Amino Acid Residue Modified during Superactivation of Neutral Proteases: Tyrosine-110 of Thermolysin†

Shmaryahu Blumberg

ABSTRACT: Acylation of thermolysin and of other neutral proteases with *N*-hydroxysuccinimide esters of acyl amino acid derivatives yields "superactive" enzyme products that are more active than the native enzymes toward a variety of peptide substrates [Blumberg, S., & Vallee, B. L. (1975) *Biochemistry 14*, 2410]. Thermolysin modified with one such active ester, the *N*-hydroxysuccinimide ester of *N*-acetyl-*p*-(2,4-dinitroanilino)-L-phenylalanine, was examined in detail to identify the site(s) of modification, responsible for the changes in activity. The chemical stability of the acyl–enzyme linkages indicates that tyrosine residues are largely modified. Although an average of about two residues can be modified by the reagent, evidence is provided that modification at only one distinct residue elicits the change in the catalytic properties. Fractionation of partially modified enzyme on an agarose–Gly_3-D-Phe affinity column yields two protein fractions, one which is highly active and the other which is lower in activity. Digestion with thermolysin and separation of 2,4-dinitrophenyl-labeled peptides reveal that two major peptides composed of Ser, Glx, Gly, Tyr and Ser, Glx, Gly, Tyr_2 constitute together about 70% of the labeled peptides in the highly active fraction, whereas in the fraction of lower activity their content is less than 20%. The N-terminal residues of these peptides are serine and tyrosine, respectively, and the C-terminal residues of both peptides are tyrosines. Since these analyses correspond uniquely to the overlapping sequences 107–110 (Ser-Gln-Gly-Tyr) and 106–110 (Tyr-Ser-Gln-Gly-Tyr) in the known sequence of thermolysin [Titani, K., et al. (1972) *Nature* (*London*), *New Biol. 238*, 35], it is concluded that modification of tyrosine-110 is responsible for superactivation of the enzyme.

Recent studies of thermolysin from *Bacillus thermoproteolyticus* and of other neutral proteases, from *Bacillus subtilis*, *Bacillus megaterium*, and *Aeromonas proteolytica*, have revealed that the enzymes may undergo large enhancements of their peptidase activities when chemically modified with *N*-hydroxysuccinimide esters of acyl amino acid derivatives (Blumberg & Vallee, 1975). Each of these enzymes is activated to a level characteristic of the particular protein and of the particular acyl group incorporated covalently. The extent of activation, moreover, depends on the substrate employed, generally being high for (poor) substrates which the native enzymes hydrolyze slowly. A variety of observations collectively suggest that these modulations of enzymic activity are due to modification at a distinct residue, presumably a tyrosine, near the active sites of the enzymes (Blumberg & Vallee, 1975; Holmquist et al., 1976).

Of all the neutral proteases, thermolysin is the only one whose amino acid sequence (Titani et al., 1972) and three-dimensional structure (Matthews et al., 1972a,b, 1974; Colman et al., 1972) are both known. Hence, it would seem possible to identify chemically the amino acid residue modified and to establish its location in the protein molecule. Toward this end, thermolysin modified with one particular reagent, Ac-Phe(DnpNH)-ONSu,[1] was chosen for study because the spectral properties of the reagent offer convenient means for quantitative analyses. Yet, some inherent properties of the modification present serious handicaps to such a study: the acyl-enzyme linkages (phenyl ester) are only moderately stable, requiring mild treatment of the derivative to avoid deacylation; the modification is only partially specific and maximal activation is achieved when more than one residue is modified, imposing problems in the assignment of the modified residue which is responsible for the activation process (Blumberg & Vallee, 1975).

The present study describes a method for the identification of the site of modification, involving fractionation of the modified enzyme, enzymic degradation, and peptide isolation procedures, all performed under acidic conditions or at neutral pH in the absence of nucleophiles, thereby minimizing deacylation of the chromophoric acyl moiety. Fractionation of partially modified enzyme into a highly active fraction and a lower activity fraction and comparison of the Dnp-labeled peptides obtained after enzymic degration allow the assignment of the labeled peptides relevant to the activation process. Analyses of the peptides lead to the conclusion that it is the phenolic side chain of Tyr-110, near the active site of the enzyme, that when acylated brings about the changes in the activity of the enzyme.

Materials

Thermolysin, 3× crystallized (A grade, lots 500839 and 501243), was obtained from Calbiochem and recrystallized before use (Latt et al., 1969). Stock solutions of the enzyme, 20 mg/mL, were made up by dissolution in 5 M NaBr–0.01 M $CaCl_2$–0.05 M Tris, pH 7.5. Furylacryloyl-Gly-Leu-NH_2 was obtained from Cyclo Chemical Corp. and Aminex A-5 and Bio-Gel P-4 were from Bio-Rad Laboratories. The acylating agent Ac-Phe(DnpNH)-ONSu was prepared as previously described (Blumberg & Vallee, 1975). The affinity adsorbent for thermolysin, agarose–Gly_3-D-Phe, was prepared by coupling Gly_3-D-Phe to Sepharose 4B by the cyanogen bromide activation method (Axēn et al., 1967) following a detailed procedure described previously (Blumberg et al., 1970). The resin contains ~3 μmol of covalently bound peptide per mL of bed resin as determined by amino acid analysis. All other reagents were of analytical grade. Buffer solutions and substrates were rendered free of heavy metal impurities by extraction with 0.01% dithizone in carbon tetrachloride (Thiers, 1957).

