Esterase Activity of Zinc Neutral Proteases†

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ABSTRACT: The hydrolysis of a series of depsipeptides demonstrates that the zinc neutral endopeptidases of bacteria are active esterases. Esters such as BzGly-OPhe-Ala, BzGly-OLeu-Ala, and FA-Gly-OLeu-NH_2 are hydrolyzed at rates three- to eightfold slower than are their exact peptide analogues, when hydrolyzed by thermolysin, *Bacillus subtilis* neutral protease and the neutral protease from *Aeromonas proteolytica*. Ester hydrolysis by zinc neutral proteases follows the characteristic preference for hydrophobic amino acids adjacent to the site of cleavage, discerned from the hydrolysis of peptide substrates. Removal of zinc from thermolysin abolishes the esterase activity of the native enzyme. Among the metals examined, only Co^{2+} and Zn^{2+} restore esterase activity to any significant extent, Co^{2+} restoring 50% and Zn^{2+} 100% of the native thermolysin activity. The hydrolysis of esters and peptides by thermolysin does not differ with respect to either the binding or catalytic steps. Substrate specificity, pH–rate profiles, inhibitor, and deuterium isotope effects are identical for both types of substrates.

The neutral proteases of bacteria are endopeptidases, have neutral pH optima, require zinc for activity, and share a common specificity (Matsubara and Feder, 1971). Thermolysin, the thermostable neutral protease from *Bacillus thermoproteolyticus*, contains one catalytically essential zinc atom as well as 4 g-atoms of calcium per mol (Latt et al., 1969; Holmquist and Vallee, 1974). Its primary sequence (Titani et al., 1972) and three-dimensional structure (Colman et al., 1972) have been determined.

Synthetic peptide substrates have served to detail the specificity of thermolysin and other neutral endopeptidases (see Morihara, 1974 for a review), particularly the marked preference of this class of enzymes for substrates which have hydrophobic amino acids contributing the amino group of the bond to be cleaved. A series of studies has failed to detect esterase activity of neutral proteases when a number of esters, e.g., hippuryl phenyllactate, benzoylarginine ethyl ester, tosylarginine methyl ester, acetyltyrosine ethyl ester, carbobenzoxyglycine *p*-nitrophenyl ester, and a series of aliphatic alkyl esters served as potential substrates (McConn et al., 1964; Morihara and Tsuzuki, 1966; Feder, 1968). We have now succeeded in synthesizing a series of depsipeptides which are excellent substrates for thermolysin and other neutral proteases. Comparisons of their specificity, pH–rate profiles, inhibitor and deuterium isotope effects, and the effect of metal replacements with those of exact peptide analogues indicate no differences in ester and peptide hydrolysis in either their binding or catalytic steps.

Materials and Methods

Commercial reagents of highest quality available were obtained as follows: 2-furanacrylic acid, hippuric acid, L-leucine, L-phenylalanine, L-α-hydroxyphenylacetic acid (Eastman Organic Chem.); *N*-benzoylglycyl-L-phenylalanine, *N*-benzoylglycyl-L-leucine, L-phenylalanyl-L-alanine, L-leucyl-L-alanine, benzoylglycyl-L-phenyllactate sodium salt, L-alanine methyl ester · HCl, and furylacryloylglycyl-L-leucylamide (FAGLA)[1] (Cyclo Chem. Co.); *N*-hydroxysuccinimide, dicyclohexylcarbodiimide (DDC), and carbonyldiimidazole (CDI) (Pierce Chem. Co.). Deuterium oxide (Bio-Rad) was 99.8% D_2O. All other materials were reagent grade. Thermolysin (Calbiochem) was recrystallized as described previously (Latt et al., 1969). Crystals of the enzyme, containing 1 g-atom of zinc/mol, were washed three times with deionized distilled water and dissolved in 5 *M* NaBr–0.05 *M* Tris–0.01 *M* Ca^{2+} (pH 7.5) and then diluted to 1 *M* NaBr with 0.05 *M* Tris–0.01 *M* Ca^{2+} (pH 7.5) yielding stock enzyme concentrations between 1 and 5 × 10^{-5} *M*. *Bacillus subtilis* neutral protease (Miles Lab.) was passed through a column, 1 × 20 cm, of Bio-Gel P-4 (pH 7.5, 0.05 *M* Tris–0.01 *M* Ca^{2+}) immediately prior to use. *Aeromonas proteolytica* neutral protease was a gift of Dr. J. M. Prescott.

The chromophoric substrate, FAGLA, served for activity measurements at pH 7.5 at a concentration of 1 m*M* (Holmquist and Vallee, 1974). Values of k_{cat}/K_m were 1.1 ● 0.1 × 10^6, 3.0 ± 0.5 × 10^5, and 1.8 ± 0.2 × 10^6 M^{-1} min^{-1} for thermolysin, the *B. subtilis*, and the *Aeromonas* enzymes, respectively.

Protein concentrations were measured by absorbance at 280 nm using a Zeiss PMQ II spectrophotometer. A molar absorptivity of 6.64 × 10^4 M^{-1} cm^{-1} was used for thermolysin (Ohta et al., 1966; Titani et al., 1972), 5.58 × 10^4 M^{-1} cm^{-1} for the *Aeromonas* neutral protease (Dr. J. M. Prescott, personal communication) and E_{cm} (1%) 280 nm = 13.5, mol wt 44800 (Tsuru et al., 1965) were used for the *B. subtilis* neutral protease.

