from the second-order rate constant of 1700 $M^{-1}$ $min^{-1}$ (Jencks and Carriuolo, 1959). [c] Calculated from the second-order rate constant for hydroxylamine catalyzed deacylation measured in 2 M hydroxylamine.

hydroxylamine, returns activity to that of the native enzyme at rates which suggest the deacylation of tyrosine. In the present instance selected modifications were employed to establish that the activation of these neutral proteases is also associated with the acylation of the enzyme.

The enhanced activities of *Bacillus subtilis* protease and *Aeromonas proteolytica* protease modified with Ac-Phe(4-DnpNH)-ONSu are unchanged on gel filtration with Bio-Gel P-4. Each of the gel filtered acyl-enzymes was subjected to deacylation with 0.1 M hydroxylamine, pH 7.5, while monitoring its specific activity. Figure 2 shows the time course both of the acylation and deacylation reactions. Analogous results have been obtained previously with thermolysin (Blumberg and Vallee, 1975). Hydroxylamine catalyzed deacylation of each activated enzyme returns activity to that of the native

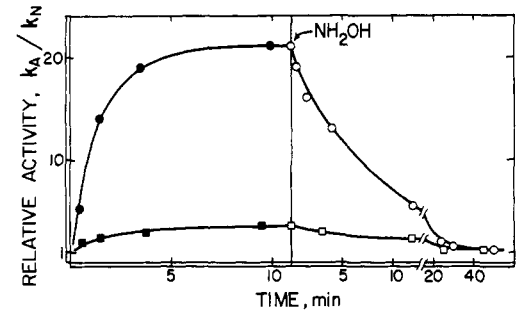


FIGURE 2: Acylation (closed symbols) and deacylation (open symbols) of *Bacillus subtilis* protease (●, ○) and *Aeromonas proteolytica* protease (■, □).. Enzyme, 2–10 × $10^{-5}$ M, was acylated with Ac-Phe(4-DnpNH)-ONSu, 0.3 to 0.5 mM, and FAGLA activity was followed as a function of time. After 11 min the enzymes were gel filtered on Bio-Gel P-4, pH 7.2, 10 mM $CaCl_2$, 2 mM Tris, 0.2 M NaCl. Deacylation was initiated by addition of 2 M hydroxylamine, pH 7.5, to the gel-filtered enzyme. The final concentration of hydroxylamine was 0.1 M.

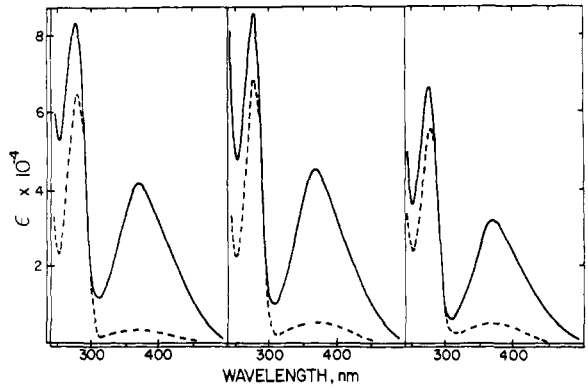


FIGURE 3: Absorption spectra before (—) and after (- - -) deacylation of Ac-Phe(4-DnpNH)-ONSu modified *Aeromonas proteolytica* protease (left), thermolysin (center), and *Bacillus subtilis* protease (right). The preparations are those described in Figure 2. The modified thermolysin was prepared as described earlier (Blumberg and Vallee, 1975). Spectra of the deacylated enzymes were measured subsequent to gel filtration of the deacylation reaction mixture of Figure 2 using the same buffer and resin.

enzyme with similar, pseudo-first order rates (Figure 2), suggesting that acylation and deacylation of tyrosine are responsible for activation and its reversal. The results with the Ac-Phe-ONSu modified enzymes are similar.