† From the Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel, and Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received November 22, 1978.* This work was supported, in part, by Grant No. GM 15003-12 to Dr. B. L. Vallee in the Biophysics Research Laboratory.

[1] Abbreviations used: Phe(DnpNH), *p*-(2,4-dinitroanilino)-L-phenylalanine; ONSu, succinimidooxy; DMF, dimethylformamide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Z, benzyloxycarbonyl; Moc, methyloxycarbonyl. All other abbreviations are according to IUPAC–IUB rules [see for example (1972) *J. Biol. Chem. 247*, 977].

0006-2960/79/0418-2815$01.00/0

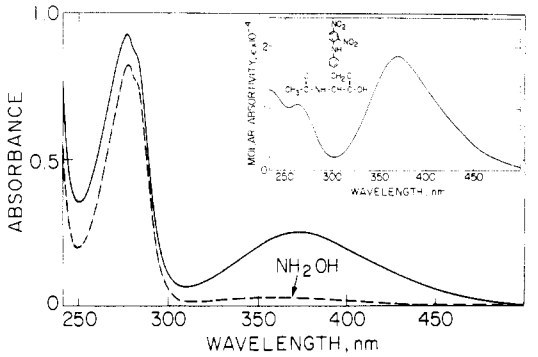

FIGURE 1: Absorption spectra of Ac-Phe(DnpNH)-ONSu-modified thermolysin before (—) and after (---) deacylation with hydroxylamine. Thermolysin, 0.3 mM, was modified with Ac-Phe(DnpNH)-ONSu, 0.55 mM in 2.5 M NaBr–0.01 M $CaCl_2$–0.025 M Tris, pH 8.0, 25 °C for 10 min, and the product was gel-filtered on a Bio-Gel P-4 column equilibrated and eluted with 0.2 M NaCl–0.01 M $CaCl_2$–0.002 M Tris, pH 7.1. A portion of the gel-filtered enzyme was deacylated by treatment with 0.1 M NH_2O-H–0.015 M Tris, pH 7.5, 25 °C for 50 min, and the product was gel-filtered. Insert: Structure and spectrum of Ac-Phe(DnpNH) in 0.2 M NaCl–0.01 M $CaCl_2$–0.002 M Tris, pH 7.1.

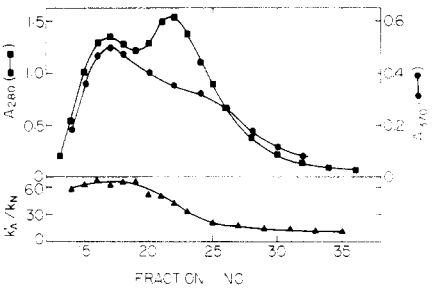

FIGURE 2: Fractionation of thermolysin, partially modified with Ac-Phe(DnpNH)-ONSu, on an agarose–Gly_3–D-Phe column (1.6 × 42 cm). Thermolysin, 20 mL of 0.3 mM, was reacted with Ac-Phe(DnpNH)-ONSu, 0.55 mM, in 2.5 M NaBr–0.01 M $CaCl_2$–0.025 M Tris, pH 8.0, 25 °C for 10 min, and the product was gel-filtered on a Bio-Gel P-4 column (2.8 × 36 cm) equilibrated and eluted with 0.2 M NaCl–0.01 M $CaCl_2$–0.002 M Tris, pH 7.1. Fifteen milliliters of the gel-filtered product, 0.14 mM, containing 1.1 groups of covalently bound Ac-Phe(DnpNH) per mol of enzyme, was chromatographed on the affinity column equilibrated and eluted with the buffer employed for gel filtration. Fractions, 7.3 mL each, were collected and absorption at 280 and 370 nm as well as activity was 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 (▲).

Methods and Results

Analytical Procedures. Amino acid analyses were carried out with a Spinco Model 120B amino acid analyzer according to the procedure of Spackman et al. (1958). Samples were hydrolyzed with 6 N HCl in sealed evacuated tubes at 110 °C for 22 h, in the presence of 10 μL of 10% phenol. The values reported are not corrected for losses during hydrolysis. The concentration of thermolysin was derived from the absorbance at 280 nm by using $A_{280}^{1\%} = 17.65$ (Ohta et al., 1966), mol wt 34 600 (Titani et al., 1972). Concentration of thermolysin modified with Ac-Phe(DnpNH)-ONSu was derived from the absorbancies at both 370 and 280 nm by using ϵ_{370} 18 500 and ϵ_{280} 7000 M^{-1} cm^{-1} for the absorbance of the Ac-Phe(DnpNH) chromophore (Blumberg & Vallee, 1975; see also Figure 1). Concentration of the Ac-Phe(DnpNH)-labeled peptides was derived from the chromophore absorbance at 370 nm. A Zeiss PMQ II spectrophotometer was used for measurements of absorbance at a single wavelength and a Cary 14 spectrophotometer for recording absorption spectra. Activity was assayed with the chromophoric substrate furylacryloyl-Gly-Leu-NH_2 at 25 °C, by monitoring the decrease in absorbance at 340 nm due to hydrolysis of the Gly-Leu bond (Feder, 1968), by using a Gilford 2400-S instrument. Assay mixtures contained 0.2 mM substrate in 0.1 M NaCl–0.01 M $CaCl_2$–0.05 M Tris, pH 7.5. Under these conditions, hydrolysis of substrate follows pseudo-first-order kinetics. Rate constants were calculated by dividing the observed first-order rate constants by enzyme concentration. N-Terminal analyses were performed with dansyl chloride (Gray, 1967). Dansyl amino acids were identified by thin-layer chromatography on polyamide sheets (Woods & Wang, 1967). C-Terminal analyses were performed with carboxypeptidase A (Ambler, 1967).