Infrared spectra were measured with a Beckman IR-20. A Gilford Model 200 optical density converter and a Heath IR-18M recorder attached to a Beckman DU monochromator equipped with double thermospacers through which

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[1] Abbreviations used are: DME, 1,2-dimethoxyethane; OLeu, 2-hydroxyisocaproic acid; OPhe, 2-phenyllactic acid; OGly, glycolic acid; MAN, 2-hydroxyphenylacetic acid; DCC, dicyclohexylcarbodiimide; CDI, carbonydiimidazole; ammediol, 2-amino-2-methyl-3,3-propanediol; Mes, morpholinoethanesulfonic acid; Tris; tris(hydroxymethyl)aminomethane; FA, 2-furanacrylic acid; FAGLA, 2-furanacryloylglycyl-L-leucylamide; PNP, *p*-nitrophenol; ONP, *o*-nitrophenol.

water was circulated from a Haake constant-temperature circulator were used for spectrophotometric rate measurements.

All rates were measured at 25 ± 0.1° in the presence of 0.01 *M* Ca^{2+}–0.05 *M* Tris (pH 7.5) unless otherwise indicated. Hydrolyses of *N*-furylacryloyl blocked substrates were followed spectrophotometrically by continuous monitoring of the decrease in absorbance at 345 nm (Feder, 1968) and that of the *N*-benzoyl blocked peptide substrates either by ninhydrin determination of dipeptide release using the automated method described by Auld and Vallee (1970) or by direct spectrophotometric monitoring at 254 nm (Schwert and Takenaka, 1955; Whitaker et al., 1966). Authentic samples of dipeptide products served as standards for the yields of the ninhydrin reaction.

Solutions for solvent isotope effect studies were prepared by lyophilizing two identical aliquots of metal-free Tris buffer, 0.05 *M*, containing 0.1 *M* NaCl and 0.01 *M* $CaCl_2$ at pH 7.5, followed by reconstituting one with H_2O and the other with D_2O. Identical aliquots of substrate dissolved in dimethylformamide were added to the reconstituted buffers to give stock substrate solutions containing 0.5% dimethylformamide, a level which does not detectably affect hydrolysis by thermolysin. The H_2O sample was then adjusted to a pH 0.4 unit greater than the pH meter reading of the D_2O sample so that pH = pD at 25° (Fife and Bruice, 1961).

Ester hydrolysis was studied either spectrophotometrically following ΔA_{254} for the *N*-benzoyl and ΔA_{345} for the *N*-furylacryloyl esters or autotitrimetrically with a Radiometer ABU-12, TT-1a pH stat system. Titrimetric rates were initiated by addition of 25–50 μl of enzyme to 3 ml of substrate equilibrated at the desired pH. Sodium hydroxide, 2–10 m*M*, calibrated against standard HCl (Fisher) served as titrant. Initial velocities were obtained from the recorder slope corresponding to less than 5% hydrolysis.

Products of hydrolysis were identified by thin-layer chromatography on Eastman 6060 fluorescent silica gel plate using butanol–acetic acid–water (9:1:1) as solvent. Quenching of fluorescence by the benzoyl and furylacryloyl blocking groups served to identify the N-terminal fragment, and ninhydrin spray was additionally used to detect the products of peptide hydrolysis. With all substrates studied, cleavage with enzyme occurred at the internal amide or ester bond to yield dipeptides or the α-hydroxylactate amide. In all instances hydrolysis was complete as judged by thin-layer chromatography. Quantitative yield of dipeptides formed was established either by means of the ninhydrin autoanalysis method or by titration of protons released upon completion of hydrolysis and was examined routinely when such procedures were used in rate studies.

Inhibition constants for Zn^{2+} and β-phenylpropionyl-L-phenylalanine were established by varying inhibitor at a fixed substrate concentration under conditions where [S] ≪ K_m. In all cases, plots of $1/k_{obsd}$ vs. [I] were linear yielding K_I as the minus *X* intercept.

Zinc-free thermolysin was prepared and reconstituted with Co^{2+} and Zn^{2+} as described earlier (Holmquist and Vallee, 1974). The resultant apoenzyme preparations contained less than 1% zinc as measured by atomic absorption spectrometry (Fuwa et al., 1964) and exhibited less than 1% activity toward FAGLA. Contamination by adventitious metal ions was minimized by extraction of all buffers and, where possible, substrate solutions, with 0.01% dithizone in CCl_4 (Thiers, 1957).

Substrates. All asymmetric α-substituted amino acid derivatives are of the L configuration. Thin-layer chromatography on Eastman 6060 fluorescent silica gel using butanol–acetic acid–water (9:1:1) showed a single uv quenching spot for all substrates.

