The Selective Isolation of an Active-Site Histidine Peptide from Chymotrypsin- α by Diagonal Peptide 'Mapping'

AN *N*^ε-CARBOXYMETHYLHISTIDINE DIAGONAL PEPTIDE 'MAP'

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1. The selective isolation of an 'active' histidine peptide from reduced and cyanoethylated chymotrypsin- α inhibited with Tos-Phe-CH₂Cl (L-1-tosylamido-2-phenylethyl chloromethyl ketone) was obtained with a His(τ Cm) (*N*^ε-carboxymethylhistidine) diagonal peptide-'mapping' technique. Performic acid vapours, used between the first and second dimensions of the diagonal peptide 'map', resulted in a peracid rearrangement of the alkylated (Tos-Phe-CH₂)-histidine-57 residue into an *N*^ε-carboxymethylhistidine residue. The consequent change in electrophoretic mobility allowed isolation of peptides that contained the 'active' histidine. 2. Peptides containing methionine or *S*-cyanoethylcysteine were oxidized to their sulphones during the treatment. Peptides in which these residues were *N*-terminal were selectively isolated on the basis of the change in electrophoretic mobility at pH 6.5 which was due to the depression of the p*K* of the terminal amino group by the inductive effect of the sulphonyl group. 3. An attempt to apply the method to subtilisin BPN' inhibited with L-1-benzoyloxycarbonylamido-2-phenylethyl bromomethyl ketone failed to yield a peptide containing *N*^ε-carboxymethylhistidine, although peptides containing *N*-terminal methionine were isolated by the procedure.

The location of disulphide bridges in chymotrypsin- α by diagonal paper electrophoresis (Brown & Hartley, 1963, 1966) introduced an elegant technique to protein chemistry. The peptides of an enzymic hydrolysate were separated by electrophoresis, after which those that contained cystine were specifically changed in mobility by oxidation to the corresponding cysteic acid peptides. These were the only peptides that did not lie on a diagonal line when the mixture was submitted to a second electrophoresis at right angles to the first. Many other proteins have since been studied by this method. The method has been modified to isolate peptides containing methionine (Tang & Hartley, 1967), histidine (Cruickshank *et al.*, 1971), lysine (Butler *et al.*, 1969; Dixon & Perham, 1968) and *O*-phosphoserine (Milstein, 1967, 1968). In addition, the technique was utilized in the selective purification of thiol-containing peptides (Perham, 1967; Weeds & Hartley, 1967, 1968; Weeds & Noda, 1968).

Smillie & Hartley (1964, 1966) applied the diagonal peptide-'mapping' technique to isolate the cystine peptides of chymotrypsin- α inhibited with Tos-Phe-

CH₂Cl*. One of these peptides contained the residue alkylated by Tos-Phe-CH₂Cl which they identified as histidine-57. Similarly, Stevenson & Smillie (1965, 1968) identified histidine-57 as the residue modified in chymotrypsin B by Tos-Phe-CH₂Cl and as the

* Abbreviations: His(τ Cm), *N*^ε-carboxymethylhistidine; Cys(-CH₂CH₂CN), *S*-cyanoethyl-L-cysteine; (O₂)Cys(-CH₂CH₂CN), *S*-cyanoethyl-L-cysteine sulphone; Cys(-CH₂CH₂CO₂H), *S*-carboxyethyl-L-cysteine; (O₂)-Cys(-CH₂CH₂CO₂H), *S*-carboxyethyl-L-cysteine sulphone; Met(O₂), methionine sulphone; Cbz-Phe-CH₂Br, L-1-benzoyloxycarbonylamido-2-phenylethyl bromomethyl ketone; Tos-Phe-CH₂Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; *m*_{sp}^{sp}, mobility of a peptide or amino acid relative to aspartic acid during high-voltage electrophoresis at pH 6.5. Measurements were taken from the neutral point denoted by alanine at pH 6.5 and by taurine at pH 2.0. Similar notations denote the mobility of a peptide (or an amino acid) relative to an amino acid during high-voltage electrophoresis at a particular pH. Symbols have been presented according to IUPAC-IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1972) 126, 773–780].

residue modified in chymotrypsin- α and chymotrypsin B by phenoxymethyl chloromethyl ketone. When the peptides were treated with performic acid vapours to oxidize cystine to cysteic acid, the alkylated histidine residue underwent the classical Baeyer-Villiger peracid rearrangement to an ester (Criegee, 1948). The labile ester was subsequently hydrolysed in the acidic conditions to yield an N^{ϵ} -carboxymethyl substituent at the site of the original alkylation by the chloromethyl ketone reagents (Stevenson & Smillie, 1965, 1968). N^{ϵ} -Carboxymethylhistidine, recovered after acid hydrolysis, was indicative of the alkylation occurring at the nitrogen- τ position of histidine-57.

