MASS SPECTROMETRY OF CYSTEINE-CONTAINING PEPTIDES

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Summary: Permethylation of cysteine-containing peptides by a modification of established methods permits the determination of their amino acid sequence by mass spectrometry.

The utility of mass spectrometry for the determination of amino acid sequences of peptides has been demonstrated (1). Permethylation is particularly useful for mass spectrometry since it enhances peptide volatility and often allows the amino acid sequences of decapeptides or even larger molecules to be determined (2). If, however, sequencing by mass spectrometry is to become a general method, permethylation must be applicable to all peptides including those containing cysteine and methionine. Mass spectra of permethylated cysteine-containing peptides have not been reported. The sequences of permethylated peptides containing methionine have been obtained by mass spectrometry up to, but not including or beyond methionyl residues; permethylation appears to form sulfonium iodides (3) or cyclopropane derivatives (4) and causes random scission of the peptide backbone during mass spectrometry.

Our studies of acetylated sulfhydryl-containing peptides permethylated according to the established procedure of Thomas (5) utilizing sodium hydride, dimethyl sulfoxide, and methyl iodide, produced poor spectra. In all cases, however, the spectra indicated that some transformation of cysteine into dehydroalanine had occurred. Assuming that formation of S-methyl cysteinyl sulfonium iodides was responsible for the complicated spectra, two approaches for improving the analysis seemed promising.

First, since thioethers are less reactive to methyl iodide than either methylsulfinyl methide or peptide anions, formation of cysteinyl sulfonium

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iodide can be prevented by using methyl iodide in amounts equimolar to all anions present. The product of such an "equimolar" reaction should be a polymethylated peptide containing S-methyl cysteine. A second approach involves deliberate formation of cysteinyl sulfonium iodide by addition of excess methyl iodide. A subsequent addition of excess methide anion should result in formation of dehydroalanine, since S-methyl cysteinyl sulfonium ions form dehydroalanine through β-elimination in alkaline media (6). The product of this reaction would be a polymethylated peptide containing dehydroalanine at positions originally occupied by cysteine.

Methods

Oxidized glutathione, synthetic S,S'-dibenzyl-oxytocin, and naturally occurring $[Lys^8]$-vasopressin were studied. All peptides were acetylated for 18 hours in methanol (adjusted to pH 8 with triethylamine) by the addition of acetic anhydride (1:4 with methanol) (3).

Reduction and permethylation reactions were carried out under argon in stoppered vessels at room temperature. A solution of acetyl oxidized glutathione (5 mg) in methanol (1 ml) was made slightly basic with triethylamine and reduced by addition of mercaptoethanol in a forty fold molar excess over disulfide. Acetyl-$[Lys^8]$-vasopressin (3 mg) was reduced in aqueous methanol (1:5) made basic with triethylamine, and containing mercaptoethanol in an eighty fold molar excess over disulfide. After eighteen hours reducing mixtures were evaporated to dryness under high vacuum and permethylated immediately.

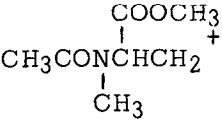
A sodium hydride oil suspension (200 mg) was rinsed x 3 with dry ether, suspended in dimethyl sulfoxide (8 ml), and heated at 65° C under argon until evolution of hydrogen ceased, to form a half-molar solution of sodium methylsulfinyl methide. In all cases 3-5 mg of peptide were permethylated and each peptide was reacted with a five fold excess of methide anion relative to the number of peptide reactive equivalents, i.e., the product of millimoles of peptide times the number of available permethylation sites. In "equimolar" reactions, an amount of methyl iodide equivalent to methide anion was added and allowed to react for one hour. In "excess methyl iodide" reactions, methyl iodide (x 20 molar excess over the number of reactive equivalents) was added. After an hour, more methide anion (x 15

molar excess over the number of "reactive equivalents") was added and the reaction left for another half hour. Reactions were terminated by addition of water (2 ml) and extraction with chloroform (5 ml). Lower phases were then washed x 4 with water (1 ml) and evaporated to dryness.

Mass spectra of products were obtained at 70 ev. with an Associated Electrical Industries MS-9. Only spectra from "equimolar" reactions will be reported here. It is of interest, however, that spectra from "excess methyl iodide" reactions showed no cleavages typical of peptide sequences.

Results

The spectrum obtained from permethylation of acetyl-glutathione, shown in Fig. 1, indicates that S-methyl cysteine partially eliminated methylmercaptan in the mass spectrometer to form a dehydroalanine peptide. Similar behavior has been reported for S-β-aminoethyl cysteine peptides (7). The spectrum also shows that in permethylated γ-glutamyl peptides, the desired peptide bond-type fragmentation occurs. In this case, the γ-glutamyl residue also undergoes a major secondary cleavage between Cβ-C γ to form



as indicated by a peak at m/e 158. The peak at m/e 231 is probably due to protonation of the cleavage product shown in the insert in Fig. 1. The absence of strong molecular ion peaks and abundance of minor peaks in that area are probably the result of excess methylation of the C-terminal glycine residue (8). Permethylation of acetyl-glutathione by the previous method (5) produced spectra containing predominantly random cleavage peaks with minor sequence peaks.

