Kinetics of the Action of Thermolysin on Peptide Substrates†

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ABSTRACT: Kinetic measurements have been performed on the action of thermolysin on a series of peptide substrates of the type A-Phe-Leu-Ala, in which A = benzyloxycarbonyl (Z), Z-Gly, Z-Gly-Gly, mansyl (Mns), Mns-Gly, or Mns-Gly-Gly, and which are cleaved solely at the Phe-Leu bond. In some of the substrates, the L-phenylalanyl residue was replaced by the *p*-nitro-L-phenylalanyl [Phe($4NO_2$)] residue, permitting spectrophotometric measurements to be made of the rate of cleavage of the Phe($4NO_2$)-Leu bond. In contrast to earlier studies of this kind with pepsin and papain, the steady-state kinetic data gave no evidence for a significant contribution of secondary enzyme–substrate interactions at a distance from the sensitive peptide bond. This suggests that the active site of thermolysin may be more rigid than those of the other two proteinases. Stopped-flow fluorescence measurements, under conditions of enzyme excess, of the rate of the association of thermolysin with mansyl peptides, and of their cleavage, were performed. The resulting data are consistent with the view that the rate-limiting step in the overall catalytic process is the decomposition of the first detectable enzyme–substrate complex. Modification of thermolysin by the removal of the four calcium atoms of the enzyme and their partial replacement by a terbium atom causes a significant decrease in k_{cat}/K_m toward Z-Phe-Leu-Ala, in confirmation of the report of previous workers who used furanacryloylglycyl-L-leucinamide (Fagla) as the substrate. It has been found in the present work that this decrease is largely a reflection of a change in K_m, without significant change in k_{cat}, suggesting that the binding of the substrate at the active site has been weakened, without significant decrease in the potential catalytic activity toward Z-Phe-Leu-Ala. Upon the modification of thermolysin by the chemical introduction of acetyl-Phe units into the enzyme it was found, in agreement with the previous findings of other workers, that such modified thermolysin exhibits "superactivation" in its action on Fagla and other substrates of the type A-Gly-Leu-B, and that this effect is abolished when the glycyl residue of these substrates is replaced by a phenylalanyl residue. The results reported in the present communication are consistent with the view that the superactivation toward substrates of the type A-Gly-Leu-B is a consequence of the contribution of an acetyl-Phe unit in the modified enzyme in meeting the specificity preference of native thermolysin for hydrophobic amino acid residues on both sides of the sensitive peptide bond.

Earlier work has shown that thermolysin, the thermostable neutral endopeptidase of *Bacillus thermoproteolyticus*, preferentially cleaves peptide bonds in which the imino group is donated by a hydrophobic amino acid residue (Leu, Phe, etc.), and that the rate of cleavage is enhanced by the presence of a hydrophobic amino acid residue as the donor of the carbonyl group of the sensitive bond (for a review, see Morihara, 1974). A widely used synthetic substrate is Fagla,[1] whose hydrolysis may be followed spectrophotometrically at 345 nm (Feder, 1968; Feder & Schuck, 1970). Thermolysin has also been shown to hydrolyze the ester analogues of suitable peptide substrates, for example, benzoyl-L-phenylalanyl-β-phenyl-L-lactyl-L-alanine, at rates (k_{cat}/K_m) that are not very different from those for the corresponding amides (Holmquist & Vallee, 1976). In specificity, therefore, thermolysin resembles pepsin and related acid proteinases (for a review, see Fruton, 1976), although the role of secondary interactions in influencing the catalytic rate appears to be less pronounced for thermolysin than in the case of pepsin (Morihara & Tsuzuki, 1970).

Like other bacterial neutral endopeptidases, thermolysin is a metalloenzyme whose catalytic activity involves the participation of a zinc atom (Latt et al., 1969). In addition, its thermostability depends on the presence of 4 atoms of calcium per molecule (Feder et al., 1971; Dahlquist et al., 1976; Voordrouw et al., 1976). X-ray crystallographic studies (Matthews et al., 1974) have shown that the zinc atom is bound at an extended active site and that the calcium atoms are bound at three other distinct sites on the enzyme surface, with one pair of calcium atoms only 3.8 Å apart. It has also been shown (Berner et al., 1975; Dahlquist et al., 1976) that this pair can be replaced by a single terbium atom.

The present studies were undertaken as an extension of our earlier work with pepsin (Fruton, 1976) and papain (Mattis & Fruton, 1976) on the relation between the conformational mobility of extended active sites of proteinases and the catalytic efficiency of these enzymes in cleaving closely related substrates. In this communication we report the preparation of new sensitive substrates for the estimation of the kinetic parameters of thermolysin, the use of analogous substrates labeled with a fluorescent probe group for stopped-flow studies of thermolysin kinetics, and the effect of removal of the calcium atoms or acylation with Ac-Phe-OSu on the kinetic behavior of the enzyme toward the new substrates.

Experimental Section

Synthesis of Peptides. The peptides used in this investigation were made by chain elongation at the NH_2 terminus, starting with the coupling of Z-Leu·DCHA with Ala-OMe·HCl with

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[1] Abbreviations used: Fagla, 2-furanacryloylglycyl-L-leucinamide; Z, benzyloxycarbonyl; DCHA, dicyclohexylammonium; OMe, methoxy; OSu, oxysuccinimido; Ac, acetyl; DCC, dicyclohexylcarbodiimide; ONp, *p*-nitrophenoxy; Phe($4NO_2$), *p*-nitro-L-phenylalanyl; Mns, mansyl, 6-(*N*-methylanilino)-2-naphthalene-1-sulfonyl; Me_2SO, dimethyl sulfoxide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane. The abbreviated designation of optically active amino acid residues denotes the L form.

