Cleavage by trypsin and by the proteinase from *Armillaria mellea* at ϵ -N-formyl-lysine residues

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Kinetic studies were made of the hydrolysis by trypsin of α -N-acetylglycyl-L-lysine methyl ester and of its neutral analogue α -N-acetylglycyl- ϵ -N-formyl-L-lysine methyl ester. The latter substance is a moderately good substrate for trypsin, and this observation is discussed in terms of the substrate specificity of the enzyme. The actions of trypsin and of the lysine-specific proteinase from *Armillaria mellea* on both a native and a formylated polypeptide substrate were compared. Both enzymes were found to hydrolyse specifically bonds to ϵ -N-formyl-lysine in the formylated substrate.

It has been generally regarded that trypsin has a narrow specificity, cleaving most readily those bonds C-terminal to the basic amino acids lysine and arginine, but not histidine. It has been observed, however, that several ϵ -N-substituted derivatives of lysine are susceptible to attack by trypsin. Both the ethyl ester and the α -N-benzoyl amide of ϵ -N-methyl-lysine are substrates (Benoitin & Deneault, 1966). This result is not surprising in that the positive charge on the side chain is not eliminated, but it does suggest that steric requirements for binding are not very restrictive. Derivatives of S-aminoethylcysteine are hydrolysed by trypsin, and this observation finds application in the determination of the amino acid sequence around cysteine residues in peptides (Lindley, 1956).

Our interest in the specificity of trypsin arose out of studies on the components of the venom of the European honey-bee *Apis mellifera*. One component is a peptide of 22 amino acids, the sequence of which is given:

It has been called peptide-401 or mast-cell-degranulating peptide. Disulphide bridges link residues 5 to 19 and residues 3 to 15 (Gauldie *et al.*, 1978). In addition to the parent peptide a variant form exists (Breithaupt & Habermann, 1968), and this has been isolated (Gauldie *et al.*, 1976). The variant form of peptide-401 is a mixture of peptides, each carrying a formyl substituent on one or more of the lysine residues 2, 17 and probably 21 (Doonan *et al.*, 1978). The formyl group arises naturally and is not an artifact of the experimental procedures used for

isolation of the peptides. During studies on the structure of the variant of peptide-401 it appeared that the peptide bonds C-terminal to the formyl-lysine residues were susceptible to attack by trypsin, despite the fact that formyl-lysine does not carry a positive charge (Doonan *et al.*, 1978).

Cleavage by trypsin of neutral substrates has been reported previously. Both acetylglycine ethyl ester (Inagami & Mitsuda, 1964) and *p*-nitrophenyl acetate (Hartley & Kilby, 1954) are substrates for trypsin, but neither interacts with the specificity-determining site on the enzyme. On the other hand, α -acetamidoadipamic acid is also a good substrate for trypsin, with a K_m of 4.5 mM compared with 0.28 mM for α -N-acetyl-lysine ethyl ester; α -N-acetyl-S-carboxamidomethylcysteine ethyl ester is also hydrolysed by trypsin (Gorecki & Shalitin, 1967). Both of these substrates may be considered as neutral analogues of lysine, and their side chains interact with the specificity-determining site on the enzyme.

The case of the variant form of peptide-401 is of particular interest because it involves specific cleavage by trypsin at a non-cationic lysine derivative within a peptide substrate. The present paper reports detailed investigations of the action of trypsin on substrates containing formyl-lysine residues. Modified peptide-401 is not a useful substrate for quantitative studies because it contains several susceptible bonds in addition to formyl-lysine. We have therefore used α -N-acetylglycyl-L-lysine methyl ester acetate and its formylated derivative for kinetic

analysis. The reaction of trypsin with melittin, the major peptide component of bee venom (Gauldie *et al.*, 1976), was also investigated qualitatively, with both the native peptide and the peptide in which all the lysine side chains were formylated.

