The Analysis of Peptides and Protein by Mass Spectrometry
Proceedings of the Fourth Texas Symposium on Mass Spectrometry
Edited by C.J. McNeal pages 21-39, 1988

THE USE OF ^{252}Cf PLASMA DESORPTION MASS SPECTROMETRY FOR THE ANALYSIS OF SYNTHETIC PEPTIDES AND PROTEINS.

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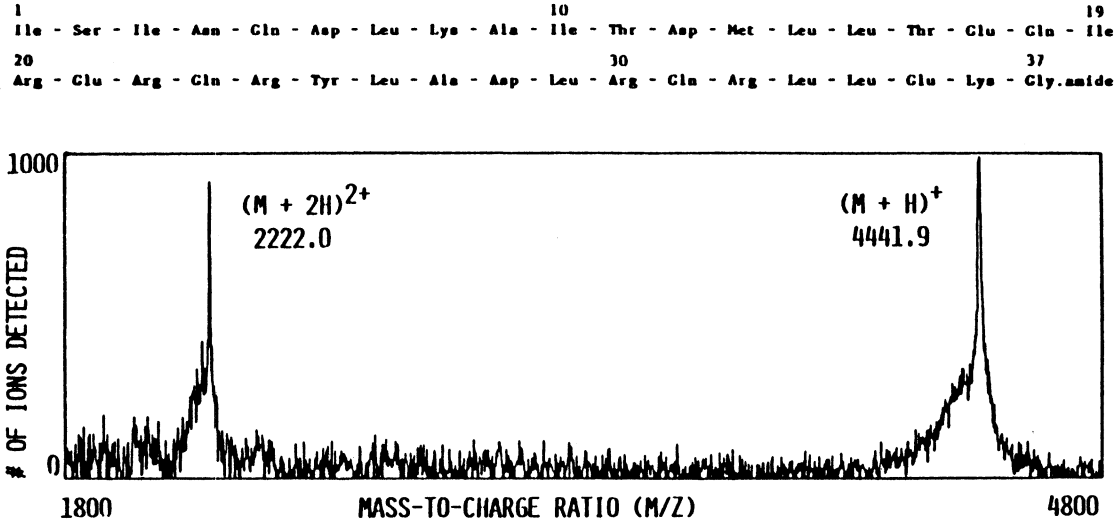
Over the past several years there has been a dramatic increase of interest in the area of synthetically produced peptides and proteins [1-3]. These materials find widespread utility in areas as diverse as the raising of antipeptide sera of predetermined specificity for a receptor protein, the development of synthetic peptide based vaccines, and the development of a fundamental understanding of the relationship of protein structure to function. The present paper describes the utility of ^{252}Cf plasma desorption mass spectrometry (PDMS) for the analysis of synthetic peptides and proteins. This utility was established over the past few years through the detailed analysis of some 800 synthetic peptides and proteins submitted to The Rockefeller University Mass Spectrometric Research Resource by 15 different laboratories in the U.S.A.

VERIFICATION OF THE CORRECTNESS OF THE COVALENT STRUCTURE

With the development of effective, commercially available, automated stepwise peptide synthesizers together with continued improvements in the chemistry of the solid phase method, it has become feasible to routinely and rapidly produce synthetic polypeptides containing up to 50 amino acid residues [3]. In response to the strong demand from the biological community, these synthetic peptides are now being produced in very large numbers. These are complex biomolecules produced by carrying out a very large number of sequential chemical operations. There are thus many opportunities for errors and modifications to occur both during and after synthesis. It is therefore imperative to have available effective means for rapidly verifying the absolute correctness of the covalent structures of these synthetic materials. At The Rockefeller University, we have found through the examination of a large number of synthetic peptides and proteins that PDMS provides an enormously useful, rapid, easy and definitive method for assessing the correctness of structure. The most useful and most easily obtained single piece of information from the ^{252}Cf plasma desorption mass spectrum is the molecular weight (MW) of the compound as determined from the peaks corresponding to the singly and multiply protonated intact molecule. In a stepwise peptide synthesis, the identity of each added amino acid is known and so a measured MW which is found to agree with the MW calculated

for the desired synthetic product provides a relatively good verification that the material has been correctly assembled. Conversely any disagreement which is observed between the measured and calculated MW's of the synthetic product immediately indicates that an error or undesired modification has occurred.

Fig. 1 shows the fission fragment time-of-flight mass spectrum of a 37 residue analogue of the egg laying hormone from the mollusk aplysia californica produced in 86% yield by automated stepwise synthesis by Kent and Schiller at California Institute of Technology [4]. The sample is prepared for mass spectrometry by absorbing ca.1 nmol of the peptide onto a specially prepared thin film of nitrocellulose as previously described [5]. The measured MW of 4441.4 u [6] agrees well with the MW of 4441.2 u calculated from the sequence shown in Fig. 1. This agreement provides a valuable initial verification of the correctness of the synthesized structure. Since the analysis shown in Fig. 1 was completed within less than 2 hours of the receipt of the sample, it can be seen that the mass spectrometric procedure is relatively rapid and straightforward. It should be emphasized that this simple molecular weight determination provides a necessary but not a sufficient condition for confirming the correctness of structure. More stringent confirmation of the proposed structure can be obtained, for example, by classical Edman sequence analysis [7] or by mass spectrometric sequence analysis [8,9]. Such detailed



MEASURED MW = 4441.4 (AVERAGE OF THE VALUES DEDUCED FROM $(M+H)^+$ & $(M+2H)^{2+}$ PEAKS)
CALCULATED MW = 4441.2
DIFFERENCE = +0.2

Fig. 1. Partial ^{252}Cf plasma desorption mass spectrum of a synthetic analogue of the egg laying hormone from aplysia californica [4]. M designates the intact molecule.

mass spectrometric sequence information is sometimes directly available from the normal ^{252}Cf plasma desorption mass spectrum. Thus, for example, we were able to use PDMS to verify the detailed identity between natural and synthetic alamethicin I, a 20 residue pore forming peptide antibiotic [10]. The mass spectrometric verification of structure was of special value in this case because alamethicin has a blocked amino terminus. Unfortunately, in many other cases, the amount and the nature of the fission fragment bombardment induced fragmentation is such as to produce weak and incomplete sequence information. In this regard it is expected that tandem mass spectrometric sequence determinations will be of great value [8,9,11].

VERIFICATION OF THE HOMOGENEITY OF THE SYNTHETIC PRODUCT - IDENTIFICATION OF UNWANTED SIDE PRODUCTS.

The ^{252}Cf fission fragment ionization mass spectrometer is also a powerful tool for determining the homogeneity of the desired synthetic product. The strength of the method resides in its high resolving power. Thus it is usually straightforward to discern in the mass spectrum side products that differ from the desired target material by as little as a fraction of a percent. In addition, if such side products are observed, their masses can be determined accurately and thus provide an important clue as to their identity. Thus, for example, the quasi-molecule ion region of the fission fragment mass spectrum obtained from a synthetic sample of the 35residue antibacterial peptide cecropin A [12] (Fig. 2) showed the presence of a small amount of undesired impurity with a MW 28 u higher than the desired material. The relative peak heights indicate that this unwanted side product is present in approximately 20% abundance. The mass difference of 28 u suggested to the synthetic chemists that the error involved a failure to fully eliminate from the molecule the formyl group which originally protected the tryptophan residue (shown circled in Fig. 2). Once the presence of such an impurity is clearly recognized and its origins established steps can be taken to eliminate its formation. Fig. 3 shows another example of a relatively subtle inhomogeneity in a small methionine containing synthetic peptide. The impurity peak has a MW 15.9 u higher than the desired material suggesting the occurrence of partial oxidation of the methionyl sulfur.