The syntheses of the intermediates Bz-Gly-L-phenyllactate (McClure, 1966), Bz-Gly-L-mandelate (Carson and Kaiser, 1966), and Bz-Gly-glycolate (Kaiser et al., 1965) have been described. Synthesis of the new ester substrates was performed by coupling *O*-(N-blocked amino acyl)-α-hydroxy acid esters to an additional amino acid derivative by the DCC–*N*-hydroxysuccinimide ester method (Anderson et al., 1964). The preparation of Bz-Gly-OPhe-Ala illustrates the general method employed. Benzoylglycyl-L-phenyllactate, sodium salt (1.5 g, 3.4 mmol), was dissolved in 50 ml of water and acidified with 1 *N* HCl, and the free acid extracted into ethyl acetate. The combined ethyl acetate fractions were dried over anhydrous $MgSO_4$ and the solvent was evaporated to an oil. To the oil dissolved in 20 ml of dimethoxyethane (DME) was added *N*-hydroxysuccinimide (0.52 g, 4.5 mmol). The resulting solution was cooled to 0°; solid DCC (0.93 g, 4.5 mmol) was added and quickly dissolved. After 16 hr of stirring at 4° the *N*-hydroxysuccinimide ester was filtered directly into 0.8 g (9 mmol) of L-alanine dissolved in 50% DME–H_2O containing 2.3 g of $NaHCO_3$. After 2 hr the solution was acidified to pH 1 with HCl and DME was removed by rotary evaporation. The aqueous suspension was extracted three times with ethyl acetate; the extracts were combined, dried over anhydrous $MgSO_4$, and evaporated to an oil. The oil was taken up in warm 2-propanol and water added to the cloud point. The product crystallized upon slow cooling. Bz-Gly-MAN-Ala, Bz-Gly-OGly-Phe, Bz-Gly-OPhe-Ala methyl ester, Bz-Gly-Phe-Ala methyl ester, Bz-Gly-OPhe-Phe, Bz-Gly-Phe-Phe, Bz-Gly-OPhe-Gly, Bz-Gly-Phe-Gly, Bz-Gly-Phe-Ala, and Bz-Gly-Leu-Ala were prepared in analogous manner. The melting points, solvent for recrystallization, characteristic ir bands, and elemental analyses of all new substrates are listed in Table I. Although the yields of all synthetic procedures were good, no attempt was made to maximize them, hence they are not reported.

FA-Gly-OLeu-NH₂. L-α-Hydroxyisocaproic acid benzyl ester was prepared by the method of Gisen et al. (1969) for the preparation of L-α-hydroxyisovaleric acid benzyl ester and exhibited the following characteristics: bp 99–100° (1 mm); ir (neat) 3500, 2960, and 1740 cm^{-1}. The benzyl ester was converted to the amide by dissolving in methanol and saturating the solution with dry NH_3 at 0°. After 18 hr, methanol was removed by rotary evaporation under vacuum to yield an oil, which was dissolved in chloroform and petroleum ether (60–90°) was added to the cloud point. The white crystalline L-α-hydroxyisocaproamide, mp 80–82°, was obtained on cooling, ir (Nujol) 3400, 3340, and 1675 cm^{-1}.

To 1.4 g (7.2 mmol) of 2-furylacryloylglycine (Feder and Shuck, 1970) dissolved in 30 ml of chloroform at 0° in a 100-ml round-bottom flask fitted with a $CaCl_2$ drying tube, 1.2 g (7.1 mmol) of CDI was added. After 20 min, 1.44 g (11 mmol) of L-α-hydroxyisocaproamide was added and the solution stirred 20 hr in the dark. Addition of 100 ml of ethyl acetate was followed by two extractions with 0.5 *M* HCl, 20 ml each, and drying of the organic phase with anhydrous $MgSO_4$. The ethyl acetate and chloroform were removed by rotary evaporation to give an oil which was taken up in chloroform. Addition of *n*-hexane followed by cooling produced tan needles exhibiting the characteristics shown in

Table I: Characteristics of Zinc Neutral Protease Ester and Peptide Substrates.

Substrate	Mp (°C)[a]	Recrystallization Solvent	Major Ir Bands[b] (cm^{-1})	Analysis[c] Calculated (%) C	H	N	Found (%) C	H	N
FA-Gly-OLeu-NH_2	130–131	Chloroform–hexane	3360, 3400, 1744, 1665, 1645, 1630	58.42	6.53	9.08	58.07	6.76	9.02
Bz-Gly-OPhe-Ala	167–168	2-Propanol–water	3300, 1750, 1670, 1610	63.30	5.56	7.03	63.22	5.83	7.15
Bz-Gly-Phe-Ala	202–203	2-Propanol–water	3420, 3300, 1740, 1675, 1655	63.46	5.83	10.59	63.39	5.99	10.37
Bz-Gly-OLeu-Ala	129–131	Methanol–water	3300, 1735, 1711, 1665, 1635	59.33	6.63	7.69	58.55	6.60	7.68
Bz-Gly-Leu-Ala	194–195	Ethyl acetate–methanol	3380, 1740, 1675, 1630	59.49	6.93	11.56	59.11	7.12	11.49
Bz-Gly-OPhe-Gly	169–171	Methanol–ethyl acetate	3260, 1750, 1710, 1660, 1640	62.49	5.24	7.29	62.05	5.16	7.03
Bz-Gly-Phe-Gly	197–198	Methanol–benzene	3240, 1740, 1665, 1650	62.65	5.52	10.96	62.65	5.27	10.75
Bz-Gly-OPhe-Phe	136–137	Ethyl acetate–hexane	3330, 3260, 1762, 1735, 1630	68.35	5.52	5.90	68.47	5.42	6.06
Bz-Gly-Phe-Phe	171–173	Ethyl acetate–methanol	3450, 1725, 1680, 1630	68.43	5.75	8.88	68.03	6.09	9.04
Bz-Phe-OPhe-Ala	178–179	Ethyl acetate–hexane	3300, 1740, 1670, 1640	68.84	5.77	5.74	68.69	5.96	5.52
Bz-Phe-Phe-Ala	224–225	Methanol	3280, 1740, 1640, 1540	68.98	6.00	8.62	68.89	5.99	8.47
Bz-Gly-OPhe-Ala methyl ester	122–123	Ethanol–water	3300, 1750, 1675, 1640	64.06	5.87	6.79	63.95	6.13	6.92
Bz-Gly-Phe-Ala methyl ester	193–194	Dimethylformamide–water	3280, 1740, 1640	64.22	6.12	10.21	63.96	6.17	10.22
Bz-Gly-MAN-Ala	193–195	Acetonitrile	3440, 3340, 1762, 1740, 1660	62.49	5.24	7.29	62.61	5.26	7.24
Bz-Gly-OGly-Phe	157–158	Ethanol–water	1779, 1736, 1754, 1680, 1650	62.49	5.25	7.29	62.98	5.62	7.48

[a] Capillary melting points are uncorrected. [b] Nujol mulls. [c] Analysis performed by PAR, Inc., Gainesville, Fla.