Reaction with the chromophoric Ac-Phe(4-DnpNH)-ONSu ester generally confers high activity on all the neutral proteases. Absorption spectrophotometry was used to quantitate the number of Ac-Phe(4-DnpNH)- groups incorporated during acylation and lost upon hydroxylamine catalyzed deacylation. In the experiment outlined above, the spectra of the activated enzymes (Figure 3) all exhibit an absorption maximum at 370 nm attributable to the dinitrophenyl group. Based on $\epsilon_{370}$ 18 500 and $\epsilon_{280}$ 7000 for the Dnp chromophore (Blumberg and Vallee, 1975), the number of groups incorporated are 2.3, 1.9, and 1.8 for thermolysin and *Aeromonas proteolytica* and *Bacillus subtilis* proteases, respectively. Figure 3 also shows the spectra of the deacylated enzymes exhibiting native activity following gel filtration subsequent to hydroxylamine treatment. For the *Bacillus subtilis* enzyme the difference in the Dnp content before and after deacylation is 1.5, for *Aeromonas proteolytica* enzyme it is 1.8, and for thermolysin it is 2.0. Thus, over 83% of the label of each enzyme is removed, concomitant with the return of native activity. Since acylated α-amino or ε-amino groups would not be expected to undergo deacylation under these conditions, the results indicate that

TABLE III: Inhibition of FAGLA Hydrolysis. Native and Ac-Phe(4-DnpNH)-Modified Enzymes.

| Inhibitor | $K_I$ (M) | | | | | |
|---|---|---|---|---|---|---|
| | Thermolysin[a] | | B.s. Neutral Proteases[b] | | A.p. Neutral Protease[b] | |
| | Native | Modified | Native | Modified | Native | Modified |
| $Zn^{2+}$ | $4.2 \times 10^{-5}$ | $5.8 \times 10^{-5}$ | $4.8 \times 10^{-5}$ | $3.0 \times 10^{-5}$ | $5.1 \times 10^{-5}$ | $11.0 \times 10^{-5}$ |
| β-Phenylpropionyl-L-Phe | $1.6 \times 10^{-3}$ | $1.0 \times 10^{-3}$ | [c] | [c] | $4.0 \times 10^{-4}$ | $8.2 \times 10^{-4}$ |

[a] Data from Blumberg and Vallee (1975). [b] Measured at 330 nm (pH 7.5) employing $2 \times 10^{-5}$ FAGLA, in 0.05 M Tris, 0.01 M $CaCl_2$ and 0.1 M NaCl. [c] Not determined.

amino groups are not modified to a significant extent.

Hydroxylamine (0.1 M) rapidly deacylates each enzyme with a half-life of between 4 and 8 min. The rates of deacylation of a number of acylated neutral proteases are compared in Table II with those of some $O^4$-acyltyrosine esters, acetylimidazole, and benzoylglycine ethyl ester. Under equivalent conditions, the rates of hydrolysis of the $O^4$-acyltyrosine esters are close to those of the acylated enzymes, while those for the acylimidazole and acyl alcohol are ~1000 times faster and ~1000 times slower, respectively.

Both β-phenylpropionyl-L-phenylalanine and zinc competitively inhibit neutral protease activity. However, neither the rate nor the extent of activation by Ac-Phe-ONSu is affected when inhibitory concentrations of these agents are present in the acylation reaction mixture, suggesting that inhibition and modification occur at different sites of the enzymes. Moreover, the values of the inhibition constants, $K_I$ (Table III), are nearly the same for the native and Ac-Phe(4-DnpNH) modified enzymes, also indicating that the site of modification is distinct from that of the inhibitory sites of these agents.

FAGLA, which has served to monitor the progress and results of the acylation of the neutral proteases, is a relatively poor substrate when compared with a number of related *N*-3-(2-furyl)acryloyl blocked substrates (Table IV). Indeed, $k_{cat}/K_m$ can be varied over four orders of magnitude by altering substrate composition through variation of the position of hydrophobic residues relative to the site of bond cleavage. When these substrates are ordered with respect to increasing rates of hydrolysis by thermolysin, the neutral proteases from *Bacillus subtilis* and *Aeromonas proteolytica* exhibit quite similar sequences, the two sole exceptions involving C-terminal Leu-Gly (Table IV, lines 4 and 7). The specificity of each of these enzymes, then, is influenced largely by the presence of hydrophobic amino acids adjacent to the site of cleavage, a characteristic feature of the neutral proteases which has been detailed extensively for thermolysin and the *Bacillus subtilis* enzyme (Morihara and Tsuzuki, 1970; Morihara, 1974). In general, the specific activity of the *Bacillus subtilis* enzyme is 2- to 10-fold less and that of the *Aeromonas* enzyme is approximately 2- to 10-fold greater than that of thermolysin. The maximal value of $k_{cat}/K_m$ is approximately $2 \times 10^6$ $M^{-1}$ $s^{-1}$, attained with three substrates for the *Aeromonas* enzyme and with one substrate for thermolysin. The *B. subtilis* enzyme is inherently less effective toward these substrates since the maximum value attained is only $2.65 \times 10^5$ $M^{-1}$ $s^{-1}$.