It is unfortunate that, presently, the most used technique for assessing the homogeneity of the purified peptide product i.e. reverse-phase high pressure liquid chromatography (RP HPLC), is also the same technique which is often used for purifying the desired compound from the crude synthetic peptide product. It is then not surprising that undesired materials which co-purify with the compound of interest are also frequently not resolved in the analytical HPLC analysis. Clearly it is desirable to check for homogeneity using an analytical technique which separates compounds by a different principle from that used in the purifi-

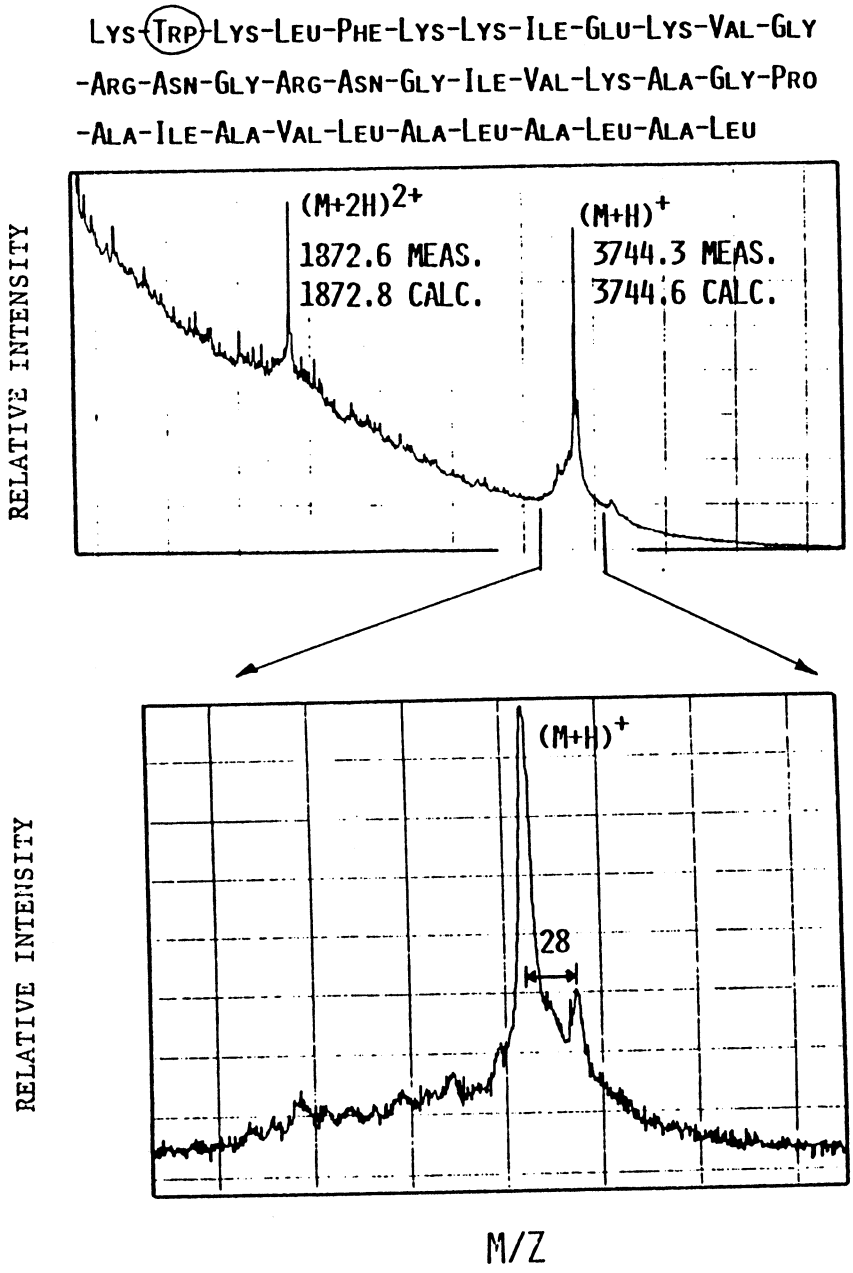


Fig. 2. ^{252}Cf plasma desorption mass spectrum of synthetic cecropin A. The bottom panel shows a detailed plot of the $(M+H)^+$ ion region. The measured and calculated m/z values for the $(M+H)^+$ and $(M+2H)^{2+}$ ions are given in the top panel.

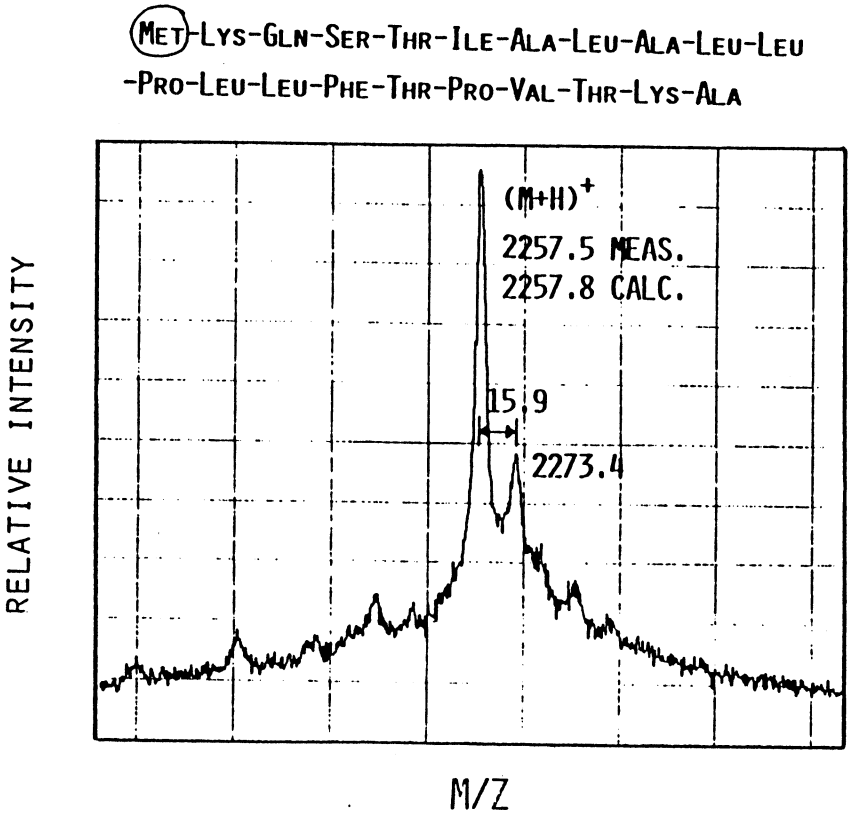


Fig. 3. Detail of the ^{252}Cf plasma desorption mass spectrum of a 21-residue synthetic peptide showing the quasi-molecular ion region.

cation. Mass spectrometry serves this function well. Fig. 4A shows the results of an HPLC analysis of a purified 39 residue cytochrome-C fragment synthesized by Kent and Selk where the analysis was made using a C4 reverse-phase column. The dominant chromatographic peak looks quite symmetrical and sharp and one might predict that the synthetic product is homogeneous. Inspection of the mass spectrum obtained from this material (Fig. 4B) shows that this is not the case. The desired material is present its experimentally determined MW agrees with the predicted MW to within 0.1 u - but so is a substantial amount of a second compound having a MW 114.0 u lower. The mass difference between this latter compound and the desired compound suggests that a deletion or deletions involving asparagine occurred during the synthesis. Subsequent HPLC analysis using a high resolution C18 reverse phase column confirmed the presence of the second unwanted compound. The cytochrome C fragment was thus carefully resynthesized and the mass spectrum from this second synthesis is shown in Fig. 4C. The peak corresponding to the deletion peptide is now no longer present in the spectrum demonstrating that this second synthesis yielded a pure product.

