STUDIES TOWARDS THE COMPLETE SEQUENCE DETERMINATION OF PROTEINS BY MASS SPECTROMETRY: DERIVATISATION OF METHIONINE, CYSTEINE AND ARGININE CONTAINING PEPTIDES

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Summary

A number of methionine, pyrimidyl ornithine, and two carboxymethyl cysteine containing peptides have given easily interpretable mass spectra following acetylation, and permethylation using a reaction time of about 60 seconds. Using this procedure involatile "salt" formation was prevented in all cases. For arginine containing peptides, refined methods are described for producing high yields of the required derivative in both dicarbonyl condensation and hydrazinolysis reactions. In the latter case in particular, a number of acidic peptides have now been derivatised without extensive peptide bond cleavage and their sequences determined.

Introduction

During the past decade there has been a continued effort to apply the mass spectrometer, a powerful tool for structural elucidation, to the singularly important field of protein sequence analysis. One of the most formidable problems has been derivative formation, a prerequisite for volatilising polar peptide molecules. This work was climaxed with the introduction of the permethylation reaction into peptide chemistry by Lederer and co-workers[1]. Since then however, despite advances in derivatisation technique[2-5], some peptides containing certain problematical amino acids have remained difficult or impossible to sequence by mass spectrometry.

The results observed may be rationalised by consideration of one or more of the following factors.

1) The guanidine grouping of arginine adversely directs fragmentation in the mass spectrometer.

2) The formation of involatile "salts", sulphonium in the cases of cysteine and methionine[6], and quaternary nitrogen in the case of histidine[4,7], may occur to a greater or lesser extent.

3) Any "salts" formed may be extracted (particularly in the case of small peptides) into the aqueous phase on work-up of the permethylation reaction, leading to little or no derivatised peptide in the chloroform layer used for mass spectrometry.

4) Thermal degradation of involatile derivatives can give rise to uninterpretable spectra.

A solution to the salt formation problem with cysteine has been described involving accurate balancing of the ratios of peptide, base and methyl iodide used in the permethylation procedure, thus preventing an excess of methylating agent responsible for salt formation.[8] Using this same "equimolar" principle successful modifications of histidine, methionine and derivatised arginine peptides have been reported [9-11], but we have been unable to consistently repeat these procedures, probably due to difficulties associated with exact peptide quantity (often unknown) base strength (variable) and precise measurement of reagents. We have recently reported a "short permethylation" procedure which has proved useful in preventing salt formation in studies on micro-quantities of histidine containing peptides[12]. The method is simple and rapid, important advantages when dealing with large numbers of peptide samples from protein digests for example.

We now wish to report the results of our application of the short permethylation procedure to peptides containing

problematical amino acids other than histidine. Refined methods for derivatising arginine-containing peptides will also be described.

Experimental

Permethylation:

By "standard" procedure we refer to an excess of base and methyl iodide over peptide, and a reaction time of 30 mins to 1 hour[3].

By "equimolar" procedure we refer to a balanced amount of base, methyl iodide and peptide, and a reaction time of 30 mins to 1 hour.

By "short" procedure we refer to an excess of base and methyl iodide over peptide, and a reaction time of about 60 seconds.

(Our arguments apply both to the normal reagents used in the Hakamori permethylation and to other reagents less commonly used, for example D.M.F./NaH/CH_3I[11].)

Acetylation, sample handling techniques and mass spectrometric conditions used were as described previously[13].

Results and Discussion

a) Methionine containing peptides:

Two 0.1μ mole samples of the peptide Met.Phe.Gly were acetylated and permethylated using the standard procedure (sample 1) and the short procedure (sample 2) respectively. The same base ($^-CH_2SOCH_3$) and other reagents were used for both experiments, and samples were run consecutively on the mass spectrometer.

Sample 1 gave no interpretable mass spectrum, with a distinct absense of signals corresponding to amino acid sequence ions.