Finally, the action of a second proteolytic enzyme at formyl-lysine residues was investigated. This enzyme, isolated from the mature fruiting body of the fungus *Armillaria mellea* (Broadbent *et al.*, 1972), is referred to as *A. mellea* proteinase. It is a metalloenzyme containing one atom of zinc per molecule of the native enzyme, and the molecular weight, calculated from amino acid analysis, is 16650 (Lewis *et al.*, 1978). It has been claimed to be an endoaminopeptidase with lysine specificity (Lewis *et al.*, 1978). In view of this specificity it was decided to test the action of the enzyme on formylated melittin. A preliminary account of some of this work has been presented (Barry *et al.*, 1979).

Materials and methods

Materials

Trypsin (type XI) was a product of Sigma Chemical Co., Poole, Dorset, U.K. The enzyme had been treated with diphenylcarbamoyl chloride to remove any contaminating chymotryptic activity. α -N-Benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester and α -N-acetylglycyl-L-lysine methyl ester acetate were products of Sigma Chemical Co. Melittin was a gift from Professor C. A. Vernon, Department of Chemistry, University College London, London W.C.1, U.K. *A. mellea* proteinase was a gift from Dr. P. L. Walton, Department of Biochemistry, Pharmaceuticals Division, I.C.I. Ltd., Alderley Park, Cheshire, U.K.

Titration of the active site of trypsin

The amount of active enzyme in the trypsin preparation used for kinetic analysis was measured by direct titration of the active site with α -N-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester as described by Bender *et al.* (1966). A 50 μ l volume of a 60 mM solution of the titrant in 80% (v/v) acetonitrile was added to 3 ml of 50 mM-sodium citrate buffer, pH 3.0, in a 1 cm cuvette. The slow non-enzymic hydrolysis of the substrate was observed at 320 nm with a Beckman Acta V double-beam spectrophotometer. After a short time 50 μ l of trypsin solution (43.4 mg/ml in 50 mM-sodium citrate buffer, pH 3.0) was added. The rapid initial burst of *p*-nitrophenol liberation, followed by the slow turnover of substrate, were recorded. Calculation of the results as described by Bender *et al.* (1966) showed the sample to contain 27.5% active enzyme.

Kinetics of the reaction of trypsin with α -N-acetylglycyl-L-lysine methyl ester and its formylated derivative

The rate of reaction of trypsin with the native and formylated dipeptide ester was measured by a pH-stat method, with a Radiometer type TTT 1C automatic titrator, attached to an auto burette (ABU 11) and a titrator (SBR 2C). The titrant used was 25 mM-KOH, held in a 0.25 ml burette. Measurements were made at 35°C. Portions (1–30 μ l) of a 0.25 M solution of the native ester in water were added to 4 ml of a solution containing 10 mM-N-ethylmorpholine/HCl buffer, pH 8.0. Reaction was initiated by adding 10 μ l of a 1 mg/ml solution of trypsin in water. With the formylated substrate, the volume of the reaction mixture was decreased to 2 ml and larger amounts of substrate were used (10–100 μ l of an approx. 0.3 M solution). The amount of trypsin was increased to 100 μ g. Initial velocities of reaction were determined from tangents drawn by eye to the pH-stat records at the start of the reaction. The errors inherent in this procedure were minimized as far as possible by using a large number of substrate concentrations (between 11 and 14) in each set of experiments. Five sets of experiments were performed with the unmodified ester and two sets with the formylated ester.

Analysis of kinetic data

This was carried out using the direct-linear-plot procedure described by Eisenthal & Cornish-Bowden (1974). Use was made of a computer program kindly supplied by Dr. A. Cornish-Bowden. This program also calculates non-parametric confidence limits for the various kinetic parameters by the method of Porter & Trager (1977) modified as described by Cornish-Bowden *et al.* (1978). In the present case, non-parametric 68% confidence limits are reported.