Fig. 4. (A) Reverse-phase HPLC of synthetic cytochrome C (66-104). Top trace obtained with 214nm irradiation. Bottom trace obtained with 254nm irradiation.
(B) Partial ^{252}Cf plasma desorption mass spectrum of synthetic spectrum of synthetic cytochrome C (66-104). First synthesis.
(C) Partial ^{252}Cf plasma desorption mass spectrum of synthetic C (66-104). Second synthesis.

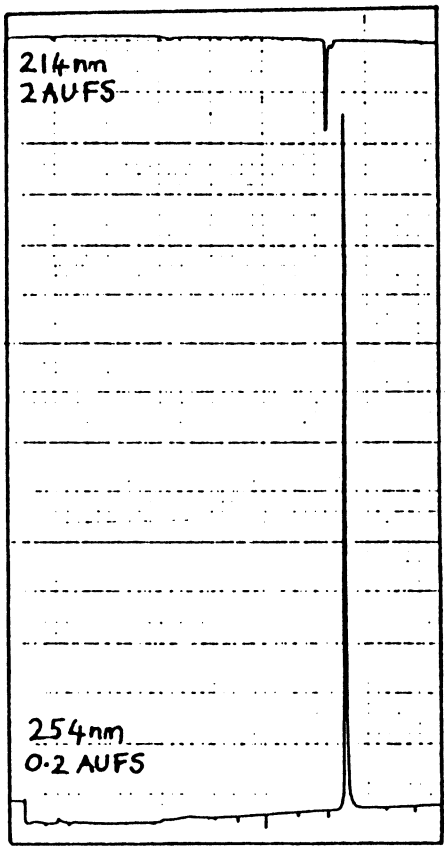
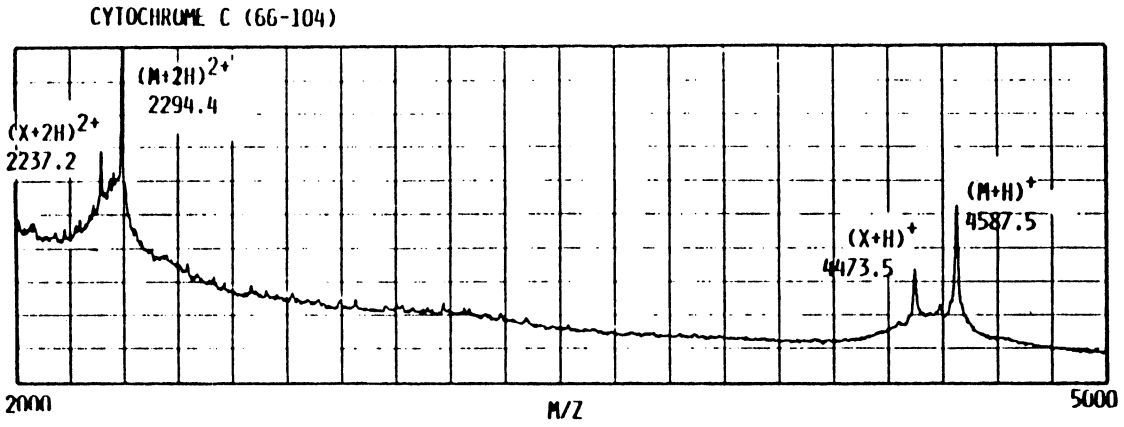


FIG. 4A.

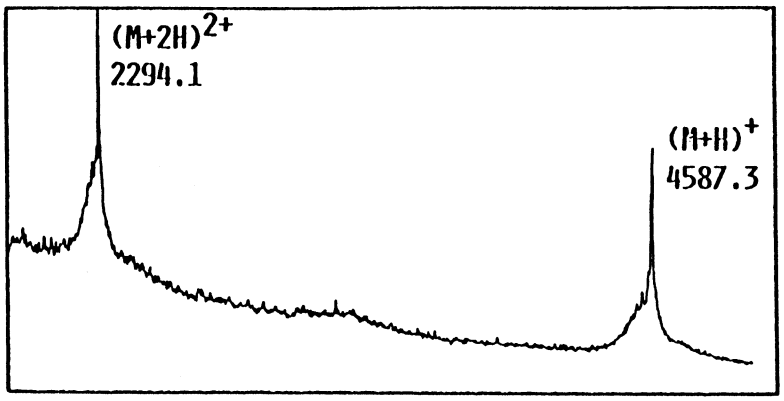
1	2	3	4	5	6	7	8	9	10
Glu -	Tyr -	Leu -	Glu -	Asn -	Pro -	Lys -	Lys -	Tyr -	Ile -
11	12	13	14	15	16	17	18	19	20
Pro -	Gly -	Asn -	Lys -	Met -	Ile -	Phe -	Ala -	Gly -	Ile -
21	22	23	24	25	26	27	28	29	30
Lys -	Lys -	Lys -	Thr -	Glu -	Arg -	Glu -	Asp -	Leu -	Ile -
31	32	33	34	35	36	37	38	39	
Ala -	Tyr -	Leu -	Lys -	Lys -	Ala -	Thr -	Asn -	Glu -	OH



ION SPECIES	MEASURED MW	CALCULATED MW	Δ
$(M+H)^+$	4586.5	4586.4	+0.1
$(X+H)^+$	4472.5	4586.4	-113.9
$(M+2H)^{2+}$	4586.8	4586.4	+0.4
$(X+2H)^{2+}$	4472.3	4586.4	-114.1

FIG. 4B.

CYTOCHROME C (66-104)

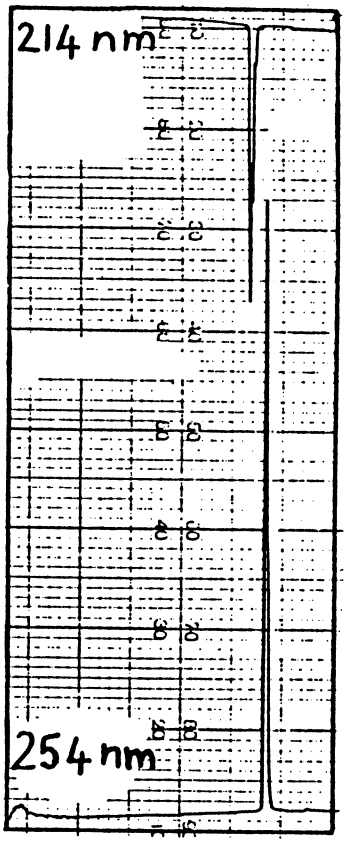


ION SPECIES	MEASURED MW	CALCULATED MW	Δ
$(M+H)^+$	4586.2	4586.4	-0.2
$(M+2H)^{2+}$	4586.3	4586.4	-0.1

Fig. 4C. See caption on previous page.