Table I.

Bz-Gly-OLeu-Ala. To 10 g (56 mmol) of benzoylglycine suspended in 100 ml of chloroform at 0° was added 9.3 g (55 mmol) of CDI. The reaction was kept dry with a $CaCl_2$ drying tube and allowed to proceed 20 min, then 12.2 g (55 mmol) of L-α-hydroxyisocaproic acid benzyl ester was added. After 3 days at room temperature the solution was washed with 1*M* citric acid, water, and saturated $NaHCO_3$, dried over anhydrous $MgSO_4$, and evaporated to an oil which could not be crystallized. The resulting depsipeptide benzyl ester was debenzylated by catalytic hydrogenolysis for 3 hr in methanol at atmospheric pressure using 10% Pd on charcoal. Removal of the catalyst by filtration and of the methanol by rotary evaporation resulted in an oil which could not be crystallized. Coupling of L-alanine to the free acid was achieved as described above for Bz-Gly-OPhe-Ala to yield the white crystalline product (Table I).

Bz-Phe-OPhe-Ala was prepared by coupling Bz-Phe (Greenstein and Winitz, 1961) to α-hydroxyphenyllactate benzyl ester as described for the preparation of Bz-Gly-OLeu. The product benzyl ester was crystallized from methanol–*n*-hexane, mp 131–132°, ir (Nujol) 3300, 1740, and 1640 cm^{-1}, and subsequently debenzylated catalytically (H_2, 10% Pd on charcoal, methanol) at atmospheric pressure. The product, Bz-Phe-OPhe, isolated as an oil after hydrogenolysis, was then coupled to alanine using the DCC–*N*-hydroxysuccinimide method. Standard work-up and recrystallization from ethyl acetate–*n*-hexane resulted in white needles, mp 178–179°. Bz-Phe-Phe-Ala was prepared by coupling Bz-Phe to phenylalanine by the DCC–*N*-hydroxysuccinimide method to give Bz-Phe-Phe as crystals from methanol–water which was then coupled to alanine by the DCC–*N*-hydroxysuccinimide technique to give Bz-Phe-Phe-Ala whose properties are listed in Table I.

Results

Thermolysin is an active esterase. Table II lists the rates of hydrolysis, expressed as k_{cat}/K_m, of a series of depsipeptides and their exact peptide analogues differing only in the chemical composition of the site of enzymatic cleavage: the NH of peptides replaces the -O- of esters. All rates of peptide and ester hydrolysis follow first-order kinetics, when $[S] \ll K_m$, so that k_{obsd}, the first-order rate constant for substrate hydrolysis, equals $k_{cat}[E]/K_m$.

The α-hydroxy acids, which contribute the alcohol component of the ester bond, include L-2-hydroxyphenyllactate (OPhe), L-2-hydroxyisocaproate (OLeu), glycolate (OGly), and 2-hydroxyphenylacetate (mandelate, MAN). In general the rates of hydrolysis of these new ester substrates are three to eightfold lower (Table II) than those of their exact peptide analogues.

The specificity of thermolysin toward these depsipeptides follows that previously established for peptide substrates. N-blocked didepsipeptides with a free carboxyl group α to the susceptible bond, e.g., Bz-Gly-OPhe or those lacking a hydrophobic side chain in the α carbon of the alcohol component, e.g., Bz-Gly-OGly-Phe, are not hydrolyzed. The contribution to specificity of the C-terminal amino acid of blocked tripeptide and depsipeptide substrates is also apparent (Table II). Thus, in this terminal position Ala is preferred over Gly or Phe, as indicated by the much more rapid hydrolysis of Bz-Gly-OPhe-Ala than that of either Bz-Gly-

Table II: Ester and Peptide Substrates for Thermolysin.[a]

	[S][b] (mM)	k_{cat}/K_m ($\times 10^{-5} M^{-1}$ min^{-1})
FA-Gly-OLeu-NH_2[c]	1.0	3.6
FA-Gly-Leu-NH_2[c]	1.0	11.0
Bz-Gly-OPhe-Ala	0.2	2.0
Bz-Gly-Phe-Ala	0.2	37.0
Bz-Gly-OLeu-Ala	0.5	8.4
Bz-Gly-Leu-Ala	0.5	40.0
Bz-Gly-OPhe-Phe	0.08	0.35
Bz-Gly-Phe-Phe	0.08	2.5
Bz-Gly-OPhe-Gly	0.1	0.32
Bz-Gly-Phe-Gly	0.1	2.5
Bz-Gly-OPhe-Ala-OMe	0.1	6.6
Bz-Gly-Phe-Ala-OMe	0.08	17.8
Bz-Phe-OPhe-Ala	0.05	300.0
Bz-Phe-Phe-Ala	0.05	1300.0
Bz-Gly-MAN-Ala	1.0	0.35
Bz-Gly-OGly-Phe	1.0	<0.0004
Bz-Gly-Gly-Phe	1.0	ND[d]

[a] Measured at pH 7.5, 0.05 M Tris, 0.01 M Ca^{2+}, 0.1 M NaCl, 25°. [b] Substrate concentration, $[S] \ll K_m$. [c] Spectrophotometric at 345 nm; all other reactions followed spectrophotometrically at 254 nm. [d] ND, not detectable.