Chemical Modification of Thermolysin with Ac-Phe(DnpNH)-ONSu. The activity of thermolysin toward furylacryloyl-Gly-Leu-NH_2 is enhanced dramatically upon acylation with the chromophoric active ester Ac-Phe(DnpNH)-ONSu. The effects of varying the concentration of the reagent and of the enzyme on the extent of modification and on the level of activation were examined in detail previously (Blumberg & Vallee, 1975). It was found that the level of activation is directly proportional to the extent of modification; a ~75-fold increase in activity was obtained for the maximal covalent incorporation of 2.4 mol of Ac-Phe(DnpNH) groups per mol of enzyme. In order to obtain an appreciable amount of thermolysin species which are modified by only one group of the reagent, the modification reaction has now been carried out under conditions which yield an enzyme product modified only partially. Thus, thermolysin, 20 mL of 0.3 mM, was reacted with the reagent, 0.55 mM, in 2.5 M NaBr–0.01 M $CaCl_2$–0.025 M Tris and 2% DMF at pH 8.0, 25 °C, for 10 min. At the end of the reaction, the enzyme was gel-filtered on a Bio-Gel P-4 column. The yellow gel-filtered enzyme contained 1.1 mol of covalently bound Ac-Phe(DnpNH) groups per mol of enzyme (Figure 1, full curve and insert) and exhibited a 36-fold higher activity than the native enzyme. A sample of the gel-filtered enzyme (1 mL) was deacylated by incubation with 0.1 M NH_2OH in 0.015 M Tris, pH 8.0, for 50 min, and the product was gel-filtered. The deacylated enzyme contained only 0.15 mol of covalently bound chromophore per mol of enzyme (Figure 1, dotted curve) and is as active as the native enzyme. The half-life for the reversal of activity to the level of the native enzyme was found to be ~4 min, the same rate that was found previously for enzyme preparations containing 2.4 mol of covalently bound reagent and exhibiting 75-fold higher activity than the native enzyme.

Fractionation of Ac-Phe(DnpNH)-ONSu-Modified Thermolysin on the Affinity Column Agarose–Gly_3-D-Phe. The gel-filtered modified thermolysin, containing 1.1 mol of covalently bound Ac-Phe(DnpNH) groups per mol of enzyme, was chromatographed on the agarose–Gly_3-D-Phe affinity column (1.6 × 42 cm). This column is longer and wider than the column used previously (Blumberg & Vallee, 1975) and the volume of the sample applied to it is smaller than before, in order to achieve a better separation of the different thermolysin species. Figure 2 shows the elution pattern of the modified enzyme, monitoring the absorption at both 370 and 280 nm. The absorptions of the chromophore and the protein are not proportional throughout the elution profile. Enzyme modified by ~1.2 mol of chromophore/mol of enzyme elutes first, followed by enzyme which is modified only partially and then by enzyme which is modified more extensively. The activity per mole of chromophore incorporated is high for the early fractions, exceeding 80% of the maximal activity achievable, and is less than 20% of the maximal activity for

the late fractions (Figure 2). Such a high activity of the early fractions indicates that modification of only one residue could be responsible for the changes in the activity of the enzyme. The highly active fractions (tubes 15–20; Figure 2) and the lower activity fractions (tubes 25–35; Figure 2) were pooled separately.

Digestion by Thermolysin and Extraction with 1-Butanol of the Dnp-Labeled Peptides. The pooled Ac-Phe-(DnpNH)-ONSu-modified enzyme fractions were precipitated with 1% trichloroacetic acid and the precipitate was collected by centrifugation. Almost no 370-nm absorbing material could be detected in the supernatant. The precipitated enzyme (4 mg/mL) was suspended in 0.2 M NaCl–0.01 M $CaCl_2$–0.002 M Tris and the pH brought to 7.0 by slowly adding 0.1 M NaOH. Thermolysin, 7% (weight of enzyme per weight of precipitate), was added with stirring and the pH kept at 7.0 by adding 0.1 M NaOH. The mixture clarified after a few minutes and was kept at 25 °C for 4 h. The solution was acidified with 1% trichloroacetic acid and the precipitate removed by centrifugation. The amount of 370-nm absorbing material in the supernatant solution was >95% of the amount in the Ac-Phe(DnpNH)-enzyme. The solution was extracted with 3 × 10 mL portions of 1-butanol and the organic layer was washed with 3 × 5 mL portions of water. The 1-butanol was evaporated in vacuo, and the residue was dissolved in 1 mL of 0.2 M pyridine–acetic acid buffer, pH 3.15. The overall yield of 370-nm absorbing material was >85% that of the Ac-Phe(DnpNH)-ONSu modified enzyme. The same procedure was applied to both the highly active and the lower activity enzyme fractions.