The present paper describes an alternative method of isolating histidine peptides derived from chymotrypsin-like proteases that were alkylated by halo-methyl ketones. The method uses the diagonal peptide-'mapping' technique of Brown & Hartley (1966) modified to avoid oxidation of disulphide bridges by prior reduction and cyanoethylation. The effect of performic acid vapours was thus restricted to the rearrangement of the ketone moiety of the alkylhistidine derivative with the ultimate formation of an N^{ϵ} -carboxymethyl derivative.

The procedure also permits the selective isolation of peptides that initially possessed methionine or S -cyanoethylcysteine as the N -terminal residue. Performic acid vapours oxidized the thioether sulphur of these amino acids to the corresponding sulphone and this lowered the pK of the α -amino group by the inductive effect enough to alter the electrophoretic mobility of the peptide during the second dimension of the high-voltage electrophoresis at pH 6.5. The modified peptides therefore lay just below the diagonal.

Experimental

Materials

Chymotrypsin- α (lot: 50C-2550), subtilisin BPN' (lot: 120C-065), L-1-tosylamido-2-phenylethyl chloromethyl ketone (lot: 41C-0440) and pepsin (lot: 15B-1370-1) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-1-Benzyl-oxy-carbonylamido-2-phenylethyl bromomethyl ketone was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Acrylonitrile was purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. S -Carboxyethyl-L-cysteine was purchased from Fluka A.G. Chemische Fabrik, Buchs, Switzerland.

Methods

Preparation of chymotrypsin inhibited with Tos-Phe-CH₂Cl. The procedure for preparing this inhibited

chymotrypsin was as described by Stevenson & Smillie (1968).

Preparation of subtilisin BPN' inhibited with Cbz-Phe-CH₂Br. The procedure for preparing this inhibited subtilisin was as described by Shaw & Ruscica (1968).

Reduction and cyanoethylation of chymotrypsin- α inhibited with Tos-Phe-CH₂Cl. Chymotrypsin- α (50mg) inhibited with Tos-Phe-CH₂Cl was dissolved in 12ml of 8M-urea in 50mM-Tris adjusted to pH 7.0 with 0.5M-maleic acid. The flask was flushed with N₂, 200 μ l (1.42mmol) of mercaptoethanol was introduced, the flask was sealed with Parafilm and left at 37°C for 16h. Maleic acid did not have a detrimental effect on the reduced protein in the presence of mercaptoethanol, since S -($\alpha\beta$ -dicarboxylethyl)cysteine was not observed on amino acid analysis. However, the use of succinic acid instead of maleic acid would avoid potential undesirable alkylations. Acrylonitrile (200 μ l; 1.5mmol) was introduced and after 40 min no thiols could be detected with sodium nitroprusside. The solution was adjusted to pH 3 with 1M-HCl, dialysed against 1mM-HCl and freeze-dried.

High-voltage electrophoresis. The vertical-strip high-voltage electrophoresis apparatus was similar to that described by Michl (1951) as modified by Ryle *et al.* (1955). The buffer systems and coolants used were as described by Smillie & Hartley (1966). Peptides were detected by the cadmium-ninhydrin dip reagent of Heilmann *et al.* (1957) and amino acids were detected with a dip reagent consisting of 0.7ml of collidine in 100ml of 0.3% ninhydrin in acetone. The presence of peptides containing histidine was determined with the Pauly reagent (diazotized sulphanilic acid) as described by Bailey (1968). Visual dye markers were used throughout high-voltage electrophoresis work as described by Stevenson (1971). Carboxymethylhistidine derivatives were synthesized as outlined by Crestfield *et al.* (1963).

Amino acid analyses. The amino acid analyses of native and modified proteases and of peptides isolated were performed by hydrolysing the material in 6M-HCl at 110°C for 24h. The acid hydrolysates were rapidly taken to dryness on an Evapomix and were analysed on either a Beckman 121 or a Phoenix M-7800 amino acid analyser by the method of Spackman *et al.* (1958). The colour constant for glycine was used to calculate N^{ϵ} -carboxymethyl-histidine (Crestfield *et al.*, 1963). The colour constant for S -carboxyethylcysteine sulphone was equivalent to 63.5% of the colour constant of aspartic acid when determined with the Beckman 121 amino acid analyser. The ratio of E_{440}/E_{570} for this compound was 0.30 which is 1.6-fold greater than the ratio for aspartic acid.

N-terminal analyses of peptides. N -Terminal residues of peptides were determined by the method

of Gray & Hartley (1963) as described by Hartley (1970).