These relationships between substrate structure and activity are considerably altered by acylation. Thus, employing three proteases modified with three different acylating agents, Ac-Val-ONSu, Ac-Phe-ONSu, and Ac-Phe(4-DnpNH)-ONSu, the extent of the resultant activity changes was explored as a function of substrate structure. Table V shows the ratio of

TABLE IV: Activities of Neutral Proteases toward Peptide Substrates.

| Substrate (FA-X), X = | Spec Act., $k_{cat}/K_m \times 10^{-3}$ ($M^{-1}$ $s^{-1}$) | | |
|---|---|---|---|
| | Thermolysin[a] | *B. subtilis* Protease | *A. proteolytica* Protease |
| Gly-Ala-Gly | 0.13 | 0.06 | 3.2 |
| Gly-Leu-$NH_2$ | 22 | 3.8 | 32.7 |
| Ala-Ala-Ala | 55 | 19.2 | 442 |
| Gly-Leu-Gly | 83 | 6.3 | 337 |
| Gly-Leu-Phe | 300 | 160.0 | 2000 |
| Gly-Leu-Ala | 870 | 265.0 | 2180 |
| Phe-Leu-Gly | 2300 | 190.0 | 1750 |

[a] Data from Blumberg and Vallee (1975).

$k_{cat}/K_m$ for the modified enzymes to that of the native enzymes. Comparisons of the effects of the acyl group structure, the substrate structure, and the enzymes employed reveal a number of features which appear to characterize the activation process.

Bulkier groups on the acylating agent, such as phenylalanine and its para substituted derivatives, generally activate thermolysin and the *Bacillus subtilis* neutral protease toward the various substrates to greater extents than the smaller Ac-Val group. However, the order is different for the *Aeromonas* enzyme, whose Ac-Phe derivative is more active toward all substrates than either the Ac-Phe(4-DnpNH) or Ac-Val modified protein. Indeed, except with FA-Gly-Ala-Gly and FA-Gly-Leu-$NH_2$, the Ac-Phe(4-DnpNH) modification actually decreases the activity of the *Aeromonas* enzyme.

The large increase in $k_{cat}/K_m$ for relatively poor substrates is an additional characteristic of the acylation of the proteases contrasting with the much lower increase for the substrates which are hydrolyzed more rapidly. Comparison of the data for FA-Gly-Ala-Gly, a poor substrate for all native enzymes, with those for FA-Gly-Leu-Ala or FA-Gly-Leu-Phe, which are excellent substrates, indicates that, with the former, activation is maximal with all acyl-enzymes whereas with the latter the rates of hydrolysis are increased only slightly or even decreased. All the modified enzymes are uniformly less active toward FA-Phe-Leu-Gly, the best substrate for native thermolysin and an excellent substrate for the other enzymes.

## Discussion

The present studies extend our earlier reports on thermolysin to the superactivation by covalent modification of the neutral endopeptidases from *Bacillus megaterium, Bacillus subtilis,* and *Aeromonas proteolytica.* These enzymes share a number of common features. All are zinc metalloenzymes (Keay, 1969;

TABLE V: Modification of Neutral Proteases by *N*-Hydroxysuccinimide Esters of Acylamino Acids.[a]