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TABLE I: Synthesis and Properties of Thermolysin Substrates.

	mp (°C)	R_f[a]	$[\alpha]_D^{26}$ [b]	calcd (%) C	calcd (%) H	calcd (%) N	found (%) C	found (%) H	found (%) N
Mns-Phe-Leu-Ala	171–174	0.82 (B)	+19.6°	65.2	6.3	8.7	64.9	6.2	8.8
Z-Gly-Phe-Leu-Ala-OMe	149–152	0.47 (A)	−24.3°	62.8	6.9	10.1	62.8	6.8	10.2
Mns-Gly-Phe-Leu-Ala	218–220	0.65 (B)	−13.5°	63.3	6.2	10.0	63.1	5.8	9.9
Z-Gly-Gly-Phe-Leu-Ala-OMe	203–205	0.54 (A)	−22.6°	60.9	6.8	11.4	60.5	6.7	11.4
Z-Gly-Gly-Phe-Leu-Ala	179–180	0.67 (C)	−15.4°	60.3	6.6	11.7	60.3	6.7	11.9
Mns-Gly-Gly-Phe-Leu-Ala	148–151	0.50 (B)	−16.0°	61.7	6.1	11.1	61.3	6.1	11.0
Z-Phe($4NO_2$)-Leu-Ala-OMe	199–201	0.52 (A)	−21.0°	59.8	6.3	10.3	59.5	6.4	10.5
Z-Phe($4NO_2$)-Leu-Ala	205–207	0.46 (B)	−14.9°	59.1	6.1	10.6	58.9	6.2	10.4
Z-Gly-Phe($4NO_2$)-Leu-Ala-OMe	191–195	0.64 (A)	−17.5°	58.1	6.2	11.7	58.0	6.1	11.8
Z-Gly-Phe($4NO_2$)-Leu-Ala	188–191	0.65 (B)	−12.5°	57.4	6.0	12.0	57.5	6.1	11.8
Z-Gly-Gly-Phe($4NO_2$)-Leu-Ala-OMe	183–185	0.49 (A)	−14.0°	56.7	6.1	12.8	56.6	6.1	12.9
Z-Gly-Gly-Phe($4NO_2$)-Leu-Ala	159–161	0.75 (C)	−9.9°	56.1	6.0	13.1	56.3	6.2	13.3

[a] The letter in parentheses denotes the solvent system used (see Experimental Section). [b] *c* 0.5–0.7, dimethylformamide.

DCC/1-hydroxybenzotriazole, to yield Z-Leu-Ala-OMe (mp 92–93 °C). The subsequent coupling reactions were mediated either by means of the *N*-hydroxysuccinimide ester or the *p*-nitrophenyl ester of the appropriate Z-protected peptide, with dimethylformamide as the solvent and triethylamine as the base, or by means of isobutyl chloroformate, with *N*-methylmorpholine as the base and tetrahydrofuran and tetrahydrofuran/dimethylformamide as the solvents for the carboxyl and amino components respectively. The coupling products were isolated in the usual manner and, if necessary, the benzyloxycarbonyl group was removed by hydrogenolysis in methanol with palladium black, or by the use of HBr in glacial acetic acid (essential with peptides containing a *p*-nitrophenylalanyl residue). Saponification of the peptide esters was conducted in methanol (or ethanol) solution with 1.1 equiv of NaOH at room temperature. Difficulty was encountered in the saponification of Z-Gly-Gly-Phe-Leu-Ala-OMe and of its *p*-nitro derivative; the corresponding free acids were prepared by coupling Z-Gly-Gly-ONp with Phe-Leu-Ala·HBr under standard conditions. For the preparation of the mansyl peptides, the protected Z-peptide esters were first treated with HBr–acetic acid and the resulting peptide esters were allowed to react with mansyl chloride under the conditions described by Sachdev et al. (1973). No difficulty was encountered in the saponification of any of the mansyl peptide esters. To conserve space, the details of the synthetic procedure are omitted; the yields and properties of the intermediates and products are listed in Table I. Z-Phe-Leu-Ala-OMe, Z-Phe-Leu-Ala, and Z-Gly-Phe-Leu-Ala have been described previously by Morihara et al. (1969a,b). The properties of the samples prepared in this investigation agreed satisfactorily with those reported by these workers. Mns-Phe and Mns-Gly-Phe were prepared in the manner described by Sachdev et al. (1973).

Chromatographic examination of the peptides was performed with Silica Gel G (Eastman Chromagram sheets 6061) in the following solvent systems: (A) chloroform–methanol (9:1, v/v); (B) chloroform–methanol–acetic acid (85:10:5, v/v); (C) 1-butanol–acetic acid–water (4:1:1, v/v). Ultraviolet light, iodine vapor, ninhydrin, and iodide–hypochlorite–starch were used to detect the chromatographic components.