Frequently, the peptide chemist does have an indication that an error may have occurred during the synthesis but has little or no information concerning the detailed nature of the error. The C18 reverse-phase HPLC analysis of a purified synthetic sample of the 26-residue, bee venom peptide, mellitin is shown in Fig. 5A. Close inspection of the top trace obtained with 214 nm irradiation shows that the intense absorption peak consists of three unresolved components indicating the presence of at least three distinct compounds in the sample. The fission fragment mass spectrum of this same sample (Fig. 5B) confirmed the presence of three main components, designated M,X, and Y with abundances of respectively 69%, 21%, and 10%. We note that these mass spectrometrically inferred abundances are consistent with the abundances of the three chromatographic components. Component M has a measured MW of 2845.9 u which corresponds to within 0.5 u to the calculated MW of 2846.4 u of mellitin. Component X has a measured MW 128.0 u lower and component Y a MW 28.0 u higher than the mellitin peak. These MW differences provide valuable clues as to the identity and origin of X and Y. The mass difference of 128 u suggests that X was produced by deletion of a lysine or a glutamine residue during the stepwise synthesis. The simple MW measurement does not, however, provide any definitive information on the actual position(s) of the deletion(s). The mass increment of 28 u suggests that Y was produced by incomplete removal of the formyl group from the tryptophan residue at position 19 during the final deprotection step, although again no direct confirmation of this can be obtained from the simple MW measurement. While this molecular weight information is clearly valuable, it would be desirable

Fig. 5. (A) C18 reverse-phase HPLC of synthetic mellitin. Top trace obtained with 214nm irradiation. Bottom trace obtained with 254nm irradiation.
(B) Partial mass spectrum of mellitin. First synthesis.
(C) Partial mass spectrum of the same sample of mellitin after 12 min digestion of the nitrocellulose-bound peptide with trypsin. The small peaks labelled F are each 28 u higher than the large adjacent peak.
(D) Partial mass spectrum of mellitin. Second synthesis.

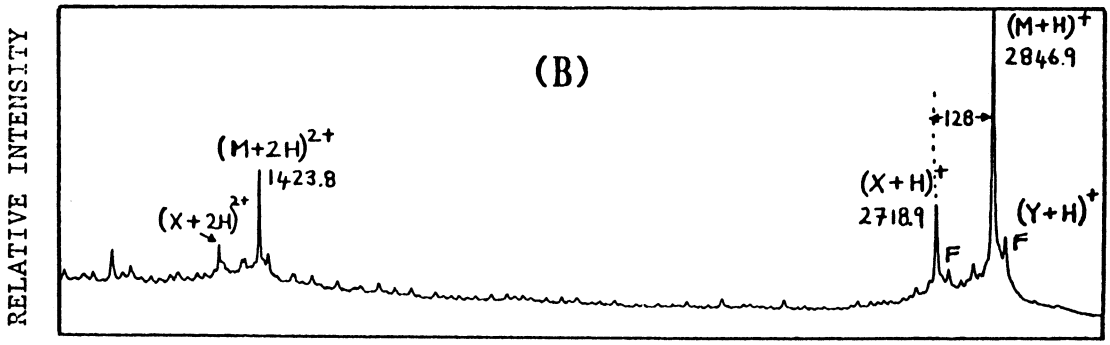


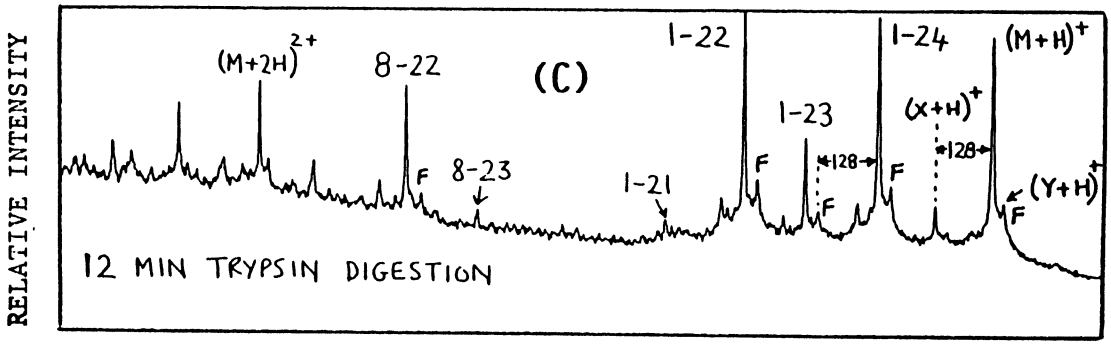
(A)

1 2 3 4 5 6 7 8 9 10
Gly - Ile - Gly - Ala - Val - Leu - (Lys) - Val - Leu - Thr -

11 12 13 14 15 16 17 18 19 20
Thr - Gly - Leu - Pro - Ala - Leu - Ile - Ser - Trp - Ile -

21 22 23 24 25 26
(Lys) - Arg - (Lys) - Arg - (Gln) - (Gln) - amide





M/Z

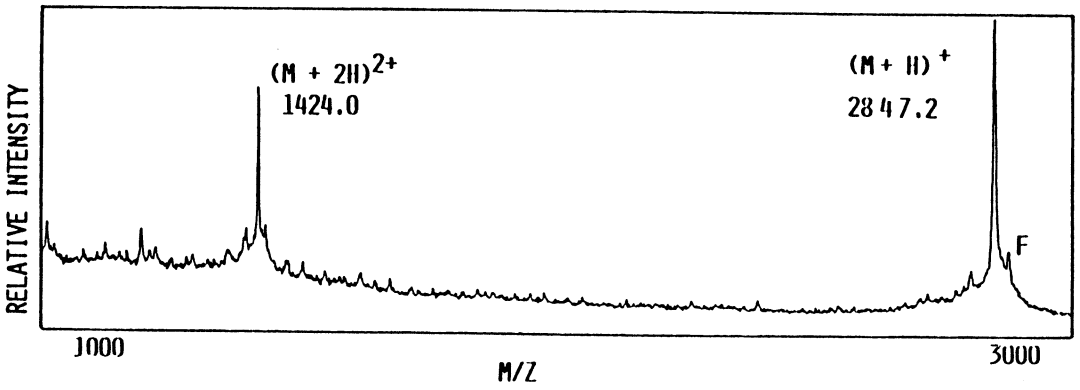


Fig. 5D. See caption on previous page.

to have available techniques which focus more tightly on the precise site in the molecule where the synthetic error or modification has occurred. Tandem mass spectrometry looks to be a good technique for this job since it provides a powerful means of directly pinpointing sites of error or modification. Indeed Biemann and Scoble have recently utilized tandem mass spectrometry to positively identify an internal cyclization of an aspartic acid sidechain in a short synthetic peptide [11].