The sequence of the first four amino acids of the peptide may be determined from the spectrum of permethylated acetyl-S, S'-dibenzyl-oxytocin, Fig. 2. In this case, benzylmercaptan was eliminated in high yield to form a dehydroalanine peptide. Peaks at m/e 161, 260, 288, and 458 are due to rearrangement and cleavage of the peptide backbone between cysteinyl and tyrosyl residues (7) to form the product shown in Fig. 2. Thomas' permethylation technique (5) yielded spectra from which the sequence of only the first three amino acids could be deduced and which contained several other non-sequence peaks.

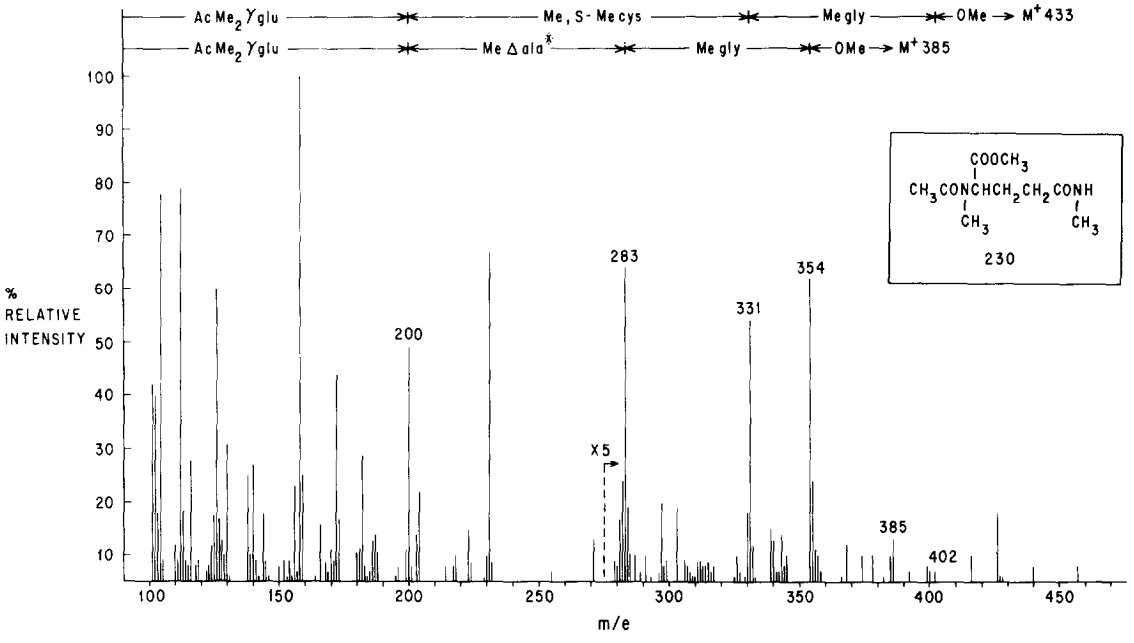


Fig. 1. Mass spectrum of permethylated N-acetyl-glutathione taken at 150° C. *N-Methyl-Dehydroalanyl