Enzyme Experiments. A commercial preparation of three times crystallized thermolysin (Calbiochem) was dissolved in Tris buffer (0.05 M, pH 7.5) containing 0.01 M $CaCl_2$ and 2.5 M NaBr to give a stock solution of 10–100 μM. In what follows, this form of the enzyme will be denoted Ca^{2+} thermolysin. Upon assay of the enzyme activity toward Fagla (Vega-Fox Biochemicals) in the manner described by Holmquist & Vallee (1974), a value of $k_{cat}/K_m = 12$ mM^{-1} s^{-1} was obtained for the commercial enzyme preparation. After purification and recrystallization of the enzyme in the manner described by these workers, this value was raised to 15.4 mM^{-1} s^{-1}. The terbium form of thermolysin (Tb^{3+}–Ca^{2+}–thermolysin) was prepared in the manner described by Berner et al. (1975) by dialysis at 0 °C of Ca^{2+}–thermolysin against a buffer solution containing 0.05 M Hepes (pH 7.5), 1 M NaCl, 0.1 M $CaCl_2$, and 0.1 mM $TbCl_3$, or incubation of the holoenzyme with a 15-fold excess of $TbCl_3$. As shown by Dahlquist et al. (1976), the remaining calcium ions in this form of the enzyme can be removed by means of EDTA to yield a form of the enzyme (Tb^{3+}–thermolysin) that retains approximately 40% of the original activity toward Fagla. The preparation of Ac-Phe-thermolysin was performed by the treatment of the enzyme with Ac-Phe-OSu under the conditions described by Blumberg & Vallee (1975). The k_{cat}/K_m value for the hydrolysis of Fagla under the conditions used for the untreated enzyme was 430 mM^{-1} s^{-1}, in agreement with the finding of these authors. Protein concentrations were estimated spectrophotometrically at 280 nm, using a value of E_{cm} (1%) = 17.65 (Ohta et al., 1966) and a molecular weight of 34 600 (Titani et al., 1972).

For peptide substrates of the type A-Phe-Leu-Ala (A = Z-$(Gly)_n$ or Mns-$(Gly)_n$, where n = 0, 1, 2), the initial rate of cleavage under conditions of substrate excess was followed by means of the reaction with fluorescamine (FLURAM, Roche Diagnostics Corp.), in the manner described previously (Sachdev et al., 1975), except that 0.01 M EDTA was present in the phosphate buffer used for the fluorescamine reaction. To estimate the extent of enzymic cleavage, a standard curve for the reaction of fluorescamine with Leu-Ala was obtained, and the fluorescence intensity was found to be proportional to the dipeptide concentration over the range 0 to 200 μM in the original reaction mixture. The kinetic studies were conducted at 25 °C in Hepes buffer (0.05 M, pH 7.5) containing 0.01 M $CaCl_2$ and 0.1 M NaBr. Because of the limited solubility of many of the peptide substrates in aqueous buffers, 10% Me_2SO was present in all the incubation mixtures; separate experiments with Z-peptides showed that this concentration of Me_2SO reduced the value of k_{cat} only slightly (10–20%) but increased the value of K_m considerably (ca. 40%). Thus with Z-Phe($4NO_2$)-Leu-Ala, k_{cat} and K_m were 1140 s^{-1} and 0.45 mM, respectively, in the absence of Me_2SO, and 951 s^{-1} and 0.65 mM in the presence of 10% Me_2SO. With Fagla, under the standard conditions of assay, the addition of 10% Me_2SO had a similar effect; the k_{cat}/K_m value for a purified enzyme preparation was lowered from 14.5 to 8.8 mM^{-1} s^{-1}.

For peptide substrates of the type Z-$(Gly)_n$-Phe($4NO_2$)-

TABLE II: Steady-State Kinetics of Hydrolysis of Thermolysin Substrates.[a]

substrate	enzyme[b]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
Z-Phe-Leu-Ala (0.09–0.73 mM)	Ca^{2+} (0.23 nM)	605 ± 100	0.55 ± 0.05	1100 ± 30
(0.25–1.66 mM)	Tb^{3+} (0.83 nM)[c]	523 ± 105	1.52 ± 0.30	345 ± 21
(0.25–1.23 mM)	Ac-Phe (0.65 nM)	362 ± 47	0.66 ± 0.05	547 ± 44
Z-Gly-Phe-Leu-Ala (0.10–1.46 mM)	Ca^{2+} (0.35 nM)	968 ± 126	0.98 ± 0.13	993 ± 40
(0.17–1.52 mM)	Tb^{3+} (0.86 nM)[c]	407 ± 90	1.39 ± 0.20	293 ± 15
Z-Gly-Gly-Phe-Leu-Ala (0.04–0.20 mM)	Ca^{2+} (3.3 nM)			419 ± 23
Z-Phe(NO_2)-Leu-Ala (90–430 μM)	Ca^{2+} (7.0 nM)	951 ± 210	0.65 ± 0.14	1431 ± 68
(90–460 μM)	Tb^{3+}–Ca^{2+} (6.7 nM)	861 ± 163	0.72 ± 0.30	1220 ± 92
(60–310 μM)	Tb^{3+} (6.7 nM)[c]			359 ± 21
(74–368 μM)	Ac-Phe (5.2 nM)			485 ± 24
Z-Gly-Phe(NO_2)-Leu-Ala (90–450 μM)	Ca^{2+} (6.0 nM)			842 ± 34
(50–270 μM)	Tb^{3+} (6.7 nM)[c]			362 ± 6
(79–393 μM)	Ac-Phe (5.2 nM)			345 ± 11
Z-Gly-Gly-Phe(NO_2)-Leu-Ala (40–210 μM)	Ca^{2+} (6.0 nM)			375 ± 8
(40–210 μM)	Tb^{3+} (33 nM)[c]			102 ± 16
(42–207 μM)	Ac-Phe (13 nM)			104 ± 7

[a] pH 7.5 (0.05 M Hepes buffer), 0.01 M $CaCl_2$, 0.1 M NaBr, 10% Me_2SO, 25 °C. [b] See Experimental Section for definition and mode of preparation of the forms of the enzymes tested. [c] Excess EDTA (6 mM) was also present in the incubation mixture.