We have developed a complementary approach to tandem mass spectrometry for rapidly extracting at least some information concerning the sites of synthetic errors. The approach has been described in detail previously (5,13). Briefly, the technique involves practically nondestructive ^{252}Cf fission fragment time-of-flight analysis of monolayer amounts of peptide molecules noncovalently bound to nitrocellulose surfaces prior to and after chemical modification. Thus, for example, inspection of the mass spectra obtained from the synthetic mellitin sample prior to (Fig. 5B) and after (Fig. 5C) incubation with trypsin provides information which constrains the regions of the molecule where the errors have occurred. Prior to any reaction, the mass spectrum of 10^{-9} mol of the sample (Fig. 5B) exhibited the three peaks discussed above. After reaction with 10^{-11} mol of trypsin, the mass spectrum (Fig. 5C) exhibited a series of additional peaks which correspond to the protonated reaction products arising from partial hydrolysis on the carboxyterminal side of residues 7, 21, 23 and 24. Thus, for example, the peak labelled 1-22 corresponds to the tryptic fragment which includes residues 1-22. Since no significant peak is observed 128 u below the 1-22 peak we can immediately deduce that the deletion side product X does not arise by deletion of either Lys 7 or Lys 21. The error then must have occurred by deletion of Lys 23, Gln 25, or Gln 26. This information was given to the peptide chemists who found upon close inspection of their records that there was indeed cause for concern during the first four synthetic cycles which were those involved in the

incorporation of residues 23-26. The compound was thus resynthesized and the new preparation gave a mass spectrum (Fig. 5D) which no longer showed the presence of any significant deletion pept. 'es. The side product which we hypothesized to arise from formyl tryptophan does, however, still appears to be present in the sample.

A great many other errors can and do occur during solid phase synthesis [1-3]. These errors range from operator or machine related errors to relatively subtle chemical modifications of the peptide during and after synthesis. We have found that the majority of these can be detected by PDMS. Thus we have, for example, been able to detect and identify the elimination of water from amino terminal glutamine as well as from other amino acid residues; S-alkylation of methionyl residues by the tertiary butyl cation; incomplete deprotection (many examples); the production of addition peptides and deletion peptides; the production of termination peptides; the production of damage products arising during the final deprotection step; and the production of a number of products which we are not yet able to interpet. The importance of the use of the PDMS technique can be gauged from our rather staggering finding that almost half of the 800 purified synthetic peptides and proteins that we examined were found to have either a molecular weight different from that calculated for the desired target material or to contain significant amounts of unwanted peptide side products.

LIMITATIONS OF THE MASS SPECTROMETRIC TECHNIQUE

While PDMS constitutes a powerful method for analyzing synthetic peptides and proteins, it should be recognized that the technique has its limitations. One important limitation concerns the ability to determine reliable abundances for the reaction side product and modification peptides. When these side products differ from the desired target peptide in their binding properties to the nitrocellulose surface or in their efficiency for desorption and ionization from such surfaces by fission fragment bombardment, then the relative peak heights observed in the mass spectrum will not faithfully reflect the relative abundances of the various components present in the sample. Such may be the case for a termination peptide which is considerably shorter than the desired target peptide or for a peptide which has not been fully deprotected. On the other hand, if the side product differs only relatively slightly from the desired peptide then the mass spectrometric quantitation is expected to be good. An example of this latter case is the single deletion peptide of mellitin discussed above (Fig. 5B).

Limitations exist at high mass where the quality of the mass spectral data may be much reduced, and limitations also exist in those cases where there are a large number of different low abundance side products. The plasma desorption mass spectrum of a synthetic sample of the 140 residue protein interleuken-3 (Fig. 6) provides an extreme illustration of both of these limit-

INTERLEUKEN-3

ALA SER ILE SER GLY ARG ASP THR HIS ARG LEU THR ARG THR LEU ASN CYS SER SER ILE
1 20

VAL LYS GLU ILE ILE GLY LYS LEU PRO GLU PRO GLU LEU LYS THR ASP ASP GLU GLY PRO
40

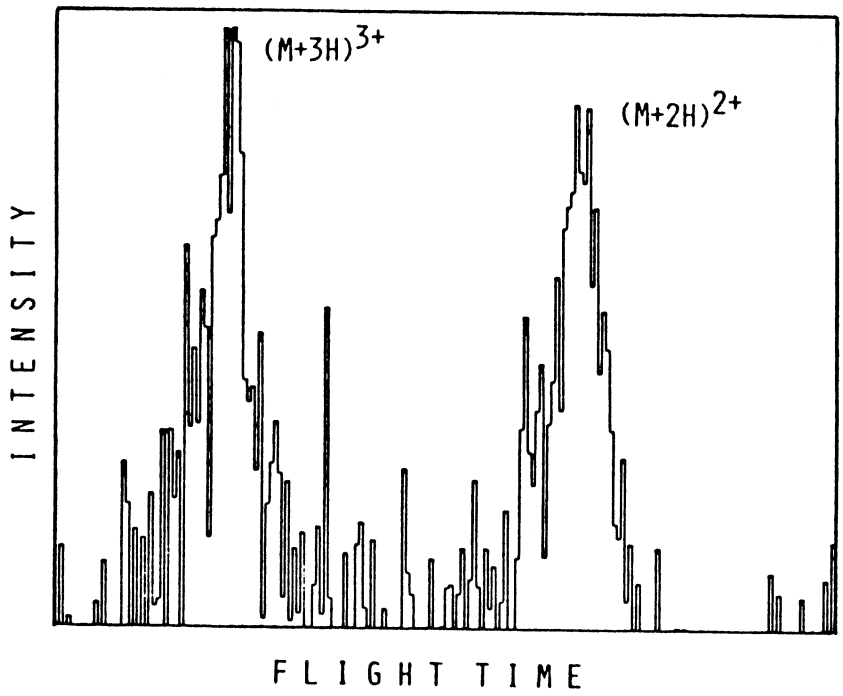
SER LEU ARG ASN LYS SER PHE ARG ARG VAL ASN LEU SER LYS PHE VAL GLU SER GLN GLY
60

GLU VAL ASP PRO GLU ASP ARG TYR VAL ILE LYS SER ASN LEU GLN LYS LEU ASN CYS CYS
80

LEU PRO THR SER ALA ASN ASP SER ALA LEU PRO GLY VAL PHE ILE ARG ASP LEU ASP ASP
100

PHE ARG LYS LYS LEU ARG PHE TYR MET VAL HIS LEU ASN ASP LEU GLU THR VAL LEU THR
120

SER ARG PRO PRO GLN PRO ALA SER GLY SER VAL SER PRO ASN ARG GLY THR VAL GLU CYS
140



ION SPECIES	MEASURED MW	CALCULATED MW	Δ
$(M+2H)^{2+}$	15,592	15,662	-70
$(M+3H)^{3+}$	15,666	15,662	+4

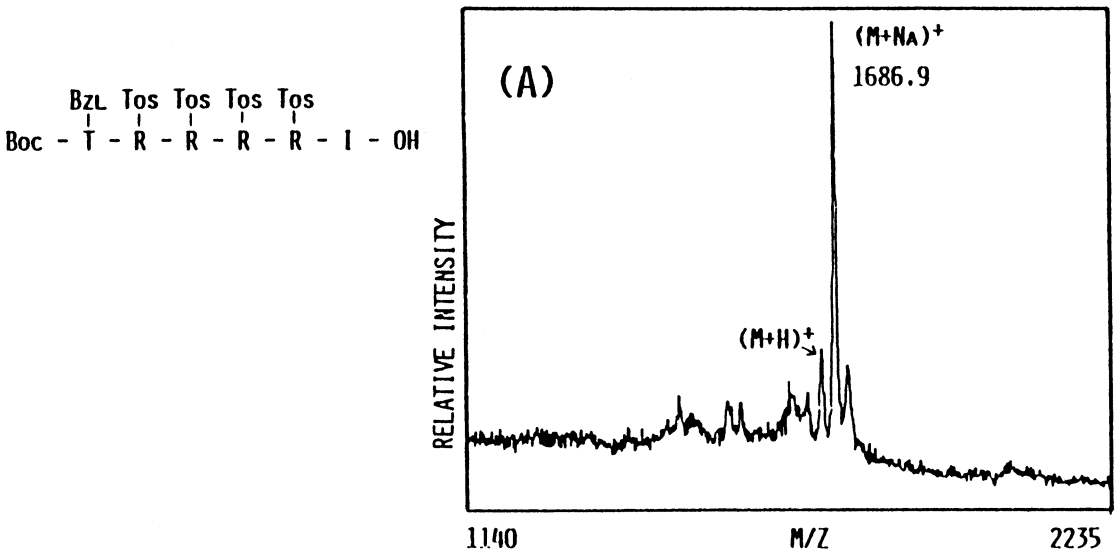
Fig. 6. Partial ^{252}Cf plasma desorption mass spectrum of synthetic interleuken-3 showing the region containing the doubly and triply protonated molecule peaks.