| Substrate (FA-X), X = | *n*-Fold Activation[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Thermolysin[c] | | | *Bacillus subtilis* | | | *Aeromonas proteolytica* | | |
| | Ac-Phe-(4-DnpNH) | Ac-Phe | Ac-Val | Ac-Phe-(4-DnpNH) | Ac-Phe | Ac-Val | Ac-Phe-(4-DnpNH) | Ac-Phe | Ac-Val |
| Gly-Ala-Gly | 400 | 100 | 4 | 45 | *d* | *d* | 5.5 | 13.2 | 2.8 |
| Gly-Leu-$NH_2$ | 70 | 20 | 2.2 | 22 | 2.9 | 1.5 | 3.4 | 11 | 2.1 |
| Ala-Ala-Ala | 2.2 | 3.3 | 1.0 | 3.5 | 2.3 | 1.5 | 0.15 | 1.3 | 1.1 |
| Gly-Leu-Gly | 22 | 20 | 2.7 | 21 | 2.3 | 1.3 | 0.80 | 5.0 | 2.2 |
| Gly-Leu-Phe | 10 | 15 | 2.6 | 7.8 | 1.8 | 1.5 | 0.70 | 2.8 | 1.5 |
| Gly-Leu-Ala | 1.8 | 3.5 | 1.5 | 3.9 | 1.8 | 1.0 | 0.40 | 1.5 | 1.5 |
| Phe-Leu-Gly | 0.22 | 0.25 | 0.16 | 0.49 | 0.33 | 0.59 | 0.18 | 0.33 | 0.27 |

[a] Modified enzymes prepared as described in text. [b] Expressed as the ratio $k_{cat}/K_m$ for the acylated enzyme to $k_{cat}/K_m$ of the native enzyme. [c] Data from Blumberg and Vallee (1975). [d] Not determined.

Keay et al., 1971; Latt et al., 1969; Griffin and Prescott, 1970), and their activities also depend on an essential His residue at the active site, as has been documented for both thermolysin (Blumberg et al., 1973, 1974; Blumberg and Holmquist, 1973; Burstein et al., 1974) and the *Bacillus subtilis* enzyme (Pangburn and Walsh, 1975). Their specificity requirements are also analogous (Matsubara and Feder, 1971; Morihara, 1974). Each prefers substrates in which bulky hydrophobic amino acids constitute both sides of the susceptible bond. Further, extended regions adjacent to the active site facilitate secondary interactions between enzyme and potential polypeptide substrates and markedly influence binding and catalytic constants (Morihara, 1974). The present study demonstrates that each of the neutral proteases also responds to reversible acylation with amino acid *N*-hydroxysuccinimide esters with marked enhancement of enzymatic activity. The rates of activation are nearly identical for a wide variety of structurally different activating esters suggesting similar or identical reactive residue(s) in all of these proteases. The stoichiometry, as monitored by reaction with Ac-Phe(4-DnpNH)-ONSu, actually implicates the modification of from 1 to 2 residues of each enzyme (Figure 3). However, studies of thermolysin (Blumberg and Vallee, 1975) suggested that modification of a single residue could account for the activity changes even though maximal activation coincides with the incorporation of approximately 2 mol of Ac-Phe(4-DnpNH) per mol of enzyme.

The acylation of tyrosyl residue(s) is consistent both with the stability of the acyl-enzymes and with their ease of deacylation by hydroxylamine. Under conditions where rapid deacylation occurs, hydrolysis of acylated $\alpha$- or $\epsilon$-amino groups would not be expected. The rates of deacylation observed are nearly identical with those found for $O^{4'}$-acyltyrosine esters. By comparison, deacylation of an amino acid alkyl ester is ~1000-fold slower, while that of an acylimidazole is ~1000-fold more rapid (Table II), excluding Thr, Ser, or His residues as those involved in any of the enzymes. Indeed, preliminary data obtained by proteolytic digestion of Ac-Phe(4-DnpNH)-labeled thermolysin have yielded a peptide containing Tyr and the major fraction of the Dnp label, but neither His nor Lys. The isolation and characterization of the labeled peptide are in progress.

The location of the enzyme residue(s) involved in modification can only be inferred at present. The lack of effect either of $\beta$-phenylpropionyl-L-Phe or $Zn^{2+}$ on the acylation process indicates that the site of modification is remote from the active center. X-Ray analysis has shown (Matthews et al., 1974) that $\beta$-phenylpropionyl-L-Phe interacts at the active site. Excess $Zn^{2+}$ may well bind to His-231, a residue close to the active site and known to bind $Ag^{2+}$. The effect of both inhibitors on the modified and native enzymes is virtually the same (Table III); their $K_I$ values are nearly identical, implying that the group(s) modified are not critical to the binding of these inhibitors.