Preparation of *S*-cyanoethyl-L-cysteine. This compound was synthesized as described by Weil & Seibles (1961) except that equimolar amounts of acrylonitrile and cysteine were used as suggested by Friedman *et al.* (1965). Elementary analyses gave the following results. Found: C, 43.0; H, 5.9; N, 15.6%; C₆H₁₀N₂O₂S requires C, 41.3; H, 5.8; N, 16.1%. Elementary analyses were done by Chemalytics, Inc., Tempe, Ariz., U.S.A.

Preparation of *S*-carboxyethyl-L-cysteine sulphone. *S*-Carboxyethylcysteine (500mg) was added to 125 ml of 100% (w/v) formic acid containing 7.5 ml of 30% (v/v) H₂O₂. The solution was left for 2 h at 25°C and was taken to dryness on a Flash-Evaporator operating

at 30°C. Water (50ml) was added to the residue and the solution was again evaporated to dryness. *S*-Carboxyethylcysteine sulphone was crystallized from 65% ethanol in 71% yield. Elementary analyses gave the following results. Found: C, 32.4; H, 5.0; N, 6.9%; C₆H₁₂NO₆S requires C, 31.8; H, 5.4; N, 6.2%. This preparation yielded a single brownish spot ($m^{A.p} = 0.24$) when subjected to high-voltage electrophoresis at pH 2.0 and developed with collidine-ninhydrin.

Results

The diagonal peptide 'map' of a pepsin digest of reduced and cyanoethylated chymotrypsin- α inhibited with Tos-Phe-CH₂Cl is shown in Fig. 1. Four major

Fig. 1. Diagonal peptide 'map' of a pepsin digest of reduced and cyanoethylated chymotrypsin- α inhibited with Tos-Phe-CH₂Cl

High-voltage electrophoresis was conducted at pH 6.5, 3 kV, 1 h in both dimensions. M denotes a mixture of alanine, aspartic acid and visual dye markers which include Xylene Cyano FF (XCF), Orange G (OG) and Crystal Violet (CV).

Table 1. Characterization of peptides derived from reduced and cyanoethylated chymotrypsin-CH₂-Phe-Tos after diagonal peptide 'mapping'

The peptides were produced by pepsin digest. Refer to Fig. 1 for numbering of the peptides. The structure of peptide I is speculative.

Peptide	Structure of peptide during first dimension of diagonal peptide 'mapping'	$m_{\text{cys}}^{\text{asp}}$	Structure of peptide during second dimension of diagonal peptide 'mapping' (after oxidation)	$m_{\text{cys}}^{\text{asp}}$	Amino acid composition	N-Terminal
I	Ala-Ala-His ⁵⁷ (τ -CH ₂ -Phe-Tos)-Cys(CH ₂ CH ₂ CN)-Gly	-0.12	Ala-Ala-His ⁵⁷ (τ -CH ₂ CO ₂ H)-(O ₂)Cys(CH ₂ CH ₂ CN)-Gly	0.12	Asp (0.18), Thr (0.35), Ser (0.43), Gly (1.80), Ala (2.00),* His(τ -CH ₂ CO ₂ H) (0.91), Val (0.43), Leu (0.61), Phe (0.35), (O ₂)Cys(CH ₂ CO ₂ H) (0.80)	—
II	Ala-His ⁵⁷ (τ -CH ₂ -Phe-Tos)-Cys(CH ₂ CH ₂ CN)-Gly-Val-Thr	0	Ala-His(τ -CH ₂ CO ₂ H)-(O ₂)Cys-(CH ₂ CH ₂ CN)-Gly-Val-Thr	0.20	Thr (1.01), Ser (0.22), Glu (0.20), Gly (1.13), Ala (1.54), His(τ -CH ₂ CO ₂ H) (0.72), Val (1.00),* (O ₂)Cys-(CH ₂ CH ₂ CO ₂ H) (0.46), Leu (0.15)	Ala
III	Ala-His ⁵⁷ (τ -CH ₂ -Phe-Tos)-Cys(CH ₂ CH ₂ CN)-Gly-Val-Thr-Thr-Ser-Asp	0.18	Ala-His(τ -CH ₂ CO ₂ H)-(O ₂)Cys-(CH ₂ CH ₂ CN)-Gly-Val-Thr-Thr-Ser-Asp	0.41	Asp (0.89), Thr (1.20), Ser (1.18), Glu (0.16), Gly (0.94), Ala (1.11), His(τ -CH ₂ CO ₂ H) (0.52), Val (1.00),* (O ₂)Cys(CH ₂ CH ₂ CO ₂ H) (0.46), Leu (0.16)	Ala
IV	Cys ¹⁹¹ (CH ₂ CH ₂ CN)-Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val	0.32	(O ₂)Cys(CH ₂ CH ₂ CN)-(O ₂)Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val	0.40	Asp (1.20), (O ₂)Met (0.81), Ser (0.94), Glu (0.26), Pro (0.78), Gly (3.23), Val (0.77), Leu (1.00),* (O ₂)Cys(CH ₂ CH ₂ CO ₂ H) (0.45)	†

* Arbitrarily taken as 1.0 residue with recoveries of other amino acids relative to this amino acid.