Leu-Ala (n = 0, 1, 2), the initial rate of cleavage of Phe($4NO_2$)-Leu bond was followed by the increase in absorbance at 310 nm (Cary 15 spectrophotometer), in the manner used for the study of the kinetics of pepsin action (Inouye & Fruton, 1967; Hollands et al., 1969). The change in molar absorptivity upon complete cleavage of the Phe($4NO_2$)-Leu bond under the above conditions was $\Delta\epsilon_{310}$ = 890 for Z-Phe($4NO_2$)-Leu-Ala, 850 for Z-Gly-Phe($4NO_2$)-Leu-Ala, and 1050 for Z-Gly-Gly-Phe($4NO_2$)-Leu-Ala. The commercial enzyme preparation was used at 25 °C in Hepes buffer (0.05 M, pH 7.5) containing 0.01 M $CaCl_2$, 0.1 M NaBr, and 10% Me_2SO.

In all cases, the steady-state kinetic data accorded with the Michaelis–Menten equation (least-squares linear plots of $1/v$ against $1/[S]$), and values for k_{cat} and K_m were estimated in the usual manner for those substrates whose solubility permitted reliable estimates of K_m. For the substrates whose solubility did not permit test at substrate concentrations near or above the apparent K_m, values of k_{cat}/K_m were estimated from the slopes of the $1/v$ against $1/[S]$ plots.

Chromatographic examination (solvent C) of complete hydrolysates of all the Z compounds tested as substrates showed that the only ninhydrin-reactive component produced was Leu-Ala, and that no Z-Gly or Z-Gly-Gly was formed from the appropriate substrates under the conditions of these studies. It may be concluded, therefore, that all the substrates tested (except for Fagla) were hydrolyzed solely at the Phe-Leu [or Phe($4NO_2$)-Leu] bond.

Stopped-Flow Fluorescence Studies. The stopped-flow kinetic measurements were performed at 25 °C with Mns-Phe-Leu-Ala as the substrate under conditions of enzyme excess, with a Durrum Model D-110 spectrophotometer, in the manner described (Sachdev & Fruton, 1975; Mattis & Fruton, 1976) for the study of the kinetics of pepsin and papain. Because of the limited solubility of the enzyme in buffer solutions of low ionic strength, it was necessary to increase the NaBr concentration to 2.5 M. Separate experiments were performed on the effect of this relatively high NaBr concentration on the fluorescence of the mansyl group. Thermolysin was found to be extremely insoluble in 10% Me_2SO, even at 2.5 M NaBr, and Me_2SO had to be omitted in the stopped-flow experiments under conditions of enzyme excess; the relatively low concentration of the mansyl peptide substrates did not require Me_2SO for their complete dissolution.

Other Spectroscopic Studies. The determination of the ultraviolet absorption spectra (Cary 15 spectrophotometer) at pH 7.5 (0.05 M Hepes) and in 10% Me_2SO showed the longer wavelength maximum to be at 330–332 nm (ϵ = 15 to 20 mM^{-1} cm^{-1}) for all the mansyl compounds used in this work. Neither the position of this maximum nor the molar absorptivity was changed significantly by increasing the NaBr concentration from 0.1 to 2.5 M. The steady-state fluorescence measurements were performed at 25 °C with a MPF-3 Hitachi Perkin-Elmer fluorescence spectrophotometer in its ratio mode, with 10-mm cells, and an excitation wavelength of 330 nm was used for the mansyl compounds. The reported emission data are uncorrected for variation with wavelength in the sensitivity of the detection system. The emission spectrum of a 1.9 μM solution of quinine sulfate in 0.1 N H_2SO_4 was determined (excitation at 360 nm; emission maximum at 450 nm) and used as a standard; its fluorescence intensity at the emission maximum was arbitrarily set at 1.0 fluorescence unit. In measuring the fluorescence of the Tb^{3+}–Ca^{2+}–thermolysin, the excitation wavelength was 280 nm, and the emission maximum was at 545 nm; the emitted light was passed through a filter with a cut off at 430 nm (Horrocks et al., 1975). The fluorescence of this form of the enzyme was not reduced significantly by the addition of excess EDTA.

The apparent dissociation constant (K_D) of the complex formed between thermolysin and Mns-Phe or Mns-Gly-Phe was estimated in the manner described by Sachdev et al. (1972).

Results and Discussion

The choice of the synthetic peptide substrates for the present studies was based on the finding of Morihara & Tsuzuki (1970) that, among the ones they tested with thermolysin, the one that gave the highest k_{cat}/K_m values was Z-Phe-Leu-Ala. In contrast to most of the other Z compounds used in their study, for which the reported K_m values were relatively large in relation to their expected solubility, this substrate gave values of K_m = 0.6 mM at pH 7.0 and 40 °C. It will be noted

in Table II that this value was also obtained in the present studies at pH 7.5 and 25 °C in the presence of 10% Me_2SO; however, the k_{cat} value of 605 s^{-1} is greater than to be expected from the value of 398 s^{-1} (pH 7.0, 40 °C) reported by Morihara & Tsuzuki (1970). The value of k_{cat}/K_m for Z-Phe-Leu-Ala obtained in the present study is approximately 100 times that found for Fagla with the same enzyme preparation. This ratio was previously found by Blumberg & Vallee (1975) for the relative k_{cat}/K_m values in the hydrolysis of FA-Phe-Leu-Gly and Fagla.