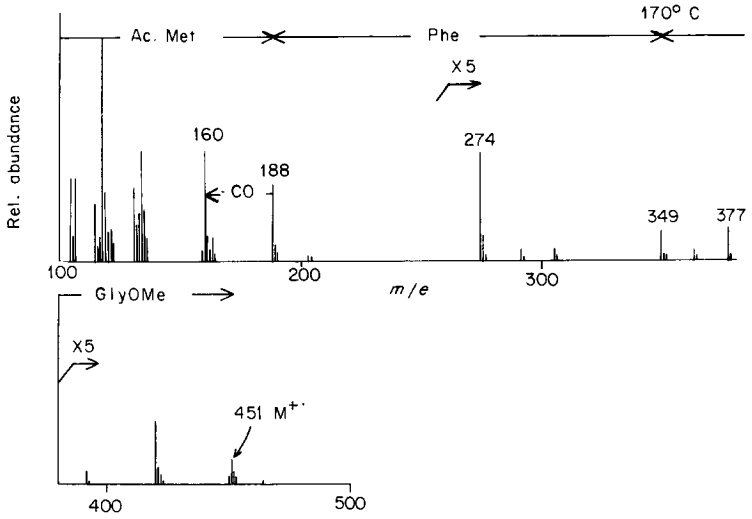


Figure 1.

Mass spectrum (above $^{m}/e$ 100) of a methionine containing peptide after acetylation and short permethylation.

Sample 2 gave the spectrum shown in Fig. 1 which is easily sequenced via the signals at $^{m}/e$ 188, 349, 420 and 451. Plausible pathways to the abundant signals at $^{m}/e$ 377 and 274 are shown in Scheme A, and these were supported by metastable transitions.

We have now applied the short permethylation procedure to a number of methionine containing peptides and protein-derived peptides of unknown sequence. All have given interpretable spectra and structures deduced are summarised in Table 1.

Our results show that as with histidine[12] extensive salt formation may be avoided even in the presence of a large excess of methyl iodide. Using this procedure therefore, methionine need no longer be thought of as a problematical amino acid necessitating either special preliminary modification (e.g. desulphurisation) or base/methyl iodide adjustment on permethylation.

b) Cysteine containing peptides:

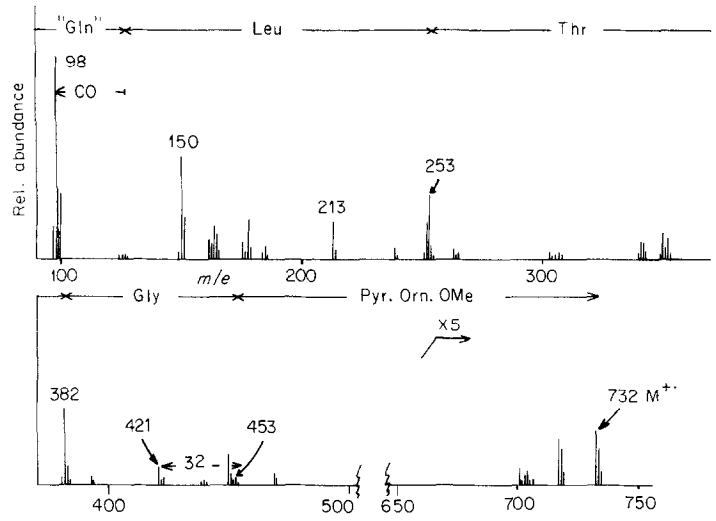


Figure 2.

Mass spectrum (above $^{m}/e$ 90) of a derivatised tryptic peptide.

Because of the reactivity of free thiols, cysteine peptides are normally isolated from proteins as derivatives; commonly carboxymethylated. The infrequent occurrence of this amino acid in our studies has limited our application of the short permethylation to just two peptides Arg.cmCys.Val. and Arg.cmCys. In both cases the peptides could be sequenced and the carboxymethyl cysteine was seen to give rise to dehydroalanine (83 mass units) exclusively in both spectra. Although previous attempts to sequence carboxymethylcysteine peptides by standard permethylation procedures have failed further examples will be needed before proper conclusions can be drawn.

c) Arginine containing peptides:

1. Condensation of the guanidine function with dicarbonyl compounds[14]: Following our failure to consistently sequence arginine containing peptides using the most recently recommended conditions[10], we have carried out a detailed study of