Table III: Ester and Peptide Substrate for Zinc Neutral Proteases.[a]

Substrate	*B. subtilis* Neutral Protease [S][b] (mM)	*B. subtilis* Neutral Protease k_{cat}/K_m ($\times 10^{-5} M^{-1}$ min^{-1})	*Aeromonas proteolytica* Neutral Protease [S][b] (mM)	*Aeromonas proteolytica* Neutral Protease k_{cat}/K_m ($\times 10^{-5} M^{-1}$ min^{-1})
FA-Gly-OLeu-NH_2[c]	1.0	0.62	1.0	3.7
FA-Gly-Leu-NH_2[c]	1.0	3.0	1.0	18.0
Bz-Gly-OPhe-Ala	1.0	0.33	0.1	650.0
Bz-Gly-Phe-Ala	1.0	1.7	0.1	493.0
Bz-Gly-OLeu-Ala	0.25	2.3	0.25	88.0
Bz-Gly-Leu-Ala	0.25	36.0	0.25	566.0
Bz-Gly-OPhe-Phe	0.08	0.43	0.2	197.0
Bz-Gly-Phe-Phe	0.20	0.39	0.2	1430.0
Bz-Phe-OPhe-Ala	0.05	8.5	0.05	*d*
Bz-Phe-Phe-Ala	0.05	280.0	0.05	260.0

[a] Measured at pH 7.5, 0.05 mM Tris, 0.01 mM Ca^{2+}, 0.1 M NaCl, 25°. [b] Substrate concentration, $[S] \ll K_m$. [c] Measured spectrophotometrically at 345 nm; all other rates of hydrolysis were followed at 254 nm. [d] Zero order at lowest [S] possible to follow.

OPhe-Gly or Bz-Gly-OPhe-Phe. These effects are entirely analogous to those observed previously in similar substitutions of peptide substrates (Morihara and Tsuzuki, 1970).

The neutral proteases from *B. subtilis* and *Aeromonas proteolytica* also readily hydrolyze ester–peptide pairs (Table III) and prefer hydrophobic amino acids adjacent to the scissile bond and in the C-terminal position though differences in specificity are apparent. Generally, the activities of these neutral proteases toward esters are lower than toward peptides.

pH Dependence of Ester Hydrolysis. At 1 mM concentrations the hydrolysis of FAGLA and FA-Gly-OLeu-NH_2 are first order between pH 4.75 and 9.5. The variation of log $[k_{cat}/K_m]$ over the pH range 4.75–9.5 for thermolysin-catalyzed hydrolysis of this ester–peptide pair is shown in Figure 1. Lines of slope one, zero, and minus one drawn through the data in the acid, neutral and alkaline pH regions, respectively, result in pK_a values of approximately 5.6 and 7.5 both for the ester and peptide. Over the entire pH range the activity toward the ester is approximately one-half that of the peptide. The pH–rate profile for FAGLA hydrolysis is similar to that observed by Feder and Schuck (1970). Bz-Gly-OPhe-Ala methyl ester also exhibits a bell-shaped pH–rate profile (not shown) which closely parallels that of the FA-Gly-OLeu-NH_2 and also exhibits pK_a values of approximately 5.6 and 7.5. Thus, groups of the protein with pK_{app}'s of 5.6 ± 0.2 and 7.5 ± 0.2 control both peptidase and esterase activity.

Metal Removal and Replacement. Figure 2 shows the effects of both zinc removal and replacement and of the substitution of Co^{2+} for Zn^{2+} on thermolysin-catalyzed hydrolysis of FA-Gly-OLeu-NH_2. Apoenzyme, prepared by dialysis of native enzyme against 1,10-phenanthroline (Holmquist and Vallee, 1974) exhibits less than 1% activity toward the ester. Addition of either Zn^{2+} or Co^{2+} restores activity stoichiometrically up to 1 g-atom/mol of enzyme and further additions have no effect. However, the maximal level of activity restored by Co^{2+}, 1.8×10^5 M^{-1} min^{-1}, is lower than that restored by Zn^{2+}, 3.6×10^5 M^{-1} min^{-1}, the latter being identical with that of the native enzyme. Results with Bz-Gly-OPhe-Ala methyl ester are quite analogous, with activities of 2.9×10^5 M^{-1} min^{-1} for the cobalt enzyme and 6.6×10^5 M^{-1} min^{-1} for the zinc enzyme.

In addition, the capacity of Ni^{2+}, Cr^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+}, Mg^{2+}, and Fe^{2+} to restore esterase activity was examined with FA-Gly-OLeu-NH_2 and Bz-Gly-OPhe-Ala methyl ester under conditions described previously (Holmquist and Vallee, 1974). Of these only Fe^{2+}, Mn^{2+}, and Cd^{2+} restored 3, 7 and 7% of the native activity, respectively.