To examine the possible effect of secondary enzyme–substrate interactions on the kinetics of thermolysin action on substrates of the type A-Phe-Leu-Ala, the A group was varied (A = Z-Gly, Z-Gly-Gly). As shown in Table II, the values of k_{cat}/K_m for Z-Gly-Phe-Leu-Ala and Z-Phe-Leu-Ala are nearly the same, with similar increases in k_{cat} and K_m upon addition of the glycyl residue. This result suggests a greater contribution of strong nonproductive binding (Hein & Niemann, 1961, 1962) in the case of Z-Phe-Leu-Ala, and indicates that, in contrast to the behavior of pepsin and papain, this structural change does not lead to a marked increase in k_{cat} without significant change in K_m. With the substrate in which A = Z-Gly-Gly, its limited solubility precluded reliable measurements of K_m and k_{cat}, but from the k_{cat}/K_m value it is evident that the addition of a glycyl residue causes a considerable decrease in the susceptibility of the sensitive peptide bond to hydrolysis. It appears likely that the decrease in k_{cat}/K_m for Z-Gly-Gly-Phe-Leu-Ala is largely a consequence of an increase in the value of K_m.

In its action on substrates of the type Z-$(Gly)_n$-Phe-Leu-Ala (n = 0, 1, 2), therefore, thermolysin differs greatly from pepsin and papain in the contribution made to the catalytic rate by secondary interactions at a distance from the sensitive Phe-Leu unit. It may be surmised that the active site of themolysin is less flexible in its response to the entry of an oligopeptide substrate than is the active site of pepsin (Fruton, 1976). The drop in the k_{cat}/K_m value for the substrate with A = Z-Gly-Gly may be a consequence of the relative rigidity of thermolysin, and the more limited area at the active site potentially available for productive interaction with an oligopeptide substrate.

Analogues of the three thermolysin substrates mentioned above were prepared in which the phenylalanyl residue was replaced by a *p*-nitro-L-phenylalanyl residue. The enzymic cleavage of such substrates can be followed spectrophotometrically (Inouye & Fruton, 1967) by taking advantage of the increase of absorbance at 310 nm when a Phe($4NO_2$)-Y bond is cleaved. This analytical procedure is less laborious than the one used above. It will be noted in Table II that the rates of cleavage of the three *p*-nitro-L-phenylalanyl peptides are similar to those found for the corresponding L-phenylalanyl peptides. Because of their sizable molar absorptivity, as well as their limited solubility, the Phe($4NO_2$) peptides with A = Z-Gly and Z-Gly-Gly, whose K_m values appear to be much larger than that of Z-Phe($4NO_2$)-Leu-Ala, could not be tested at a sufficiently high concentration to provide reliable values of k_{cat} and K_m, and only k_{cat}/K_m data are given in Table II.

Effect of Replacement of Calcium by Terbium. To test the hypothesis that the conformational flexibility of the active site is related to its catalytic efficiency, two forms of thermolysin were prepared in which the calcium was either partially replaced by terbium or the resulting Tb^{3+}–Ca^{2+}–thermolysin was treated with EDTA to produce a calcium-free Tb^{3+} enzyme. Dahlquist et al. (1976) have reported that replacement of the calcium pair by a single terbium ion yields a form of the enzyme that retains full catalytic activity toward Fagla, and that the addition of 5 or more equiv of EDTA produces a form of the enzyme which still contains the catalytically essential zinc atom and exhibits approximately 40% of this activity. With the substrates used in the present study, both of the conclusions drawn by Dahlquist et al. (1976) are confirmed. As will be noted in Table II, with Z-Phe($4NO_2$)-Leu-Ala as the substrate, the kinetic parameters for Ca^{2+}–thermolysin and for Tb^{3+}–Ca^{2+}–thermolysin are the same within the precision of the data. Moreover, with all the substrates tested, the value of k_{cat}/K_m for the calcium-free Tb^{3+}–thermolysin is approximately 30–35% of that found for the fully active all-calcium enzyme. Of special interest is the finding that, with Z-Phe-Leu-Ala as the substrate, this decrease is entirely attributable to a marked increase in the value of K_m, whereas, for Z-Gly-Phe-Leu-Ala, there is a significant decrease in k_{cat}. Further work is needed to explain this apparent difference in the behavior of the two substrates, but a possibility that merits consideration is that the Phe-Leu-Ala portion of both compounds is recognized in a similar manner by Tb^{3+}–thermolysin, and the difference lies in the lowered ability of the Tb^{3+}–enzyme to interact by hydrogen bonding with the additional CO–NH group of the larger substrate; this additional hydrogen bonding may also be a factor in the relative rates of cleavage of the two substrates by Ca^{2+}–thermolysin. Weaver et al. (1977) have provided evidence for such bonding upon the interaction of Ca^{2+}–thermolysin with oligopeptide substrates. The results presented above are consistent with the view that the active site of Tb^{3+}–thermolysin is less rigid than that of Ca^{2+}–thermolysin.

Stopped-Flow Studies. It should be noted, however, that the interpretation of changes in K_m as reflecting differences in the binding of substrates such as Z-Phe-Leu-Ala is based on the assumption that the rate-limiting step in the catalysis of oligopeptide substrates by thermolysin is the decomposition of the enzyme–substrate complex whose dissociation constant to free enzyme and substrate is given by K_s (i.e., $K_m = K_s$). To test the validity of this assumption, experiments were performed under conditions of enzyme excess with an analogue of Z-Phe-Leu-Ala in which the benzyloxycarbonyl group was replaced by the mansyl group. Such Mns peptides have been used in earlier stopped-flow spectrophotometric studies (Sachdev & Fruton, 1975; Mattis & Fruton, 1976) on the kinetics of the interaction of pepsin and papain with appropriate substrates under conditions of enzyme excess. With these enzymes, an initial rapid increase in fluorescence, arising from enzyme–substrate interaction, was followed by a slower first-order decrease in fluorescence, associated with the cleavage of the substrate and the release of products. In applying this approach to thermolysin, a difficulty was encountered arising from the limited solubility of the enzyme in buffer solutions of low ionic strength, and it was necessary to increase the NaBr concentration from the level of 0.1 M used in the steady-state kinetic experiments to 2.5 M. This causes a partial quenching of the fluorescence of the mansyl group. Thus, in a solution of Mns-Phe-Leu-Ala in 10% Me_2SO at pH 7.5, and containing 60% dioxane (to lower the dielectric constant), the fluorescence intensity at the emission maximum (485 nm) is decreased from 2.4 arbitrary fluorescence units when the NaBr concentration is 0.1 M to 1.5 units for a solution at 2.5 M NaBr. A similar decrease was noted for the other mansyl peptides prepared in this study. In the absence of dioxane, the emission maxima were near 415 nm (0.2–0.3 fluorescence unit), and the addition of 10% Me_2SO did not alter markedly the position of the emission maximum and the fluorescence intensity either in the presence or the absence of 60% dioxane. When the fluorescence of mansyl compounds that are