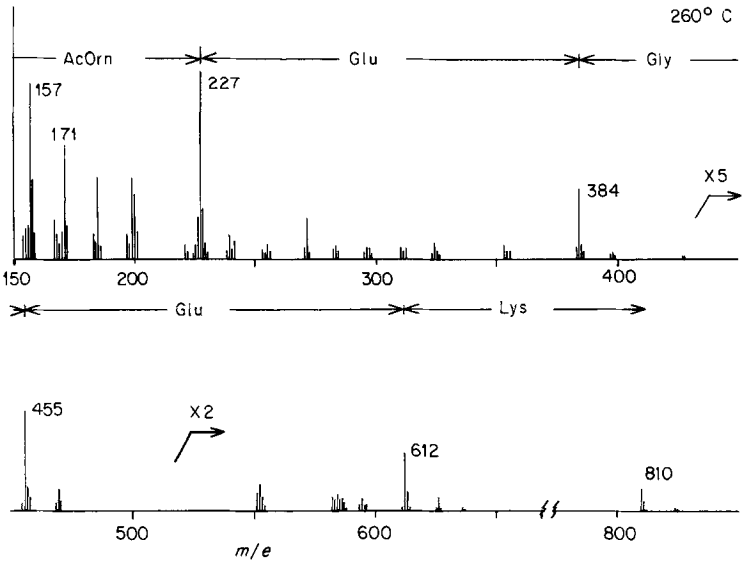
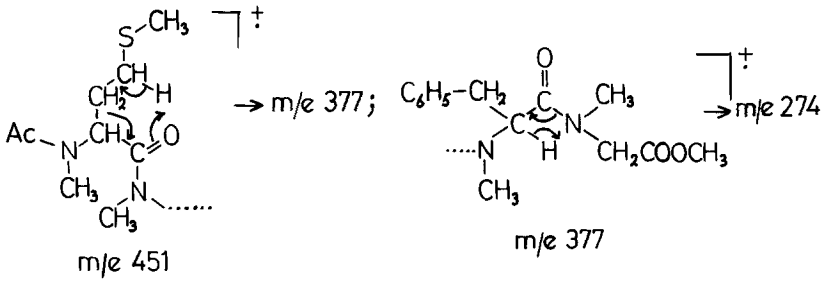


Figure 3.

Mass spectrum (above $^{m}/e$ 100) of a derivatised tryptic peptide.



Scheme A.

Plausible fragmentation pathways to $^{m}/e$ 377 and $^{m}/e$ 274.

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the factors affecting the yield of pyrimidyl ornithine on condensation of acetylacetone with the guanidine function. The following standardised procedure has been found to be most successful.

The peptide is heated under reflux with water (0.1ml.) ethanol (0.2ml.) triethylamine (0.1ml.) and acetylacetone (0.2ml.)

Table 1.

Methionine containing peptides sequenced following acetylation and short permethylation.

Sequence Deduced	Origin
Met. His	Synthetic
Met. Phe.Gly	
Trp. Met. Asp. Phe	
Ala. Leu. Gln. Val. Asp. Leu. Met	Protein
Met. Val. Thr. Arg	
Met. Asp. Glu. Ala. Leu	
Met. Gln. Pro. Leu. Glu. Val. Ala	

for 4 hours. The reaction mixture is rotary evaporated, taken up in water (0.5ml.) and acidified to pH4 with glacial acetic. This is then heated at 100°C for 10 mins, cooled and extracted with ether to remove excess acetylacetone and its self condensation product 2-acetyl-3,5-dimethylphenol. The aqueous phase is then dried in vacuo. The use of a volatile buffer is stressed since it was found that the large quantities of inorganic salt resulting from the use of a sodium bicarbonate buffer[10] badly affected the subsequent permethylation reaction. Following acetylation and 60 second permethylation (to avoid quaternising the pyrimidyl ornithine) a typical peptide spectrum, which can be easily interpreted, is shown in Fig 2.

2. Hydrazinolysis conversion to ornithine: Experiments on standard peptides using the two previously described procedures[14,6] have often resulted in peptide chain cleavage particularly in peptides containing acidic amino acids. (Neither of the above publications contained examples of this type of peptide.) We

Table 2.

Arginine containing peptides sequenced following a) condensation with acetyl acetone or b) hydrazinolysis, acetylation and short permethylation.

Arg. Glu. Gly. Glu. Lys

Arg. Leu. Asp. Leu. Phe

Thr. Gly. Arg. Leu. Asp. Leu. Phe

Asp. Arg. Val. Leu

Arg. CmCys. Val

Arg. CmCys.

Val. Arg. Ser. Val. Leu

Val. His. Thr. Thr. Arg

Met. Val. Thr. Arg

Arg. Gly. Gly. Arg

Leu. Arg. Pro. Gly

Asn. Arg. Val. Tyr. Val. His. Pro. Phe

Gln. Leu. Thr. Arg

Ser. Phe. Asn. Arg

have been able to minimise this cleavage using the following milder conditions.

The peptide is heated in hydrazine: water 1:1 (0.5ml.) for 15 mins at 75°. This is followed by dilution with water and removal of reagents under vacuum with warming.

Using one or in some cases both of the above two procedures the peptides shown in Table 2 have been sequenced, most as protein derived unknowns at the 0.1μ molar level, and an example is shown in Fig 3.

Acknowledgement

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