Synthesis of α -N-acetylglycyl- ϵ -N-formyl-L-lysine methyl ester

Synthesis of the formyl derivative of the dipeptide ester was essentially by the method of Sheehan & Yang (1958). Acetic anhydride (0.83 ml) was added slowly with stirring at room temperature to 2.5 ml of a 0.4 M solution of the ester in 90% formic acid. A further 1 ml of acetic anhydride was added over 10 min. The reaction was allowed to proceed for 24 h and was quenched by addition of 1 ml of water. Unchanged dipeptide was removed by ion-exchange chromatography on sulphopropyl-Sephadex C-25 in 1% acetic acid. The formylated ester passed through the column unretarded. The product showed a single spot on cellulose t.l.c. with the solvent system butan-1-ol/pyridine/acetic acid/water (15:10:3:12,

by vol). The product had an R_F value of 0.86, whereas the native dipeptide had an R_F value of 0.65. Detection of samples after chromatography was by the starch/chlorine/KI method, since the formylated dipeptide was negative to ninhydrin. That the substituent group was formyl was demonstrated by ^1H n.m.r. at 100 MHz with $^2\text{H}_2\text{O}$ as solvent and sodium trimethylsilylpropionate as reference. The spectra of the native and modified peptides were very similar, except that the latter contained a signal at $\delta = 7.95$ characteristic of the formamido group. Integration of the spectrum showed the presence of one formyl proton compared with the three protons of the acetyl and methyl ester groups.

Synthesis of α -*N*-acetylglycyl- ϵ -*N*-formyl-L-lysine methyl ester acetate was sometimes performed by the method of Okawa & Hase (1963). The peptide was treated with *p*-nitrophenyl formate, synthesized by reaction of *p*-nitrophenol with formic acid in the presence of *NN'*-dicyclohexylcarbodi-imide. *p*-Nitrophenol was removed by extraction with ether. The reaction was done in tetrahydrofuran.

Amino acid analysis

The concentrations of the solutions of formylated dipeptide obtained by the methods outlined above were measured by amino acid analysis, with a Locarte Amino Acid Autoanalyser, after hydrolysis of a sample at 105°C *in vacuo* for 24 h. Norleucine was used as an internal standard.

Analysis of primary structure

The identity and purity of the melittin preparation used were checked by determination of its amino acid sequence with an LKB 4020 Solid Phase Peptide Sequencer.

Action of trypsin and *A. mellea* proteinase on native and formylated melittin

The reaction mixture contained 1 ml of a 4 mg/ml solution of the native peptide in 10 mM-*N*-ethylmorpholine/HCl buffer, pH 8.0, or 5 ml of a 0.8 mg/ml suspension of the formylated peptide in the same buffer. Trypsin (25 μg) or 20 μl of the *A. mellea* proteinase solution was added to the native melittin. With the formylated peptide the quantities were increased 10-fold. Reaction was allowed to proceed for 12 and 24 h for the native peptide and formylated peptide respectively. Reaction was quenched by addition of acetic acid. Fragments obtained after digestion were separated by high-voltage paper electrophoresis in a Locarte flat-bed apparatus. The sample was concentrated by vacuum desiccation and the entire sample was applied as a 10 cm band to Whatman 3MM paper (46 cm $\times$ 57 cm) 20 cm from the short edge. Separations were conducted at pH 3.5 with the buffer system 0.5% (v/v) pyridine/

5% (v/v) acetic acid. Time of separation was 60–80 min at 3000 V with a plate pressure of 24 kPa (3.5 lbf/in²).

Separated fragments were eluted from the paper with 5% (v/v) acetic acid and subjected to *N*-terminal analysis and amino acid analysis.

N-Terminal analysis

The dansyl method of Hartley (1970) was used for peptides. The sample of trypsin used for kinetic studies was also analysed by using the modified method described by Gray (1972).

Formylation of melittin

Melittin was formylated by using one of the two methods outlined above. To check that formylation was complete, the modified melittin was dansylated by the method of Hartley (1970). The absence of ϵ -monodansyl-lysine was taken as verification that all of the lysine groups were formylated. Similarly, no dansylglycine was produced, indicating that formylation of the *N*-terminal residue was also complete.