ations. The sample of interleuken-3 was produced by Clark-Lewis, Kent and co-workers [14] using automated stepwise solid phase synthesis and represents perhaps the largest biologically active synthetic protein produced to date. It is apparent from the partial time-of-flight mass spectrum shown in Fig. 6 that it is not possible to extract the kind of detailed information that we are able to obtain from lower molecular weight compounds. The multiply protonated molecule peaks are broad and weak and have mass uncertainties of 50-100 u. Thus while this molecular weight information is much superior to that which can be obtained using SDS gel electrophoresis - and in this respect is really quite valuable - the peak is so broad and poorly defined that we cannot use it to extract information about the impurities, especially the deletion peptides, which are certainly present in this sample.

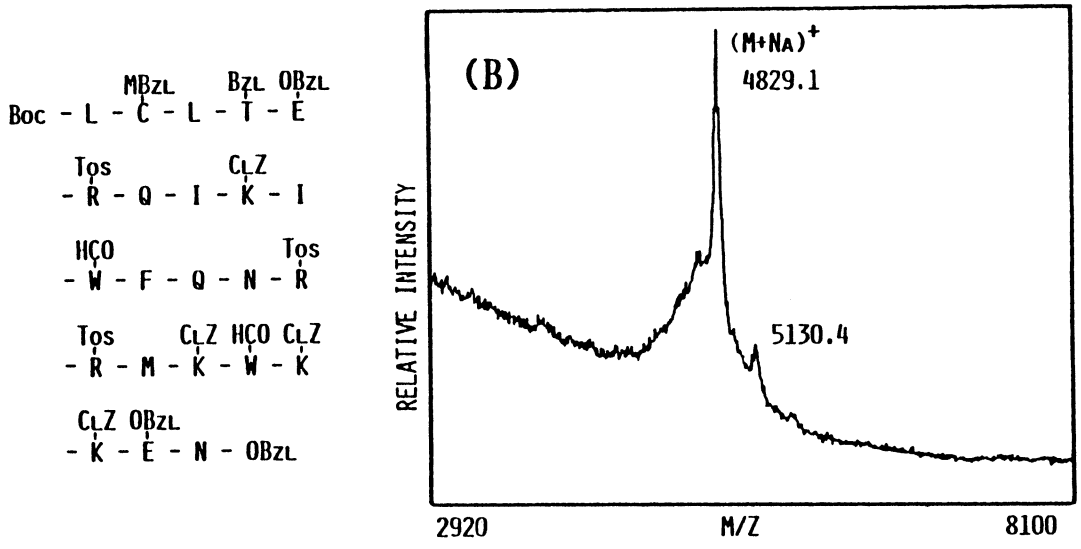
MASS SPECTROMETRY OF FULLY PROTECTED SYNTHETIC PEPTIDES

During the synthesis of a peptide one would ideally like to monitor progress at each stage during chain assembly. Mass spectrometrically this is a fairly large undertaking at present. There are, however, several situations where limited mass spectrometric monitoring during assembly proves to be highly valuable. One such example concerns the synthesis of small proteins by the segment condensation method [15]. In this technique protected peptide segments containing fewer than 10 amino acid residues are produced by stepwise solid phase synthesis in a fairly homogeneous form and are then further purified by various chromatographic methods. These fully protected segments are then successively condensed to form larger and larger fully protected segments with purification of the resulting segment after each condensation step. Finally when the whole protected protein has been assembled the protecting groups are removed. Since this is a fairly lengthy procedure involving many purification steps, it is desirable to ensure that the segments are correctly synthesized and that they have not, for example, lost a protecting group. It is also desirable to check that the homogeneity of these difficult to purify fully protected peptide segments is sufficiently good. Mass spectrometrically these materials are also difficult to handle because they are rather insoluble and tend to aggregate and because they are bristling with protecting groups which have a tendency to fall off upon desorption and ionization in the mass spectrometer. We have found that this tendency to fragment can be minimized by desorbing the protected peptides from nitrocellulose surfaces and by arranging for the molecule to be ionized by a sodium cation [16]. To utilize this technique it is necessary to find solvents which readily dissolve the protected peptides but at the same time do not attack the nitrocellulose adsorption surface. We have found trifluoroethanol, trifluoroacetic acid and highly diluted DMSO to be quite effective.

Fig. 7 shows examples of plasma desorption mass spectra obtained of three fully protected peptide-intermediates produced during the fragment condensation synthesis of the homeo box protein



MEASURED MW = 1686.9 ± 0.2
CALCULATED MW = 1686.9
Δ = 0.0



MEASURED MW = 4806.1 ± 1.5
CALCULATED MW = 4807.3
= - 1.2

Fig. 7. Partial ^{252}Cf plasma desorption mass spectra of protected peptide fragments used in the segment condensation synthesis of the homeo box protein of drosophila [17].
(A) 6-residue protected peptide.
(B) 23-residue protected peptides.
(C) 40-residue protected peptide.

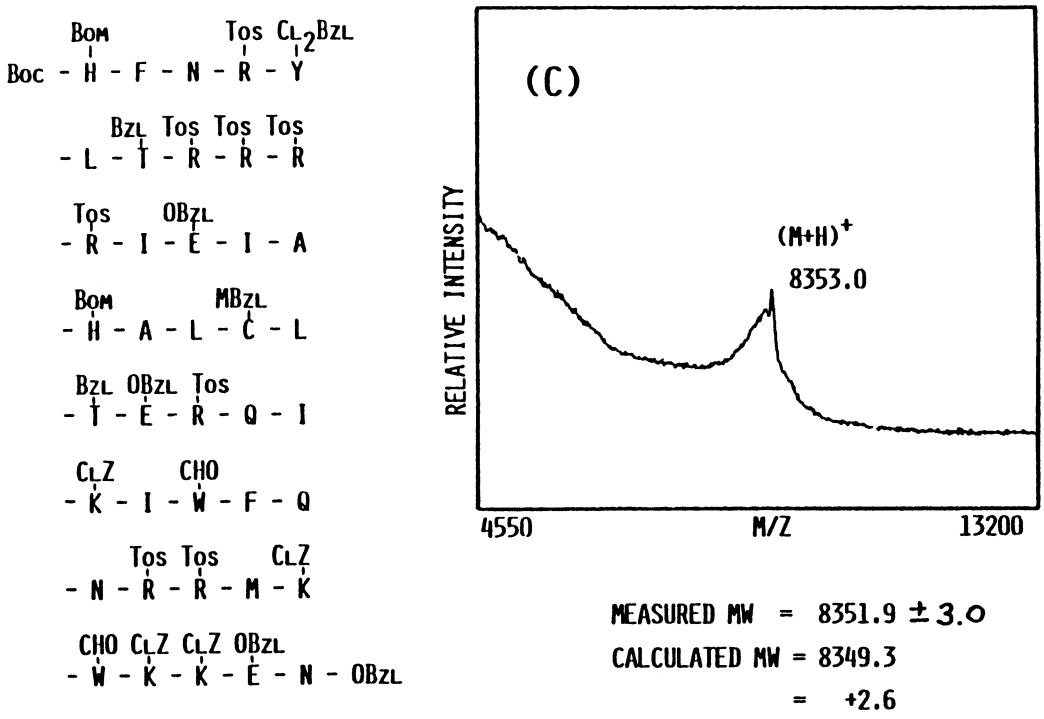


Fig 7C. See caption on previous page.