Separation of the Dnp-Labeled Peptides on Aminex A-5 Column: Assignment of the Dnp-Labeled Peptides Relevant to the Activation Process. The mixture of Dnp-labeled peptides obtained after digestion with thermolysin and extraction with 1-butanol was chromatographed on an Aminex A-5 column (0.9 × 14.5 cm). Figures 3A and 3B, respectively, compare the 370-nm absorbing material, eluted from the column, from digests of the highly active protein fraction (fractions 15–20, Figure 2) and of the lower activity protein fraction (fractions 25–35, Figure 2). The yields of 370-nm absorbing material in these separations are >95%. It is apparent from the elution profiles that only bands 2 and 3 are larger in their amount in Figure 3A than in Figure 3B, while all the other bands are larger in Figure 3B than in Figure 3A. The insert of Figure 3 shows that the yellow material of band 1 is Ac-Phe(DnpNH), the product of deacylation of the labeled peptides, as will also be apparent below. The amount of this deacylation product varies from experiment to experiment and it can be minimized by speeding up the isolation procedure. The insert of Figure 3 also shows that the combined peaks 2 and 3 are homogeneous with respect to their yellow component. Assuming that deacylation of all the acylated peptides, to yield Ac-Phe(DnpNH), occurs at approximately the same rate, then bands 2 and 3 together represent ~70% of the total acylated peptides in Figure 3A, but less than 20% of the total acylated peptides in Figure 3B. Thus, the yellow components of bands 2 and 3 must be the peptides containing the acylated tyrosine, which is responsible for the activation process.

It should be noted that similar separations of Dnp-labeled peptides on Aminex A-5 were also performed on digests of the modified enzyme which had not been fractionated on the affinity column. In these instances, bands 2 and 3 constitute together only ~40% of the total Dnp-labeled peptides. These findings agree well with the previous observations that maximal enhancements of the activity were found when 2.4 residues

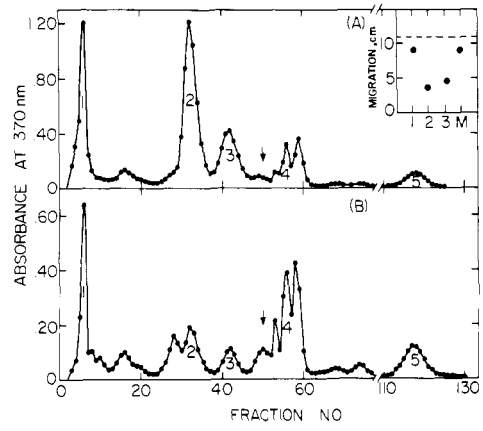

FIGURE 3: Chromatography on Aminex A-5 column (0.9 × 14.5 cm) of Dnp-labeled peptides from thermolytic digests of thermolysin modified with Ac-Phe(DnpNH)-ONSu. Modified thermolysin was precipitated with 1% trichloroacetic acid, the precipitate was digested with 7% thermolysin at pH 7.0, 25 °C for 4 h, and the Dnp-labeled peptides were extracted with 1-butanol (see text). The solvent was evaporated and the residue was applied on the Aminex A-5 column. The column was developed first with 0.2 M pyridine, pH 3.15 (adjusted to pH 3.15 with acetic acid) at 30 mL/h and 52 °C. Fractions, 1.6 mL each, were collected and the absorbance at 370 nm was recorded. After 50 fractions, the eluting buffer was changed to 1 M pyridine, pH 4.1 (adjusted to pH 4.1 with acetic acid), and 80 additional fractions were collected until no yellow material could be seen on the column or eluted from it. (A) Chromatography of the digest of the highly active fraction obtained from the affinity column (tubes 15–20, Figure 2). (B) Chromatography of the digest of the lower activity fraction obtained from the affinity column (tubes 25–35, Figure 1). The amount of 370-nm absorbing material applied on the column in A was ca. twice the amount applied in B. The recovery of the Dnp chromophore in each case exceeded 95%. Insert: Thin-layer chromatography of the yellow Dnp-labeled fragments on silica gel plate in 1-butanol:acetic acid:water, 4:1:1. 1, 2, and 3 are aliquots taken from the combined peaks 1, 2, and 3, respectively, and M is an authentic sample of Ac-Phe(DnpNH). Each sample yielded only one yellow spot at the position indicated.

were modified per mol of enzyme (Blumberg & Vallee, 1975).