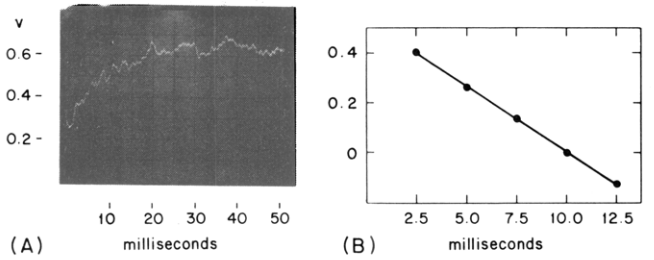

FIGURE 1: Time course of the change in fluorescence during the binding of Mns-Phe-Leu-Ala by thermolysin. $[E]_t = 19.25\ \mu M$; $[S]_0 = 1.25\ \mu M$; pH 8.5 (0.05 M Tris buffer); 2.5 M NaBr; 0.01 M $CaCl_2$; 25 °C. (A) Oscilloscope trace of the reaction. Time constant, 0.5 ms. The ordinate denotes the fluorescence as photomultiplier voltage (V) in volts (50 mV per division). (B) Evaluation of the time course as a plot of log ($V_{max} - V_t$) against time.

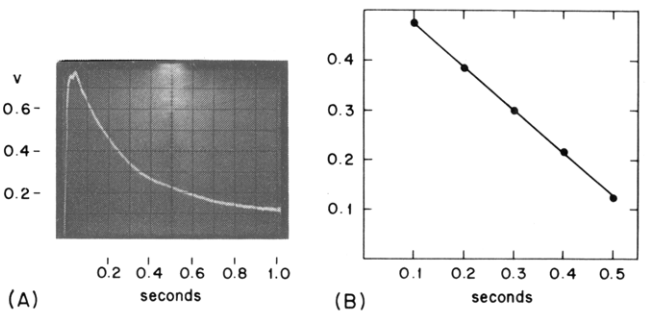

FIGURE 2: Time course of the change in fluorescence during the cleavage of Mns-Phe-Leu-Ala by thermolysin. Experimental conditions the same as for Figure 1, except that $[E]_t = 27.4\ \mu M$. (A) Oscilloscope trace of the reaction. Time constant, 10 ms. The ordinate denotes the fluorescence as photomultiplier voltage (V) in volts (100 mV per division). (B) Evaluation of the time course of the decrease in fluorescence as a plot of log (ΔV) against time, calculated by means of the Guggenheim method (Guggenheim, 1926), with a Δt of 400 ms.

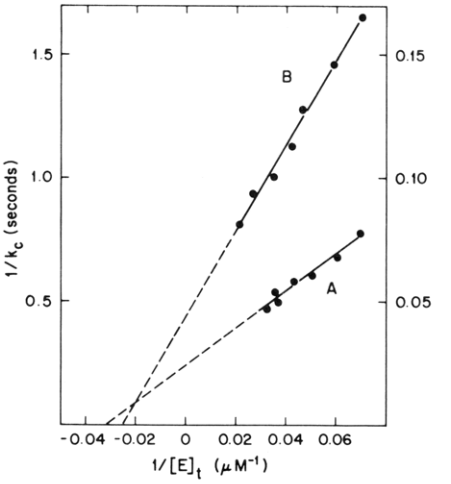

FIGURE 3: Plots of $1/k_c$ against $1/[E]_t$ for the cleavage of mansyl peptides by thermolysin. (Curve A) Mns-Phe-Leu-Ala (left ordinate); (curve B) Mns-Gly-Gly-Phe-Leu-Ala (right ordinate). $[E]_t = 15$–$40\ \mu M$; $[S]_0 = 1.25\ \mu M$; pH 8.5 (0.05 M Tris buffer); 2.5 M NaBr; 0.01 M $CaCl_2$; 25 °C.

not substrates (Mns-Phe and Mns-Gly-Phe) was examined under the above conditions (pH 7.5, 10% Me_2SO, 2.5 M NaBr), a similar large shift in the position of the emission maximum (from 425 nm to 470 nm) was observed upon the addition of 60% dioxane, but the enhancement of fluorescence was much less (from 0.3 to 0.6 unit at the respective emission maxima) than that observed for Mns-Phe-Leu-Ala.

A further difficulty encountered in attempts to perform stopped-flow experiments with Mns-Phe-Leu-Ala at pH 7.5 under conditions of enzyme excess arose from the fact that the fluorescent cleavage product (Mns-Phe) is bound to the enzyme considerably more tightly than is the substrate. As a consequence, the extent of the fluorescence decrease during the course of the hydrolytic process was too small to permit reliable estimates of the rate to be made. Examination of the effect of pH on the cleavage of Mns-Phe-Leu-Ala by thermolysin indicated a less unfavorable ratio of the dissociation constants of the enzyme–product and enzyme–substrate complexes at pH 8.5, and this pH value was chosen for stopped-flow experiments.