Kinetic Parameters. The low solubility, ~2.5 mM, and high K_m values, ~50 mM, of FAGLA (Feder and Schuck, 1970) and its ester analogue, FA-Gly-OLeu-NH_2, preclude the documentation of Michaelis–Menten kinetics and measurement of binding and catalytic constants. Hence, the dependence of hydrolysis rates on the concentration of two readily soluble substrate pairs was examined. Lineweaver–Burk plots for the hydrolysis of Bz-Gly-OPhe-Ala are linear and can be extrapolated to obtain reliable values of k_{cat} and K_M. The hydrolysis of the analogous ester–peptide pair, Bz-Gly-OLeu-Ala and Bz-Gly-Leu-Ala, also conforms to Michaelis–Menten kinetics. Values of the kinetic parameters for these two ester–peptide substrate pairs are given in Table IV.

Inhibition. β-Phenylpropionyl-L-phenylalanine and Zn^{2+} reversibly inhibit the peptidase activity of thermolysin when examined with FAGLA (Holmquist and Vallee, 1974). The K_I for β-phenylpropionyl-L-phenylalanine, 1.6×10^{-3} M, is identical for the inhibition of FAGLA, FA-Gly-OLeu-NH_2, Bz-Gly-Leu-Ala, and Bz-Gly-OLeu-Ala. Similarly, the K_I of 2×10^{-5} M for the zinc inhibition of FAGLA hydrolysis is identical with that for FA-Gly-OLeu-NH_2, Bz-Gly-Leu-Ala, and Bz-Gly-OLeu-Ala. The competition of these inhibitors with both esters and peptides further substantiates that esters and peptides bind to identical sites of the enzyme.

Salt Effects. The concentration of salt in the assay medium markedly affects thermolysin activity. When assayed at pH 7.5, 0.05 M Tris–0.01 M $CaCl_2$, 25° in 3 M NaBr, $CaCl_2$, LiBr, NaCl, or KCl, the activities of thermolysin toward FAGLA or its ester analogue FA-Gly-OLeu-NH_2 increase by 1000, 220, 270, 520, and 510%, respectively,

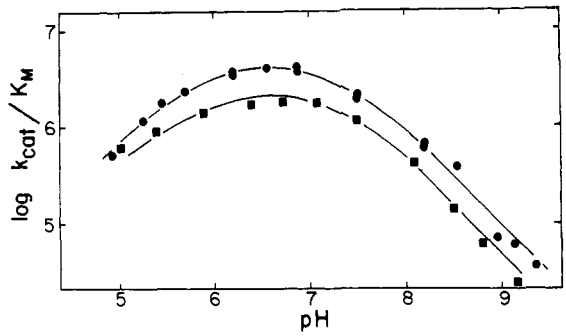

FIGURE 1: pH–rate profiles for the thermolysin-catalyzed hydrolysis of the peptide FA-Gly-Leu-NH_2 (●) and the ester FA-Gly-OLeu-NH_2 (■), both 1 mM, in 0.01 M $CaCl_2$, 25°. Mes, Tris, and ammediol, each 0.05 M, maintained pH in the ranges from 5 to 7, 7 to 9, and 9 to 9.5, respectively. Ionic strength was held constant at 1 M with NaCl.

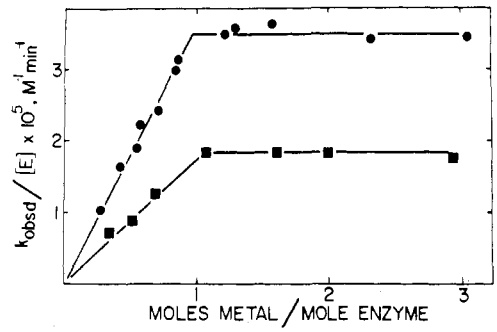

FIGURE 2: Restoration of esterase activity by titration of apothermolysin (1.82×10^{-6} M) with Zn^{2+} (●) and Co^{2+} (■). Rates of hydrolysis of FA-Gly-OLeu-NH_2, 1 mM, were followed spectrophotometrically at 345 nm, at pH 7.5, 0.05 M Tris–0.01 M $CaCl_2$. Apoenzyme exhibited <1% activity before addition of metal.

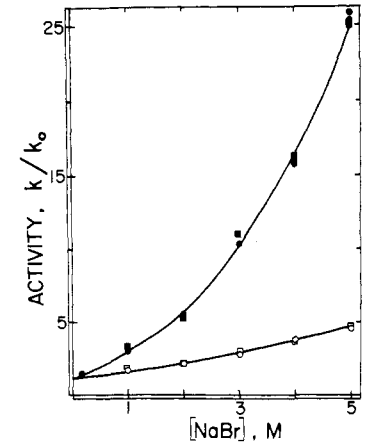

FIGURE 3: Effect of sodium bromide on the esterase and peptidase activities of thermolysin measured at pH 7.5 in 0.05 M Tris–0.01 M $CaCl_2$ at 25° at a substrate concentration of 2×10^{-4} M: FA-Gly-Leu-NH_2 (●); FA-Gly-OLeu-NH_2 (■); Bz-Gly-Phe-Ala (○); Bz-Gly-OPhe-Ala (□).

Table IV: Values of k_{cat} and K_m for Two Ester–Peptide Substrate Pairs with Thermolysin Determined from Lineweaver–Burk Plots.[a]

Substrate	k_{cat} (min^{-1})	K_m (M)
Bz-Gly-Phe-Ala	1750	1.4×10^{-4}
Bz-Gly-OPhe-Ala	424	1.1×10^{-3}
Bz-Gly-Leu-Ala	11000	1.4×10^{-3}
Bz-Gly-OLeu-Ala	16000	7.8×10^{-3}

[a] Measured at pH 7.5, 0.01 M Ca^{2+}, 1 M NaCl, 25°.