Purification of the Dnp-Labeled Peptides by Paper Chromatography and High-Voltage Electrophoresis. The different 370-nm absorbing bands obtained by chromatography on the Aminex A-5 column were lyophilized and the residue from each band was dissolved in 0.5 mL of 10% acetic acid. The yellow solution was applied on a Whatman No. 3 paper and descending chromatography in 1-butanol–acetic acid–water (4:1:1) was carried out for 17 h until the solvent front migration exceeded 50 cm. The yellow component of band 1 migrates close to the front (R_f 0.9). It is ninhydrin negative and it cochromatographs with an authentic sample of Ac-Phe(DnpNH). It is therefore the deacylation product of the modification, liberated during the steps of proteolytic digestion, 1-butanol extraction, and solvent evaporation. The yellow components of bands 2 and 3, the relevant Dnp-labeled peptides, migrate half-way (R_f 0.46 and 0.52, respectively). These ninhydrin positive yellow bands were extracted with 10% acetic acid in 65% and 55% yield, respectively, and lyophilized. Band 5 was also chromatographed in the same way and the yellow component (R_f 0.48) was extracted and lyophilized. Purification of the yellow peptide of band 4 was accomplished in an alternative way, by high voltage electrophoresis (60 V/cm) at pH 1.9 for 4 h.

Analysis of the Dnp-Labeled Peptides. As was noted above the yellow band, 1, of the Aminex column is Ac-Phe(DnpNH), the product of deacylation of the Dnp-labeled peptides. Amino acid analyses of the yellow peptides 2 and 3, the peptides relevant to the activation process, are shown in Table I. The

Table I: Amino Acid Composition and End Groups of Dnp-Labeled Peptides from Thermolytic Digest of Ac-Phe(DnpNH)-ONSu-Modified Thermolysin[a]

	peptide[b]			
group	2	3	4	5
Dnp[c]	1.0	1.0	1.0	1.0
His			1.0	1.0
Asx		0.1	1.0	
Ser	0.9	0.9	0.1	0.1
Glx	1.0	1.0		
Gly	1.0	1.0	0.2	0.1
Ala		0.1	1.0	1.0
Val			1.0	
Tyr	1.0	1.8	1.0	1.0
N terminal	Ser	Tyr	ND[d]	ND
C terminal	Tyr	Tyr	ND	ND

[a] Amino acid analyses were performed after hydrolysis in 6 N HCl at 110 °C for 22 h in the presence of phenol. The values are uncorrected. [b] The peptides in the table are the purified Dnp-labeled peptides of the corresponding bands eluted from the Aminex A-5 column (Figure 3). [c] Dnp content estimated from the absorbance at 370 nm by using ϵ_{370} 18 500 M^{-1} cm^{-1} for the chromophore. The values for the amino acids are relative to the Dnp content. [d] ND, not determined.

compositions of these peptides are Ser, Glx, Gly, Tyr and Ser, Glx, Gly, Tyr_2, respectively. These two analyses fit uniquely the compositions of the peptides 107–110 (Ser-Gln-Gly-Tyr) and 106–110 (Tyr-Ser-Gln-Gly-Tyr) in the known sequence of thermolysin, suggesting that Tyr-110, common to both peptides, is the residue acylated. This conclusion was substantiated by end-group analyses. C-Terminal analyses of peptides 2 and 3 were performed with carboxypeptidase A by incubation in 0.2 M *N*-ethylmorpholine, pH 8.5, at 37 °C for 16 h. Under these conditions, deacylation of acylated phenolic groups is likely to occur. Indeed, the major amino acid product of these digestions was tyrosine, for both peptides, with smaller amounts of glycine and Ser + Gln, the latter two amino acids appearing at the same position of the amino acid chromatogram. The N-terminal residues of peptides 2 and 3 were found to be Ser and Tyr, respectively (Table I). The dansyl derivative obtained from peptide 2 was Dns-Ser, whereas the major dansyl derivative obtained from peptide 3 was Dns-Tyr(Dns) with only slight amounts of Dns-Tyr. This suggests further that the phenolic side chains of the N-terminal Tyr of peptide 3 are not acylated and reinforces the deduction that, in peptide 3, as well as in peptide 2, the C-terminal tyrosine is the residue acylated.

Amino acid analyses of two additional peptides, 4 and 5, yielded the compositions His, Asx, Ala, Val, Tyr and His, Ala, Tyr, respectively (Table I). These two analyses are compatible with the sequences 71-75 (Val-Asp-Ala-His-Tyr) and 73-75 (Ala-His-Tyr), suggesting that Tyr-75 is also modified to some extent, although this modification does not lead to enhancement in activity.