Results and discussion

Kinetics of hydrolysis of the dipeptide esters

Rates of hydrolysis of α -*N*-acetylglycyl-L-lysine methyl ester and its ϵ -*N*-formyl derivative were measured at pH 8.0 by using a pH-stat as described. The amount of trypsin used for the formyl derivative was 10-fold higher than with the native peptide because of the lower rate of hydrolysis of the former. The data were analysed by using the direct-linear-plot method of Eisenthal & Cornish-Bowden (1974). The median values of the parameters obtained from each set of kinetic runs are given in Table 1; the non-parametric 68% confidence limits are also given.

In the case of the formyl dipeptide the non-parametric 68% confidence limits covered a wide span of values of the kinetic parameters, particularly in the estimates of K_m . This presumably reflects the high value of K_m (about 60 mM) and the fact that, for practical reasons, the maximum substrate concentrations used in the two sets of experiments were 13.65 mM and 18.05 mM. Results with the native peptide were much more satisfactory; in this case substrate concentrations in excess of the K_m value could be used.

Although, for the reasons outlined above, the mean values of the estimates of the kinetic parameters for the formylated dipeptide cannot be considered to be very accurate, gross comparisons with the parameters for the native dipeptide can still be made. The K_m values for the two substrates differ by about two orders of magnitude. The extent to which the increase in K_m for the formylated substrate is due to loss of the positive charge on the one hand, and to an increased bulk of the side chain

Table 1. *Kinetic parameters for the hydrolysis of native and formylated α -N-acetylglucyl-L-lysine methyl ester by trypsin*
 The pH was 8.0 and temperature was 35°C. The numbers of substrate concentrations for each set is given in parentheses. Results for each set are given with non-parametric 68% confidence limits; mean values are given $\pm$ S.D.

Substrate	Set	K_m (mM)	V_{max} (mmol/min per mg)	$10^{-3} \times k_{cat}$ (min ⁻¹)	$10^{-3} \times k_{cat}/K_m$ (min ⁻¹ ·mM ⁻¹)
Native	1 (13)	0.56 (+0.03, -0.12)	1.74 (+0.04, -0.24)	41.8 (+0.9, -5.8)	75.1 (+4.7, -2.5)
	2 (11)	0.32 (+0.05, -0.06)	1.38 (+0.12, -0.10)	33.1 (+2.9, -2.4)	104.5 (+11.2, -9.2)
	3 (12)	0.79 (+0.10, -0.10)	1.88 (+0.08, -0.07)	45.1 (+1.9, -1.7)	57.4 (+5.1, -3.5)
	4 (13)	0.40 (+0.09, -0.10)	1.23 (+0.16, -0.15)	29.5 (+3.9, -3.6)	73.8 (+8.0, -8.0)
	5 (13)	0.23 (+0.09, -0.07)	1.01 (+0.17, -0.21)	24.2 (+4.1, -5.0)	107.9 (+22.7, -21.2)
	Mean ...	0.46 ($\pm$ 0.22)	1.45 ($\pm$ 0.36)	34.7 ($\pm$ 8.6)	83.7 ($\pm$ 21.7)
Formyl ester	1 (14)	37.5 (+23.2, -12.9)	0.065 (+0.035, -0.017)	1.56 (+0.84, -0.41)	0.042 (+0.004, -0.002)
	2 (14)	84.8 (+114.9, -32.0)	0.152 (+0.171, -0.052)	3.65 (+4.10, -1.25)	0.043 (+0.003, -0.003)
	Mean ...	61.2 ($\pm$ 33.4)	0.109 ($\pm$ 0.062)	2.61 ($\pm$ 1.48)	0.043 ($\pm$ 0.001)

on the other, cannot be assessed. It is clear, however, that a positively charged side chain is not a prerequisite for binding of a substrate to trypsin. This point has been made previously by Gorecki & Shalitin (1967), who emphasize the importance of substrate hydrogen atoms capable of forming hydrogen bonds with the protein. Indeed, it has been shown (Huber & Bode, 1978) that all three hydrogen atoms of the lysine ammonium group are involved in hydrogen-bonding in the active site of trypsin. One such hydrogen atom is retained in formyl-lysine, and this may provide the dominant interaction between the formylated substrate and the active site of the enzyme.