Upon the addition of 15 μM thermolysin to 1 μM Mns-Phe-Leu-Ala (in 0.05 M Tris buffer, pH 8.5, and in the presence of 0.01 M $CaCl_2$ and 2.5 M NaBr), the position of the emission maximum did not change significantly (from 435 nm to 425 nm), but the fluorescence intensity at the maximum increased from 0.5 to 2.9 units. Similar behavior was shown by Mns-Phe and by Mns-Gly-Phe. From Scatchard plots of the fluorescence data obtained at 1.25 μM Mns-Phe (or Mns-Gly-Phe) and various concentrations of thermolysin, the value of the dissociation constant (K_D) of the enzyme–ligand complex was estimated to be about 10 μM under the above conditions. Similar determinations of the K_D for Mns-Phe-Leu-Ala could not be performed by steady-state fluorescence measurements because of the rapid cleavage of the substrates at such high enzyme concentration.

Upon mixing Mns-Phe-Leu-Ala (1.25 μM) with various concentrations (10–30 μM) of thermolysin in the stopped-flow apparatus, a monophasic first-order increase in fluorescence was observed, and it was sufficiently slow (a representative oscilloscope trace is given in Figure 1) to permit estimates to be made of the magnitude of the parameters k_1, k_{-1}, and K_s

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

$$k_s = k_{-1}/k_1$$

in the association process. Under the conditions of these experiments, where the equation $k_{obsd} = k_{-1} + k_1([\bar{E}] + [\bar{S}])$ applies, it may be assumed that only a small fraction of the total enzyme concentration $[E]_t$ is in the bound form, and that the equilibrium concentration $[\bar{E}] \cong [E]_t$. Since $[E]_t \gg [S]_0$, a plot of k_{obsd} against $[E]_t$ should give a straight line with the intercept k_{-1} and slope k_1. Replicate determinations gave average values of $k_{-1} = 55 \pm 10\ s^{-1}$ and $k_1 = 3.0 \pm 0.5\ \mu M^{-1}\ s^{-1}$, giving an approximate value of $K_s = 18\ \mu M$. Similar data could not be obtained for Mns-Gly-Phe-Leu-Ala and Mns-Gly-Gly-Phe-Leu-Ala because the association reaction was too fast for reliable estimates to be made with our apparatus.

The rapid increase in fluorescence associated with the association process is followed by a first-order decrease in fluorescence (Figure 2). On the minimal assumption that, under conditions of enzyme excess, the over-all catalytic process is described by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products}$$

the observed first-order rate constant (k_c) for the fluorescence decrease should equal $k_2[E]_t/([E]_t + K_s)$ (Kezdy & Bender, 1962). A plot of $1/k_c$ against $1/[E]_t$ at constant substrate concentration (1.25 μM) and enzyme concentrations ranging from 14 to 38 μM are shown in Figure 3 for Mns-Phe-Leu-Ala; the values of k_2 and K_s were $4.0 \pm 1.0\ s^{-1}$ and $28 \pm 10\ \mu M$,

respectively. It will be noted that the estimated value of K_s is in reasonable agreement with that calculated from the kinetics of the association process.

Steady-state kinetic measurements ($[E]_t$ = 8.4 nM; $[S]_0$ = 13–77 μM) gave values of k_{cat} and K_m for the cleavage of Mns-Phe-Leu-Ala by thermolysin of 2.5 ± 0.4 s^{-1} and 66 ± 19 μM, respectively, at pH 8.5 (0.05 M sodium barbital buffer), and in the presence of 0.075 M NaBr, 0.01 M $CaCl_2$, and 10% Me_2SO (needed to attain substrate concentrations near the value of K_m). In the absence of Me_2SO, k_{cat}/K_m did not change greatly with an increase in NaBr concentration from 0.08 to 2.5 M for substrates used in this study, in agreement with the data of Holmquist & Vallee (1976) on the cleavage of benzoyl-Gly-Phe-Ala. In view of the effect of Me_2SO on the kinetic parameters for the action of thermolysin on Z-peptide substrates and on Fagla (see Experimental Section), the values of k_{cat} and K_m, when corrected for this effect, are sufficiently close to the values of k_2 and K_s obtained under conditions of enzyme excess to warrant the conclusion that k_{cat} approximates k_2 and that K_m approximates K_s. It may be inferred, therefore, that the rate-limiting step in the overall catalytic process is the decomposition of the first detectable enzyme–substrate complex.

Stopped-flow experiments with Mns-Gly-Gly-Phe-Leu-Ala (1.25 μM) as the substrate and with various concentrations of thermolysin in excess (14–39 μM) at pH 8.5 gave values of k_2 and K_s of 22 ± 9 s^{-1} and 41 ± 11 μM, respectively (Figure 3). It will be noted that, while the value of K_s approximates that for Mns-Phe-Leu-Ala, the value of k_{cat} is considerably higher. This result suggests a contribution of secondary enzyme–substrate interaction to catalytic efficiency (Fruton, 1976), but is at variance with the decreased k_{cat}/K_m values for the comparable Z-peptide substrates. Steady-state kinetic measurements of the hydrolysis at pH 8.5 of Mns-Gly-Gly-Phe-Leu-Ala (10–50 μM) by thermolysin (1.7 nM) in the presence of 10% Me_2SO gave a k_{cat}/K_m value of 70 ± 6 mM^{-1} s^{-1}. Under the same conditions, the steady-state parameters for the hydrolysis of Mns-Gly-Phe-Leu-Ala (15–75 μM) by thermolysin (4.2 nM) were found to be k_{cat} = 6.1 ± 1.2 s^{-1} and K_m = 98 ± 20 μM. Because of the relatively high K_m value for this substrate, reliable stopped-flow data under conditions of enzyme excess could not be obtained.