† The N-terminal determination was equivocal. Authentic Dns-(O₂)Cys-(CH₂CH₂CO₂H) migrated just ahead of Dns-Glu in the system proposed by Hartley (1970) and was unchanged after acid hydrolysis.

peptides were observed lying below the peptide diagonal. Some very minor peptides were also noted but were not investigated. The sequential alignment of the major peptides to the structure of chymotrypsin- α (Hartley, 1964) was based on amino acid and *N*-terminal analyses as presented in Table 1. Peptide I was not pure but did contain His(τ Cm) on amino acid analyses. Its composition suggested that it contained the 'active' histidine residue. Similarly, the amino acid compositions of peptides II and III were indicative of the amino acid sequences of the 'active' histidine-57 peptide (Smillie & Hartley, 1966). Two alterations in the peptide structure occurred during performic acid oxidation, the formation of His(τ Cm) and the formation of (O₂)Cys-(CH₂CH₂CN). These derivatives markedly changed the mobility of the peptides during high-voltage electrophoresis (Table 1). Peptide IV did not possess His(τ Cm) residues and thus was not associated with the amino acid sequence near histidine-57. On the basis of its amino acid composition this peptide was assigned the sequence of half-cystine-191 to valine-200, thus encompassing the 'active' serine-195.

The position of peptide IV below the diagonal was attributed primarily to the transformation of a suspected *N*-terminal *S*-cyanoethylcysteine residue into its sulphone. The oxidation resulted in an enhanced inductive effect and this caused a lowering in the p*K* value of the α -amino group so that this was partly deprotonated at pH 6.5. Similar effects for *N*-terminal methionine sulphone residues have been discussed by Brown & Hartley (1966) and for *N*-terminal half-cystine residues by Ryle *et al.* (1955).

An attempt was made to apply the method to subtilisin BPN' inhibited with Cbz-Phe-CH₂Br, but the only peptides found off the diagonal were also present in the 'map' from subtilisin not treated with Cbz-Phe-CH₂Br. Their composition suggested that they possessed the sequences methionine-222 to alanine-230 and methionine-222 to alanine-232 and were therefore off the diagonal because of their *N*-terminal methionine. They contained no *N*^ε-carboxymethylhistidine. This result is not surprising in view of the low yield of *N*^ε-carboxymethylhistidine found in similar methods by Shaw & Ruscica (1968) and Markland *et al.* (1968). The failure to readily form *N*^ε-carboxymethylhistidine could be associated with the acid-labile nature of the Cbz group which could have been released in the presence of performic acid, thereby altering the rearrangement.

About 0.46 residue of a theoretical maximum of 1.0 residue of *S*-carboxyethylcysteine sulphone was present in the acid hydrolysates of peptides II, III, and IV (Table 1). Authentic *S*-carboxyethylcysteine sulphone was stable to acid hydrolysis and was eluted from the Beckman 121 amino acid analyser, in 0.1M-sodium citrate buffer, pH 3.25, 55°C, after

29 min, well in advance of aspartic acid which was eluted at 43 min. *S*-Carboxyethylcysteine co-eluted with glutamic acid after 63 min in this system. The lower recoveries of *S*-carboxyethylcysteine sulphone obtained from the isolated peptides suggested that performic acid oxidation of *S*-cyanoethylcysteine residues resulted in partial rearrangement and degradation of the thioether side chain. Further degradations probably occurred during acid hydrolysis leaving about 50% of the initial *S*-cyanoethylcysteine sulphone. Similar losses were observed on performic acid oxidation of *S*-cyanoethylcysteine. Partial breakdown of a thioether upon exposure to performic acid was also observed with *S*-4-pyridylethylcysteine by Stevenson (1973).

The successful formation of *N*^ε-carboxymethylhistidine from an alkylated histidine is markedly dependent on the migratory aptitude of the alkyl derivatives comprising the ketone group (Hassall, 1957; Stevenson & Smillie, 1970) since cleavage of a carbon-carbon bond adjacent to the ketone moiety of the inhibitor can occur on either side. Thus the applicability of the diagonal peptide-'mapping' technique presented for the isolation of peptides containing His(τ Cm) residues must be ascertained by experiment when other halomethyl ketone reagents are used (Shaw, 1970) to alkylate the 'active' histidine residue of enzymes.

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