Once bound to the active site of the enzyme, the formylated dipeptide ester is rapidly hydrolysed with a k_{cat} only one order of magnitude less than that of the non-formylated substrate. Hence binding of the formylated substrate must be such as to orientate the scissile bond accurately with respect to the catalytic groups of the enzyme. A more realistic comparison of the two substrates may perhaps be considered to be provided by the ratio k_{cat}/K_m , which is a measure of the second-order rate constant for the reaction at very low substrate concentrations. The values of this ratio are given in Table 1, and the means for the two substrates differ by about 2000-fold. On this basis, the formylated dipeptide can only be considered as a moderately good substrate for trypsin.

For interpretation of the results quoted above it was important to establish that hydrolysis of the formylated ester was indeed occurring at the ester bond and that deformylation followed by hydrolysis of the regenerated native ester was not occurring. At complete reaction the product of hydrolysis was still ninhydrin-negative, but positive to the starch/chlorine/KI stain, after cellulose t.l.c., and the R_F was 0.63. Treatment of the reaction products with elastase liberated ϵ -N-formyl-lysine, which was identified by chromatography against an authentic sample (obtained from Sigma Chemical Co.). These

results taken together establish that the product after hydrolysis with trypsin was α -N-acetylglucyl- ϵ -N-formyl-L-lysine.

The sample of trypsin used in these investigations was of low activity, and possible complications arising from this should be considered. Whereas α -trypsin (produced by cleavage of the bond between lysine-131 and serine-132 of β -trypsin) has the same specificity as the β -form, further proteolysis of the bond between lysine-176 and aspartic acid-177 yields pseudo-trypsin, which has the ability to cleave peptide substrates at neutral aromatic residues (Keil-Dlouhá *et al.*, 1971). A possible interpretation of the results quoted here might then be that pseudo-trypsin is responsible for hydrolysis at the formyl-lysine residues. That this is not the case was shown by examination of the N-terminal amino acid residues of the trypsin sample used by dansylation in the presence of sodium dodecyl sulphate (Gray, 1972). Analysis of the products by t.l.c. showed the presence of dansyl-isoleucine (and dansyl-isoleucylvaline from incomplete acid hydrolysis) and comparable amounts of dansyl-serine (from α -trypsin), but only very small amounts of dansyl-aspartic acid, the expected extra N-terminal residue from pseudo-trypsin. It should also be noted that extended treatment of melittin (see Fig. 1) with the trypsin sample did not result in cleavage of the bond C-terminal to tryptophan-19 in this peptide. Hence it may be concluded that the trypsin sample did not contain significant quantities of pseudo-trypsin activity and that the cleavage at formyl-lysine is a property of α - and β -trypsins.

Action of trypsin and A. mellea proteinase on native and formylated melittin

Having established that trypsin is capable of hydrolysing the bond C-terminal to formyl-lysine in a model substrate, it seemed to be worth while to extend the study to a polypeptide substrate as a more stringent test of the specificity of the cleavage.

For this purpose we chose to use the bee-venom peptide melittin, the structure of which is shown in Fig. 1 (Habermann & Jentsch, 1967). At the same time, the ability of the lysine-specific proteinase from *A. mellea* to cleave at formyl-lysine residues was tested.

The identity and purity of the sample of melittin used were established by solid-phase sequencing after attachment of the peptide to aminopropyl-glass by using the phenylene di-isothiocyanate method. The sequence was confirmed up to arginine-22. A sample of the melittin was formylated as described above; failure of the *N*-terminus or the side chains of lysine residues to react subsequently with dansyl chloride was taken as evidence of complete formylation.