Table V: Deuterium Solvent Isotope Effect of Thermolysin Hydrolysis of Esters and Peptides.

	$(k_{cat}/K_m)H_2O/(k_{cat}/K_m)D_2O$	$(k_{cat})H_2O/(k_{cat})D_2O$	$(K_m)H_2O/(K_m)D_2O$
FAGLA[a]	0.36		
FA-Gly-OLeu-NH_2[a]	0.874		
Bz-Gly-Leu-Ala[b]	0.43,[c] 0.42[d]	1.0	2.3
Bz-Gly-OLeu-Ala[b]	1.00,[c] 1.27[d]	1.0	1.0

[a] Measured at pH 7.56 (pD 8.03) in 0.05 M Tris, 0.01 M Ca^{2+}, 0.1 M NaCl, and 1 mM substrate. [b] Measured at pH 7.49 (pD 7.98) in 0.05 M Tris, 0.01 M Ca^{2+}, and 0.1 M NaCl. [c] Calculated from data obtained by analysis of Lineweaver–Burk plots based on the spectrophotometric method of assay. [d] Calculated from rate data determined at 1 mM substrate, $[S] \ll K_m$.

when compared with identical assays without added salt. Increasing the NaBr concentration progressively increases activity until at 5 M, the maximum concentration employed for both the peptide and ester substrate, it becomes 25-fold that observed in the absence of salt (Figure 3). The effect of sodium bromide on Bz-Gly-Phe-Ala and Bz-Gly-OPhe-Ala, another substrate pair, is analogous, though there is considerably less activation (Figure 3).

Deuterium Solvent Isotope Effect. The effect of D_2O on the hydrolysis of esters and peptides was compared directly using four substrates, assayed at a single pH, 7.56 (Table V). Identical samples of buffer were lyophilized and reconstituted with D_2O and H_2O to ensure reliability. In accord with earlier results (Feder and Schuck, 1970), FAGLA hydrolysis exhibits an inverse isotope effect of 0.36 (H_2O/D_2O) for k_{cat}/K_m. In contrast, there is virtually no D_2O effect on the hydrolysis of the ester analogue, FA-Gly-OLeu-NH_2, indicating either nearly complete absence or nearly exact compensation of isotope effects on k_{cat} and K_m. The benzoyl substrates, Bz-Gly-Leu-Ala and Bz-Gly-OLeu-Ala, exhibit an inverse isotope effect on K_m for the peptide, but not for the ester (Table V). Importantly, for either benzoyl substrate, D_2O does not affect k_{cat}, as would be expected if general base catalysis were the rate-determining step.

Discussion

Based on the residue believed to be critical to the catalytic mechanism of proteolytic enzymes, carboxyl-, thiol-, seryl-, and metalloproteases have been recognized. Enzymes of each group are known to exhibit esterolytic activity, e.g., pepsin, streptococcal proteinase, chymotrypsin, and carboxypeptidase A. For seryl proteases, detailed comparisons of ester and peptide hydrolysis have led to different mechanistic proposals for esters and peptides (Hess, 1971). A number of zinc exopeptidases, among these leucine aminopeptidase and carboxypeptidase B, also hydrolyze esters efficiently. Replacement of the zinc of carboxypeptidase A with other metals as well as chemical modification, studies of inhibition and substrate binding have shown that esters and peptides bind differently to this enzyme (Vallee et al., 1968), hence precluding identity of their respective mechanisms of hydrolysis (Auld and Holmquist, 1974).

The bacterial neutral proteases are zinc metalloenzymes (McConn et al., 1964; Tsuru et al., 1966; Hiramatsu, 1967; Matheson and Armstrong; 1967; Keay, 1969; Griffin and Prescott, 1970; Keay et al., 1971; Nakajima et al., 1974).

Thermolysin, *B. subtilis* neutral protease, and the neutral protease of *Aeromonas proteolytica* all actively hydrolyze esters, a feature hitherto unrecognized. This implies that esterase activity is a general property of zinc neutral proteases. We have now examined some kinetic parameters and the effects of metal replacement, inhibition, salt, and D_2O on this newly recognized esterolytic activity.

Apparently, esters and peptides both interact with the same binding sites and catalytic residues of thermolysin, and the specificity requirements seem identical; only those esters are substrates whose structures conform to those of readily hydrolyzed amides and where oxygen replaces the NH of the susceptible bond (Ohta, 1966; Feder, 1967; Morihara and Tsuzuki, 1970). The enzyme does not hydrolyze simple esters, such as *p*-nitrophenyl acetate, Z-Gly-ONP, Bz-Arg-OEt, Bz-Tyr-PNP, and alkyl esters (Morihara and Tsuzuki, 1966; Matsubara, 1967) nor the ester substrates of carboxypeptidase, such as Bz-Gly-Gly-OPhe.