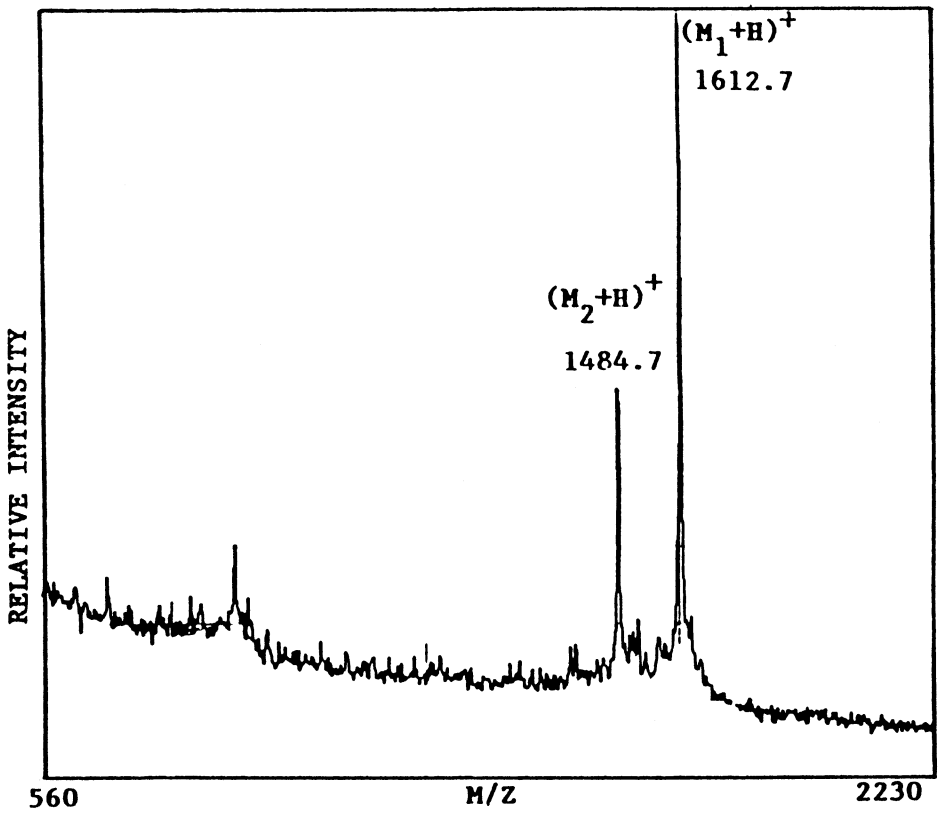
of drosophila [17] carried out by Mihara and Kaiser at Rockefeller University [18]. In each case, the mass-to-charge ratio (m/z) of the dominant spectral peak was found to agree with the calculated m/z value to within the estimated experimental errors (see Fig. 7). It should be noticed (compare Fig. 7C with Fig. 1) that the higher MW protected peptides yield mass spectra that are lower in quality than those obtained from unprotected peptides having equivalent numbers of residues. The lower quality spectra result in somewhat reduced mass determination accuracies for these large protected peptides. However, even with this reduced quality, the data are highly useful for confirming that the condensation reaction proceeded correctly and for checking the degree of homogeneity. These mass spectral data are especially important since no other technique appears to be available for directly analyzing large peptides which are fully protected.

MASS SPECTROMETRY OF UNUSUAL SYNTHETIC PEPTIDES.

We have found plasma desorption mass spectrometry to be very useful for the analysis of unusual synthetic peptide-containing molecules which cannot readily be analyzed by techniques such as Edman sequencing or nuclear magnetic resonance. Examples of such compounds are: peptides modified at the amino terminus as by an acetyl or a myristyl group; a large octally branched

synthetic peptide (MW = 10645 u) containing eight identical 12-residue terminal peptides (a synthetic malaria vaccine); and a MW = 8599 u synthetic enzyme mimic consisting of four 15-residue peptides attached to a central porphyrin core. In all these cases the mass spectrometric determination of the molecular weight proved crucial in verifying the integrity of the synthetic product. Fig. 8 shows the mass spectrum of a 13-residue peptide which has been myristylated at the amino terminus. It is readily apparent from the spectrum that in addition to the desired material designated M_1 (measured MW = 1611.7, calculated MW = 1611.9), a relatively high abundance single deletion side product is present in this preparation. In the absence of this mass spectrometric measurement, it would have been difficult to discover and convincingly demonstrate the presence of this side product.

$CH_3(CH_2)_{12}$-CO-NH-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Asp-Pro-Ser-Gln-Arg



	Measured MW	Calculated MW	Δ
M_1	1611.7	1611.9	-0.2
M_2	1483.7	1611.9	-128.2

Fig. 8. Partial ^{252}Cf plasma desorption mass spectrum of a synthetic amino terminally myristylated peptide.

DISULFIDE BONDS IN SYNTHETIC PEPTIDES AND PROTEINS.

When the target synthetic protein contains internal disulfide bonds, then the initially produced linear peptide chain must be caused to fold correctly and the thiol groups caused to oxidize and form the required linkages. Even though the folding and oxidation is carried out at low concentrations we are frequently asked to determine whether the species produced is monomeric or dimeric, i.e. whether intermolecular disulfide linkages have been inadvertently produced. Sometimes this question is prompted by the observation of atypical migration of the protein during SDS gel electrophoresis. For example, a sample of what was thought to be fully oxidized transforming growth factor-alpha (TGF_{α}) (Fig. 9) prepared by Woo, Clark-Lewis and Kent [19] migrated upon SDS gel electrophoresis with an apparent molecular weight more than three times the expected value of 5446 u. The question of inadvertent intermolecular disulfide formation, which may be difficult to resolve using amino acid or sequence analysis, is readily resolved by simple inspection of the plasma desorption mass spectrum. The mass spectrum of the TGF_{α} preparation (Fig. 9A) shows peaks corresponding to the singly, doubly, and triply protonated monomer. The observation of these peaks together with the absence of peaks corresponding to the singly and triply protonated dimer confirms that the compound is present exclusively as the monomer. It should be noticed that the doubly charged dimer has the same m/z value as the singly charged monomer so that it is not sufficient to inspect only this latter species in order to resolve the question of dimer formation. The close correspondence between the measured and calculated MW's confirms that this preparation is indeed a fully oxidized form of the growth factor. We obtained further confirmation that all three disulfide linkages were intact by carrying out an additional experiment on the sample which gave the spectrum shown in Fig. 9A. In this experiment, the nitrocellulose-bound sample was removed from the mass spectrometer, reduced by applying a solution containing dithiothreitol to the nitrocellulose surface, dried and then reintroduced into the mass spectrometer for re-analysis. Fig. 9B shows the resulting spectrum of the reduced TGF_{α}. The observed mass increase of 5.9 u over the oxidized form corresponds closely with the expected increase of 6.0 u if all three disulfides were intact. We have not yet used PDMS to assist in the assignment of the disulfide pairings in synthetic peptides, but we have used PDMS in conjunction with Edman sequencing to examine enzymatically generated disulfide containing peptide fragments to assist in the assignment of the seven previously unknown S-S pairings in neurophysin, a naturally occurring, 95-residue protein [20,21]. Our success with this difficult case leads me to believe that PDMS used in conjunction with enzyme degradation will be of general utility for the confirmation of the disulfide pairings in synthetic proteins as well.