Samples of the native peptide and of the formylated material were digested with trypsin or with *A. mellea* proteinase for times sufficient for the patterns of peptides obtained after high-voltage paper electrophoresis at pH 3.5 to become stable. Digestion products were then isolated and characterized by *N*-terminal analysis and by amino acid analysis.

Fig. 1 shows the major peptides isolated after digestion of native melittin with trypsin and with *A. mellea* proteinase. Trypsin cleaved at least partially at all lysine and arginine residues, with the possible exception of lysine-23, although the presence of free arginine and lysine in the digest may indicate that cleavage had occurred at that residue as well. The cluster of four basic residues at the *C*-terminus produced, as expected, a somewhat complex digestion pattern. The bond between lysine-7 and valine-8 appeared to be cleaved quantitatively, since no

peptide was found containing lysine-7 internally. With *A. mellea* proteinase a simpler pattern was obtained. Again complete hydrolysis appeared to have occurred at lysine-7; it should be recalled that *A. mellea* proteinase cleaves peptide bonds *N*-terminal to lysine (Lewis *et al.*, 1978; Doonan *et al.*, 1975). Partial cleavage occurred at lysine-21 and lysine-23.

The major fragments isolated after digestion of formylated melittin with trypsin are shown in Fig. 2. Hydrolysis occurred at formyl-lysine-7 and also at arginine-22 and arginine-24. At least partial hydrolysis must have occurred at formyl-lysine residues 21 and 23, as judged by the presence of free arginine and free formyl-lysine in the digest. The digest was only partially soluble, which probably accounts for failure to isolate a peptide with valine-8 as the *N*-terminal residue. The essential point that emerges from these results is that formyl-lysine residues provide points for specific cleavage by trypsin within a polypeptide substrate; no other bonds were cleaved except those *C*-terminal to arginine residues. This again emphasizes that a positively charged side chain is not an essential prerequisite for tryptic action. It should be recalled that the variant form of peptide-401 from bee venom contains ϵ -*N*-formyl-lysine residues (Doonan *et al.*, 1978). Given that these residues are cleaved by trypsin and moreover that formyl-lysine would be identified as lysine during sequence analysis of peptides because of the lability to acid of the formyl group, it may be that formyl-lysine residues exist in other peptides or proteins but have previously escaped detection.

With *A. mellea* proteinase, peptides were isolated

Fig. 1. Structure of melittin and the origins of the major peptides isolated after digestion with trypsin (Tr) or with *A. mellea* proteinase (AM)

Fig. 2. Origins of the major peptides isolated after digestion of formyl-melittin with trypsin (Tr) or with *A. mellea* proteinase (AM)

resulting from hydrolysis of formyl-melittin *N*-terminal to residues 7 and 23; at least in the latter case cleavage was incomplete. Failure to isolate the *N*-terminal peptide was again probably due to its insolubility. Hence, in this case also, a proteinase that was thought to be highly specific for the positive side chain of lysine is able to recognize and cleave at neutral formyl-lysine residues. Again it is tempting to speculate that hydrogen-bonding potential is an important factor in dictating the specificity of the enzyme.

One puzzling feature of the digest with *A. mellea* proteinase was the occurrence of free formyl-lysine. A possible explanation for this is provided by the observation (Doonan *et al.*, 1975) that the enzyme will cleave *C*-terminal to arginine residues in certain circumstances. It may be that the mode of interaction between formyl-lysine and the binding site of the enzyme renders the bonds on either side of the formyl-lysine residue susceptible to attack. This could not be tested by using the formylated dipeptide ester as a substrate, since it is known (Broadbent *et al.*, 1972) that *A. mellea* proteinase is inactive on dipeptide esters and other model substrates of low molecular weight. Clearly this and other features of the activity of *A. mellea* proteinase merit further study.

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