It would appear from the above kinetic data that the specificity of thermolysin, as regards the effect of secondary enzyme–substrate interactions, is different for the series $Mns(Gly)_n$-Phe-Leu-Ala at pH 8.5 from that for the corresponding benzyloxycarbonyl compounds at pH 7.5.[2]

Effect of Treatment with Ac-Phe-OSu. In addition to studies on the effect of the replacement of Ca^{2+} by Tb^{3+} in thermolysin on the kinetics of its action on the substrates used in this investigation, experiments were conducted to determine the effect of chemical modification of the enzyme by treatment with Ac-Phe-OSu. Blumberg & Vallee (1975) have shown that such modification enhances the value of k_{cat}/K_m for the hydrolysis of Fagla approximately 20-fold over the value for purified thermolysin, and this result was confirmed in our work with a sample of the modified enzyme prepared according to their procedure. Holmquist et al. (1976) showed, however, that, when FA-Phe-Leu-Gly was used as the substrate, the k_{cat}/K_m value for the action of Ac-Phe-thermolysin was only approximately 25% of that for the untreated enzyme. As is reported in Table II, our substrates, in all of which the Phe-Leu bond is the one cleaved by the enzyme, were also cleaved much more slowly by the modified enzyme, in complete agreement with the conclusion drawn by Holmquist et al. (1976) that the introduction of a phenylalanyl residue in place of the glycyl residue in substrates of the type X-Gly-Leu-Gly decisively alters the response of the enzyme to chemical modification with Ac-Phe-OSu. Holmquist et al. (1976) have provided strong evidence for the chemical modification of 2–3 tyrosyl residues (out of 28 in thermolysin), and from the failure of β-phenylpropionyl-Phe to inhibit the reaction, have suggested that these residues are not in the active site, but may be close to it. The results presented in this communication, taken together with the extensive data reported from Vallee's laboratory, suggest the possibility that an Ac-Phe unit of the modified thermolysin (perhaps located on Tyr-157; see Weaver et al., 1977) provides a group that fills a space at the active site that is occupied in the productive enzyme–substrate complex by the phenylalanine side chain of substrates such as X-Phe-Leu-Y. Such substrates are cleaved by thermolysin much more rapidly than analogous X-Gly-Leu-Y substrates, and the differential effect of chemical modification with Ac-Phe-OSu may be plausibly explained, as suggested by Holmquist et al. (1976), by a contribution of an Ac-Phe unit of the modified thermolysin in meeting the optimum specificity requirements of the enzyme when an X-Gly-Leu-Y substrate is used, or in blocking the entry into the extended active site of substrates of the type X-Phe-Leu-Y. In some respects, this conclusion is analogous to that offered to explain the effect of the ethylammonium ion on the hydrolysis of Ac-Gly-OEt by trypsin (Inagami & Mitsuda, 1964).

It should be noted, however, that the value of K_m for the hydrolysis of Z-Phe-Leu-Ala by Ac-Phe-thermolysin is the same, within the precision of the data, as with the unmodified enzyme. The introduction of the Ac-Phe group into the enzyme does not appear, therefore, to hinder the binding of Z-Phe-Leu-Ala, and it is reasonable to surmise that the marked decrease in k_{cat} is a consequence of a less favorable positioning, in Ac-Phe-thermolysin, of the sensitive peptide bond of the substrate. On the other hand, with substrates such as Fagla, for which a K_m of 30 mM has been estimated (Feder, 1968), the effect of the introduction of an Ac-Phe group into thermolysin is to markedly lower the value of K_m. Use of the integrated Michaelis–Menten equation for the evaluation of the kinetic constants for the hydrolysis of Fagla by the Ac-Phe-thermolysin prepared in this laboratory gave a value of 1.3 mM, in agreement with the finding (Blumberg & Vallee, 1975) that it was necessary to lower the concentration of Fagla from 1 mM to about 0.2 mM in order to obtain pseudo-first-order kinetics for the action of Ac-Phe-thermolysin on this substrate.

It should be noted that Kester & Matthews (1977) and Weaver et al. (1977) have proposed that Trp-115 (one of three tryptophan residues in thermolysin) is in a location that permits it to form hydrogen bonds with the CO and NH groups of an amino acid residue linked to the amino group of the sensitive dipeptidyl unit. It may be expected, therefore, that resistant analogues of thermolysin substrates of the type $Mns(Gly)_n$-Phe-Leu-Ala will be useful for the study of the efficiency of fluorescence energy transfer from Trp-115 to the fluorescent probe group in the substrates. Preliminary experiments have shown that Mns-Phe-D-Leu-Ala, which is resistant to hydrolysis by thermolysin, causes a significant quenching of the tryptophan fluorescence when an excitation wavelength of 285 nm is used. These studies are being continued.

[2] At pH 7.5, under the conditions given in Table II, the k_{cat}/K_m values (in mM^{-1} s^{-1}) for the hydrolysis by thermolysin of the mansyl peptides used in this study were as follows: Mns-Phe-Leu-Ala, 450 ± 14; Mns-Gly-Phe-Leu-Ala, 962 ± 65; Mns-Gly-Gly-Phe-Leu-Ala, 1590 ± 144 (enzyme concentration, 0.5–1.2 nM substrate concentration, 14–93 μM).

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