Discussion

The results presented above demonstrate that, among the 28 tyrosine residues of thermolysin, it is Tyr-110 that when acylated by active esters of aromatic amino acid derivatives brings about large enhancements in activity and changes in the specificity of the enzyme. The principal observations and deductions leading to this conclusion are outlined below. The activity of thermolysin toward furylacryloyl-Gly-Leu-NH_2, as well as toward other peptide derivatives, is enhanced markedly upon acylation with Ac-Phe(DnpNH)-ONSu or with other *N*-hydroxysuccinimide esters of aromatic acyl amino acid derivatives (Blumberg & Vallee, 1975). The modifications and their functional consequences are both reversible. Thus, treatment of Ac-Phe(DnpNH)-ONSu-modified thermolysin with NH_2OH, 0.1 M, pH 7.5, deacylates ~85% of the Ac-Phe(DnpNH) label concomitant with the return of activity to that of the native enzyme (Figure 1). The half-life for the reversal of activity to native activity is ~4 min (Blumberg & Vallee, 1975), very similar to the rate of deacylation of O^4-acyltyrosine model compounds but very different from the rate of deacylation of acylated α- or ϵ-amino groups, alkyl ester, acylimidazole, and acid anhydride linkages (Holmquist et al., 1976), strongly indicating that a phenolic side chain of tyrosine has been modified. The activity of thermolysin modified with Ac-Phe(DnpNH)-ONSu, toward furyl-acryloyl-Gly-Leu-NH_2, is directly proportional to the extent of modification, reaching an ~75-fold higher activity than the native enzyme for the maximal covalent incorporation of 2.4 Ac-Phe(DnpNH) groups per mol of enzyme. This relationship raises the question whether or not modification of only 1 tyrosine residue, among the 2.4 residues modified, brings about the changes in activity. Fractionation on an agarose–Gly_3-D-Phe affinity column, of partially modified enzyme, into a highly active fraction and a lower activity fraction, the former exhibiting ~80% of the maximal activity achievable per mol of Ac-Phe(DnpNH) groups incorporated covalently (Figure 2), strongly suggests that modification of only 1 residue causes the activity changes. Enzymic degradation of the two protein fractions and separation of the Dnp-labeled peptides by ion-exchange chromatography allow the assignment of the peptides relevant to the activation process: only two of the peptides (peptides 2 and 3 in Figure 3) are larger in their amount in the digest of the highly active fraction (Figure 3A) compared with the digest of the lower activity fraction (Figure 3B), while all the other peptides are larger in the digest of the lower activity fraction. Furthermore, these two peptides constitute together about 70% of the total Dnp-labeled peptides in the digest of the highly active fraction but less than 20% in the digest of the lower activity fraction. Finally, amino acid and end-group analyses of these two peptides (Table I) reveal that they are overlapping peptides and lead to the conclusion that Tyr-110, common to both peptides, is the residue modified which is responsible for the changes in activity.

The procedures employed here allow identification, in a protein, of a tyrosine residue which is acylated by a carboxylic acid derivative. Phenyl ester linkages are rather unstable as is apparent from the rates of deacylation by 0.1 M NH_2OH of phenyl ester model compounds (Holmquist et al., 1976) and of acylthermolysins (Blumberg & Vallee, 1975), as well as from the partial deacylation of Ac-Phe(DnpNH) during the peptide isolation procedure (Figure 3). However, by speeding up the isolation procedure, and performing it under acidic conditions or in the absence of nucleophiles, the extent of deacylation was minimized, allowing isolation of acylated peptides and identification of the tyrosine residue modified.

The selectivity of modification of tyrosine residues in thermolysin by Ac-Phe(DnpNH)-ONSu is somewhat surprising since *N*-hydroxysuccinimide esters of amino acid derivatives, employed in peptide syntheses, react generally with amino groups. Similar selectivities of tyrosine modification by the same reagent were, however, also found for other neutral proteases (Holmquist et al., 1976), as well as for acetylcholinesterase (Blumberg & Silman, 1978). On the other hand, the structurally related active ester Moc-Phe-(DnpNH)-ONSu modifies thermolysin preferentially at lysine residues (S. Blumberg, unpublished results). These differences between such related reagents could reflect a tendency of

N-acetyl amino acid *N*-hydroxysuccinimide esters to undergo a conversion, in aqueous media, to oxazolone derivatives which also act as acylating agents, whereas *N*-methyloxycarbonyl amino acid active esters are resistant to such a conversion. The cyclic and hydrophobic oxazolones formed may have a higher reactivity toward the hydrophobic tyrosines than toward the hydrophilic lysines. Furthermore, binding of the oxazolones to a hydrophobic area on the enzyme may precede the covalent reaction. The potential usefulness of such oxazolone derivatives for selective tyrosine modification of proteins deserves further attention.

Fractionation of partially modified thermolysin on the affinity absorbent agarose–Gly_3-D-Phe is essential for the definitive assignment of Tyr-110 as the amino acid residue modified. Hence, the mechanism by which such a fractionation takes place is of a considerable interest. The enzyme modified at Tyr-110 elutes first from the column, followed by the native enzyme and then by enzyme species modified at other sites or at several sites (Figures 2 and 3). Although the enzyme modified at Tyr-110 is "superactive" toward various peptide substrates, its affinity for the reversible inhibitors tested is similar to that of the native enzyme (Blumberg & Vallee, 1975). It appears that the affinity of the enzyme modified selectively at Tyr-110 for a resin-bound inhibitor is even lower than that of the native enzyme. Enzyme species modified at sites other than Tyr-110 or at several sites, on the other hand, are retarded on the column more effectively than the native enzyme. This effect may be attributed to charge transfer or hydrophobic interactions between the Ac-Phe(DnpNH) groups bound to the enzyme and the Phe groups of the agarose–Gly_3-D-Phe inhibitor.