Secondary interactions between enzyme and substrates which govern the overall rate of substrate hydrolysis are important, common factors for all the enzymes which have been activated in this manner. Indeed, "substrate mapping" (Schechter and Berger, 1967) has proven feasible to identify such interactions in thermolysin and the *Bacillus subtilis* neutral protease (Morihara and Tsuzuki, 1970, Morihara et al., 1969) in order to delineate subsites affecting both binding and catalysis. While the chemical identity of the amino acid contributing the amino group of the bond cleaved exerts the predominant influence on catalysis, there are significant contributions from amino acids which are as far as 3 residues removed from the susceptible bond. When hydrophobic residues occupy sites of the substrate which correspond to the respective subsites on the enzyme, turnover is most "efficient" and rapid. On this basis, it has been suggested (Morihara, 1974) that both binding and catalysis of the *Bacillus subtilis* and *Bacillus thermoproteolyticus* neutral proteases are maximal when substrates conform optimally to the enzyme's subsite structure. The alterations of proteolytic activity in response to acylation appear to modulate such secondary enzyme–substrate interactions. An enzyme subsite is altered, thereby augmenting the "fit" of a given substrate, usually to potentiate hydrolysis, though attenuation of hydrolysis is observed also. Collectively, the inhibitor studies suggest that the site of modification is not in the active site (Table III). However, the dependence of the modified activity on the structure of the substrate and of the acylating agent indicates that the modified residue must be sufficiently close to the active site to be capable of interacting with some group on the substrate to induce such wide variations of activity. The acylated enzymes actually hydrolyze those substrates less efficiently which exhibit the highest $k_{cat}/K_m$ values and thus optimally fulfill the subsite requirements of the native enzyme, as is apparent when phenylalanine occupies the position donating the carboxyl group of the susceptible bond (Table V). The hydrolysis of two additional substrates, FA-Phe-Ala-Gly and FA-Phe-Gly-Gly, also follows this trend when attacked by Ac-Phe(4-

DnpNH)-thermolysin. The $k_{cat}/K_m$ values of these substrates for native thermolysin, $21.5 \times 10^3$ and $2.8 \times 10^2$ $M^{-1}$ $s^{-1}$, respectively, are decreased by 75% in the modified enzyme. Similar considerations pertain to the attentuation of the activity for the majority of substrates when hydrolyzed by Ac-Phe(4-DnpNH)-ONSu modified *Aeromonas proteolytica* protease, since the catalytic coefficients of this particular native enzyme are in general very high, indicative of optimal secondary interactions (Tables IV and V). Conversely, substrates which "fit" the enzyme subsite structure poorly and, thus, are hydrolyzed slowly, are potentiated maximally, as exemplified best by the hydrolysis of FA-Gly-Ala-Gly, whose hydrolysis is increased maximally in all cases examined.

Acylating agents containing bulky aromatic substituents generally elicit the greatest activation. Thus, the nonaromatic Ac-Ala, Ac-Val, or Ac-Ile esters moderately affect the FAGLA activity of any of the proteases conferring an increase of no more than 2.5-fold (Table I), whereas the larger Ac-Phe ester and its para-substituted counterparts are much more effective (Table I). Table V further illustrates this for the Ac-Val, Ac-Phe, and Ac-Phe(4-DnpNH) derivatives of three of the enzymes. For the *Bacillus subtilis* enzyme and thermolysin, activation increases in the order, Ac-Val, Ac-Phe, Ac-Phe(4-DnpNH) with all substrates examined. However, while the Ac-Phe-modified *Aeromonas* enzyme is more active than that modified with Ac-Val, it is also more active than the Ac-Phe(4-DnpNH) enzyme. In this instance, placing the 4-substituent on the aromatic ring of phenylalanine is less than optimal as compared with the smaller Ac-Phe derivative, perhaps due to overextension of the enzyme subsite undergoing modification and its subsequent, less favorable interactions with substrate.

The changes in activity induced here by chemical modifications with acyl amino acid derivatives are reminiscent of the control and regulation of an increasingly large number of enzymes by enzymatically mediated covalent, reversible modification of tyrosyl or seryl residues with phosphoryl, adenyl, or ADP ribosyl groups (Stadtman, 1970). In those instances, the concomitant gains and losses of activity constitute important physiological mechanisms for the regulation of complex metabolic events. Such functional responses signal a remarkable potential of the enzymes to seemingly minor changes in structure.

Synthetic chemical modifications such as those presented here which generate systematic changes in enzyme function may serve as models for such processes. Such modulations of function likely represent perturbations of features essential to catalytic mechanisms and may provide a potentially powerful tool for the study of other mechanistic aspects of enzyme action.

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