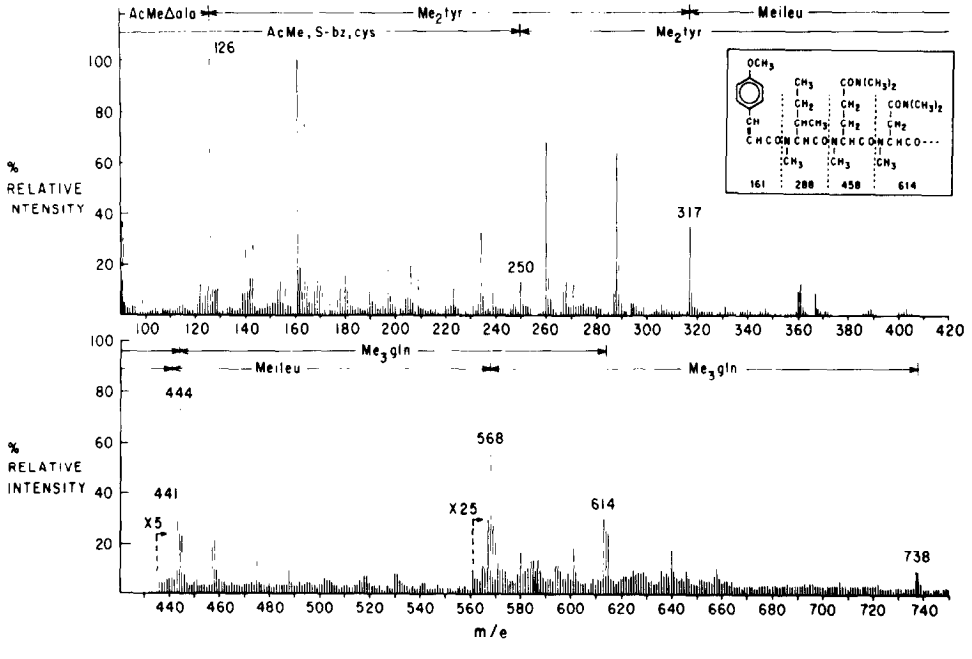


Fig. 2. Mass spectrum of permethylated N-Acetyl-S, S'-dibenzyl-oxytocin taken at 235° C.

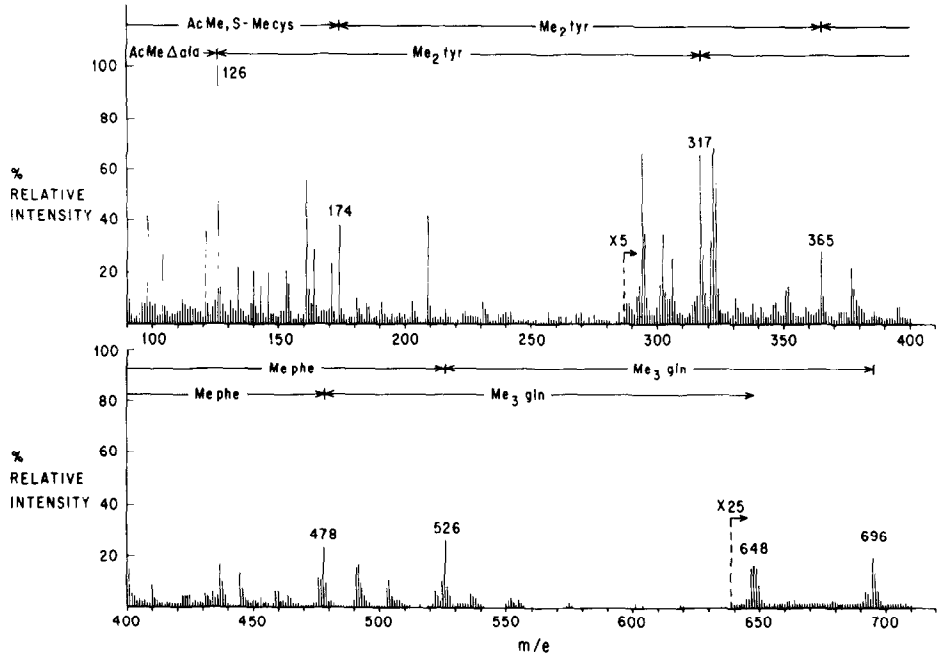


Fig. 3. Mass spectrum of reduced, permethylated N-Ac- $[Lys^8]$-vasopressin taken at 245° C.

Permethylated acetyl- $[Lys^8]$-vasopressin gave the spectrum shown in Fig. 3, from which the sequence of the first four amino acids may also be determined. In this case, methylmercaptan was partially eliminated to form a dehydroalanine peptide. A non-peptide cleavage analogous to that occurring in oxytocin produced peaks at m/e 161, 294, 322, and 492. Acetyl- $[Lys^8]$-vasopressin, permethylated according to the method of Thomas (5), also gave spectra with sequence peaks through only the first three amino acids.

Discussion

In the work described above, acetyl peptides containing underivatized cysteine have been permethylated by a modification of Thomas' technique to yield products suitable for sequencing by mass spectrometry. Failure to obtain the sequence of amino acids beyond the fourth residue in both oxytocin and vasopressin may be due to the asparagine residue at position five which has been reported to hinder peptide sequencing (2). Back-titration of cysteinyl sulfonium iodide peptides with methide anion did not produce the corresponding

dehydroalanine peptides as judged from the spectra. We have no explanation for this failure since facile β-elimination of methylmercaptan from S-methyl sulfonium halides in alkaline media has been reported (6).

We also found that N-acetyl cysteinyl peptides, on treatment with diazomethane, were S-methylated as well as esterified. When permethylated by the "equimolar" technique, such methionine homologues gave sequence spectra compatible with the presence of an intact thioether plus a small amount of the corresponding dehydroalanine peptide. Therefore, our procedure appears to be applicable to methionine peptides, as well as to cysteine peptides. Permethylation by this technique may also be applicable to histidine-containing peptides; these probably have not been amenable to sequence determination by mass spectrometry because of formation of N, N'-dimethylimindazolium ions during permethylation.

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