Previous studies with *N*-hydroxysuccinimide esters of different acyl amino acid derivatives had indicated that all of them modify thermolysin at the same residue, now identified as Tyr-110, albeit with different functional consequences (Blumberg & Vallee, 1975). Thus, active esters of aromatic acyl amino acids, i.e., Ac-Phe-ONSu and Ac-Phe-(DnpNH)-ONSu, cause large enhancements in the activity of the enzyme toward the standard substrate of thermolysin furylacryloyl-Gly-Leu-NH_2, whereas active esters of aliphatic acyl amino acids, i.e., Ac-Ala-ONSu and Ac-Val-ONSu, cause only small enhancements in activity. It was also found that the extent of activation by each aromatic active ester is substrate dependent; it is generally small for good tripeptide substrates and large for poor substrates. A 400-fold activation was observed for Ac-Phe(DnpNH)-thermolysin, relative to native thermolysin, when acting upon furylacryloyl-Gly-Ala-Gly. However, for substrates having a Phe residue on the carboxyl side of the scissible peptide bond, i.e., furylacryloyl-Phe-Leu-Gly and furylacryloyl-Phe-Gly-Gly, it was found that modification by the active esters decreases activity toward the poor as well as the good substrates. Most of the activity changes that were measured so far reflect an overall change in k_{cat}/K_m. In those examples where detailed kinetic measurements were carried out, i.e., in the case of β-phenylpropionyl-Phe-thermolysin acting on Bz-Gly-Phe-Ala and on Z-Gly-Leu-Gly, the increase in k_{cat}/K_m was due to both an increase in k_{cat} and a decrease in K_m.

Recent X-ray crystallographic studies of thermolysin and of its complexes with reversible peptide inhibitors led to a proposal as to the mode of binding to the enzyme of peptide substrates (Kester & Matthews, 1977; Weaver et al., 1977). In this proposal, the substrate residues designated P_2, P_1, and P_1' (according to the nomenclature of Schechter & Berger, 1967) interact with the enzyme backbone between residues Trp-115 and Ala-113 as in an antiparallel pleated sheet, whereas the residue P_2' is hydrogen bonded to the side chain carboxamide group of Asn-112. The phenyl group of Phe-114 is thought to participate in the formation of subsite S_1. It is likely that acylation of the nearby Tyr-110 might affect the above interactions. In particular, the phenolic oxygen atom of Tyr-110 is 4–5 Å apart from the side chain carboxamide group of Asn-112 or from the phenyl group of Phe-114, and the covalent incorporation of acyl moieties at this position seems to optimize interaction of these side chains with a variety of substrates. The findings that activity toward substrates having Gly or Ala in P_1 is enhanced by the modification, whereas activity toward substrates having Phe in P_1 is diminished, suggest that attachment of the acyl moieties reduces the flexibility or causes a "shrinkage" of subsite S_1 of the enzyme.

While providing a first indication as to the possible mechanism of the activity changes, the presently available data are yet insufficient to completely characterize the phenomenon. Detailed studies with tri- and tetrapeptide substrates and inhibitors should allow more precise correlation of the kinetic and thermodynamic aspects of the activity changes with the structure of the substrate and of the acyl group incorporated covalently into the enzyme. The fractionation procedure described above for obtaining acylthermolysin species selectively modified at Tyr-110 is particularly useful in this regard. Such selectively modified thermolysin derivatives may also serve in structural and spectroscopic studies, for determining the exact allignment of the acyl moiety and its interaction with the active center residues.

The enhancements in activity subsequent to modification with active esters of acyl amino acid derivatives and their reversal by nucleophiles are not limited to thermolysin but apply to all the neutral proteases that had been examined. Analogous enhancements in activity were observed for a series of neutral proteases from different types of bacillus and for the neutral protease from *Aeromonas proteolytica* (Blumberg & Vallee, 1975; Holmquist et al., 1976) as well as for neutral proteases from *Streptomyces griseus* (S. Blumberg, unpublished results). These suggest that all these enzymes might possess a reactive tyrosine residue, located with respect to the active site, similarly to Tyr-110 in thermolysin. The location of Tyr-110 in a turn of the polypeptide chain near the active site might account for its conservation in different neutral proteases. It is interesting to note that neutral protease A from *Bacillus subtilis* NRRLB3411, which was found to be homologous with thermolysin, has indeed a tyrosine in position 110 (Levy et al., 1975). The methods described above, for isolation of acylated peptides, now allow characterization of neutral proteases by means of the reactive tyrosine residue near their active sites and the amino acid sequence around it.

Acknowledgments

I express my thanks to Dr. B. L. Vallee for his encouragement and interest and to Drs. B. Holmquist, L. Cueni, and J. L. Bethune for their helpful suggestions. I am indebted to N. Tal for his skilled technical assistance.

References

Ambler, R. P. (1967) *Methods Enzymol. 11*, 155–166.

Axēn, R., Porath, J., & Ernback, S. (1967) *Nature (London) 214*, 1302–1304.

Blumberg, S., & Vallee, B. L. (1975) *Biochemistry 14*, 2410–2419.