The subsite specificity of thermolysin is extensive and allows for interaction with up to six residues of a polypeptide substrate (Morihara and Tsuzuki, 1970). The tripeptide substrates and their ester analogues employed here conform to this subsite specificity (Table II). The k_{cat}/K_m is highest when hydrophobic amino acids donate the amino and carboxyl group of the bond cleaved. Thus, Bz-Gly-Phe-Ala and Bz-Gly-Leu-Ala and their ester analogues are excellent substrates and Bz-Phe-Phe-Ala and Bz-Phe-OPhe-Ala with two hydrophobic amino acids adjacent to the bond cleaved are even more active (Table II). Bz-Gly-Gly-Phe and its ester analogue are not hydrolyzed. The neutral proteases from *B. subtilis* and *Aeromonas proteolytica* also prefer hydrophobic substrate residues (Table III). The active center of *B. subtilis* neutral protease has been shown earlier to have six subsites which accommodate an equal number of amino acid residues of peptide substrates (Morihara et al., 1969). Such studies have yet to be performed for the *Aeromonas* enzyme, although the present data for the hydrolysis of both ester and peptide substrates are quite similar to those for the *B. subtilis* enzyme, indicating analogous subsite properties.

Both the peptidase and esterase activities of thermolysin absolutely depend on a metal at the active site. Reconstitution of the apoenzyme with 1 g-atom of Zn^{2+}/mol of enzyme fully restores esterase (Figure 2) and peptidase activities (Holmquist and Vallee, 1974). Co^{2+} stoichiometrically restores both activities to the apoenzyme (Figure 2). The Co^{2+}, Fe^{2+}, and Mn^{2+} enzymes exhibit 200, 60, and 10% of the peptidase activity of the native enzyme, respectively, but 50, 3, and 7% of the esterase activity. Cadmium restores 7% of the native esterase, but none of the peptidase activity. Such differences in metal dependence likely reflect the stereochemical or electronic differences between esters and peptides.

The same enzyme residue(s) seems to be essential to substrate and inhibitor binding and catalysis of esters and peptides, as judged from the identity of the pK_a values controlling k_{cat}/K_m values of esters and peptides and the identity of the K_I values for β-phenylpropionyl-L-phenylalanine and Zn^{2+} inhibition of esters and peptides (Holmquist, 1970). By analogy, this zinc inhibition may well be due to interaction with His-231, a residue near the active site which binds Ag^{2+}, a metal which inhibits the enzyme (Colman et al., 1972).

Salt, a general perturbant of protein structure and function, fails to differentially affect ester and peptide hydrolysis (Figure 3). While high ionic strength can increase k_{cat}/K_m significantly, the extent depends markedly on the substrate employed, varying from 4.5- to 25-fold in 5 m*M* NaBr, perhaps due to alterations of protein structure inducing tighter binding or more efficient catalysis.

The ratios of esterase to peptidase activities of each of these bacterial neutral proteases are unusually low. The ratios of k_{cat}/K_m vary from 1.3 to 0.03, the activity of the esters generally being about fivefold lower (Tables II and III) primarily due to weaker binding (Table IV). Liu and Elliot (1971) have classified proteolytic enzymes exhibiting esterase and peptidase activities into those which hydrolyze esters at a rate at least 10^3 times faster than that of the corresponding amide, such as trypsin, chymotrypsin, and streptococcal proteinase, and those which hydrolyze esters and peptides at comparable rates, such as papain, pepsin, and ficin. For the very active esterases, a His residue is believed to be the principal catalytic residue while a carboxyl group is believed to be essential for those whose ester and peptide hydrolytic rates are similar. Thermolysin and other neutral proteases would seem to belong to this latter category, consistent with chemical and structural studies demonstrating the participation of a carboxyl group in the mechanism of thermolysin action (Colman et al., 1972; Burstein et al., 1974). X-ray structure analysis has pointed to Glu-143 and its proximity to the active site zinc atom (Colman et al., 1972). A His residue is involved in catalysis (Blumberg et al., 1973, 1974; Burstein et al., 1974); but it has been suggested that—in addition—the function of Glu-143 may be analogous to that of Glu-270 carboxypeptidase, a residue believed to serve as a general base or nucleophile in the catalytic mechanism of that enzyme.

The absence of a D_2O effect on k_{cat} of either peptides or esters could denote that proton transfer is not rate limiting. Hence, hydrolysis would not be expected to proceed by general base catalysis, although Jencks (1969) has outlined the reasons for caution in interpreting the absolute magnitude of D_2O effects on enzymes. A deuterium isotope effect on association constants of peptides but not of esters may be ascribed to a difference between esters and peptides of a potential hydrogen bonding interaction between the N-H of the scissile bond with an acceptor of the protein. In D_2O, all substrate amide hydrogens undergo rapid exchange for deuterium (Hvidt and Neilsen, 1966). The hydrogen bonding of deuterium, weaker compared to hydrogen, could thus account for the D_2O effect on peptides. For esters this interaction is not present, of course, perhaps an additional reason for overall weaker binding of esters compared to peptides (Table IV).

The present study documents the hydrolysis of esters by neutral proteases and compares it with that of their exactly analogous peptide substrates. The marked similarity of the two activities indicates that ester and peptide hydrolysis proceed by identical binding and catalytic mechanisms in contrast to carboxypeptidase A (Auld and Holmquist, 1974).

Studies of inhibitor binding to thermolysin based both on x-ray crystallographic analysis of the zinc enzyme (Colman et al., 1972) and magnetic resonance relaxation studies of the Mn^{2+} substituted enzyme, (Bigbee and Dahlquist, 1974), have suggested that the amide carbonyl group of the productively bound inhibitor, Phe-Phe amide, displaces water at the 4th coordination site of the metal. Thus, there appears to be direct interaction of the carbonyl with the metal. If peptide substrates bind in analogous manner, the

metal may function as a Lewis acid activating the carbonyl carbon of both esters and peptides for either nucleophilic catalysis, consistent with the absence of a D_2O effect, or general base catalysis.

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