Blumberg, S., & Silman, I. (1978) *Biochemistry 17*, 1125–1130.

Blumberg, S., Schechter, I., & Berger, A. (1970) *Eur. J. Biochem. 15*, 97–102.
Colman, P. M., Jansonius, J. N., & Matthews, B. W. (1972) *J. Mol. Biol. 70*, 701–724.
Feder, J. (1968) *Biochem. Biophys. Res. Commun. 32*, 326–332.
Gray, W. R. (1967) *Methods Enzymol. 11*, 139–151.
Holmquist, B., Blumberg, S., & Vallee, B. L. (1976) *Biochemistry 15*, 4675–4680.
Kester, W. R., & Matthews, B. W. (1977) *Biochemistry 16*, 2506–2516.
Latt, S. A., Holmquist, B., & Vallee, B. L. (1969) *Biochem. Biophys. Res. Commun. 37*, 333–339.
Levy, P. L., Pangburn, M. K., Burstein, Y., Ericsson, L. H., Neurath, H., & Walsh, K. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4341–4345.
Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., & Dupourque, D. (1972a) *Nature (London), New Biol. 238*, 37–41.
Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., & Neurath, H. (1972b) *Nature (London), New Biol. 238*, 41–43.
Matthews, B. W., Weaver, L. H., & Kester, W. R. (1974) *J. Biol. Chem. 249*, 8030–8044.
Ohta, Y., Ogura, Y., & Wada, A. (1966) *J. Biol. Chem. 241*, 5919–5925.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Thiers, R. T. (1957) *Methods Biochem. Anal. 5*, 273–335.
Titani, K., Hermodson, M. A., Ericsson, L. H., Walsh, K. A., & Neurath, H. (1972) *Nature (London), New Biol. 238*, 35–37.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.
Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 113*, 369–370.

Cross-Linking of Iodine-125-Labeled, Calcium-Dependent Regulatory Protein to the Ca^{2+}-Sensitive Phosphodiesterase Purified from Bovine Heart†

David C. LaPorte, William A. Toscano, Jr.,‡ and Daniel R. Storm*

ABSTRACT: The calcium-dependent regulatory protein (CDR)·Ca^{2+} sensitive cyclic nucleotide phosphodiesterase was purified to apparent homogeneity from bovine heart by using ammonium sulfate fractionation, DEAE-cellulose chromatography, and CDR–Sepharose affinity chromatography. The enzyme was purified 13 750-fold with a 10% yield and a specific activity of 275 μmol of cAMP min^{-1} mg^{-1}. The purified enzyme ran as a single band during sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an apparent molecular weight of 57 000. Phosphodiesterase activity was stimulated 10-fold by Ca^{2+} and CDR with half-maximal activation occurring at 9 ng/assay. [^{125}I]CDR was cross-linked to the purified phosphodiesterase by using dimethyl suberimidate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the cross-linked products revealed a number of discrete ^{125}I-labeled bands. The molecular weights of the cross-linked products indicate that the stoichiometry of the phosphodiesterase complex is A_2C_2, where A is the phosphodiesterase catalytic subunit and C is the calcium-dependent regulatory protein.

The calcium-dependent regulatory protein (CDR)[1] was independently discovered by Cheung (Cheung, 1970) and Kakiuchi (Kakiuchi et al., 1970). These investigators discovered that CDR stimulates the Ca^{2+}-sensitive isozyme of cyclic nucleotide phosphodiesterase. It was subsequently demonstrated that CDR binds Ca^{2+} (Teo & Wang, 1973) and both Ca^{2+} and CDR must be present for stimulation of phosphodiesterase activity (Teo & Wang, 1973; Wolff & Brostrom, 1974; Lin et al., 1974; Teo et al., 1973). It is well established that CDR forms a complex with the phosphodiesterase which is stable during Sephadex G-200 chromatography and electrophoresis on nondenaturing gels in the presence of Ca^{2+}, but not in the presence of EGTA (Teshima & Kakiuchi, 1974; Lin et al., 1975; LaPorte & Storm, 1978). These observations support the proposal that CDR binds Ca^{2+}, forms a complex with the phosphodiesterase, and stimulates the enzyme (Kakiuchi et al., 1973).

The Ca^{2+} and CDR sensitive phosphodiesterase has been purified 5250-fold from bovine heart with a 7% yield (Ho et al., 1977). It was estimated that the enzyme was approximately 80% pure. This preparation was quite unstable in the absence of Ca^{2+} and CDR, although less purified preparations were reasonably stable. In this report, we describe a procedure for purification of the Ca^{2+}-sensitive phosphodiesterase which results in a 13 750-fold purification with a 10% yield. This

† From the Department of Pharmacology, University of Washington, Seattle, Washington 98195. *Received February 6, 1979.* This investigation was supported by National Science Foundation Grant PCM 78-0318 and a grant from the Washington Heart Association.
* Recipient of National Institutes of Health Research Career Development Award AI 00120.
‡ Recipient of National Institutes of Health postdoctoral fellowship GMO 6875.

[1] Abbreviations used: CDR, calcium-dependent regulatory protein; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-2820$01.00/0