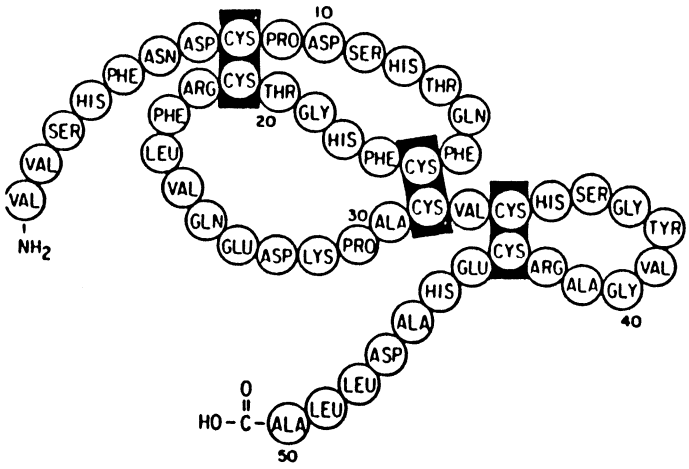
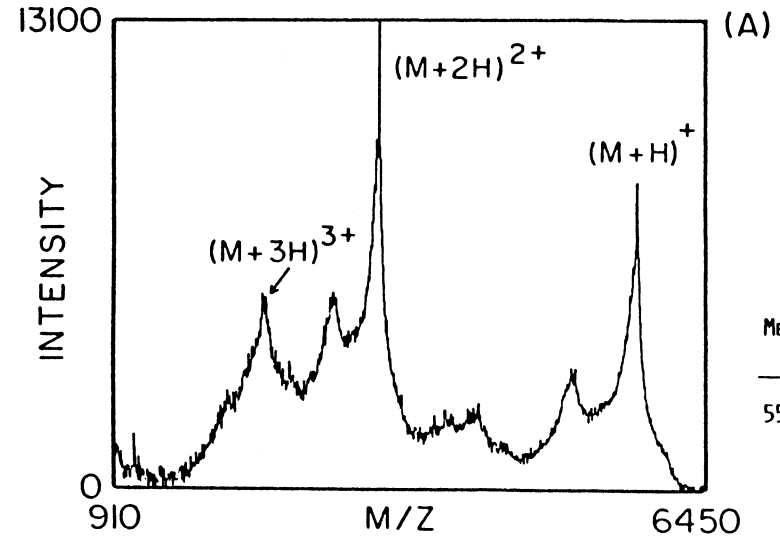


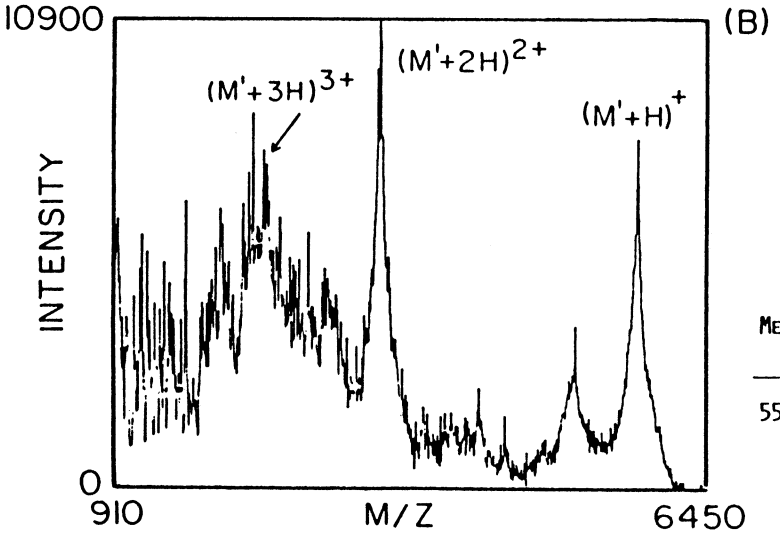
Fig. 9. Mass spectra of synthetic TGF$_\alpha$ taken (A) prior to and (B) after reduction with dithiotreitol.

TGF$_\alpha$: 1×10^{-9} MOLE DEPOSITED ON NITROCELLULOSE



PRIOR TO REDUCTION

MEASURED MW	CALCULATED MW	Δ
5546.2	5546.3	-0.1



AFTER REDUCTION BY DITHIOTHREITOL FOR 9 MIN AT 25°C.

MEASURED MW	CALCULATED MW	Δ
5552.1	5552.3	-0.2

OPTIMIZATION OF PEPTIDE CHEMISTRY

In collaboration with Merrifield and Singer at Rockefeller University [22], we have recently developed a sensitive and precise new technique for identifying and quantitating reaction byproducts produced during peptide synthesis. It was our intention that the rapid, high quality mass spectrometic information be used to fine-tune and optimize the synthetic chemistry in order to obtain the highest possible stepwise yields. In this technique, model oligopeptides containing 10 or 20 alanine residues ($Ala_{10}Val$, $Ala_{20}Val$) were synthesized by automated solid phase methods using a variety of protocols, and the levels of peptide byproducts were measured by PDMS where the total, unfractionated, synthetic product was deposited on a film of nitrocellulose and analyzed. The use of alanine, which lacks a third functionality, essentially eliminated the production of branched chains or modification peptides. Thus the observed byproducts were almost exclusively deletion and insertion peptides. The 10 or 20 alanine residues provided a large amplification factor for observing these deletion and insertion peptides. The introduction of D-alanine at every third residue of the model eliminated peptide conformation problems that led to incomplete reactions in the all L model. Couplings with preformed symmetrical anhydrides in dimethylformamide gave rise to significant levels of both deletion peptides and insertion peptides. The best of the protocols examined was a double coupling of Boc-alanine by in situ activation with dicyclohexylcarbodiimide in dichloromethane. In this case $[D\text{-}Ala^{3,6,9,12,15,18}]Ala_{20}Val$ was synthesized with an average deletion of only 0.036% per step and an average insertion of only 0.029% per step, which is equivalent to a stepwise yield of 99.93% for the target peptide. We are presently extending the technique to the study of model oligopeptides containing up to 50 alanine residues and also to other potentially more problematic amino acids like lysine. It appears that the use of polyamino acid peptides and mass spectrometry will continue to have considerable utility for the improvement of peptide synthesis.

CONCLUSION

We have found through the examination of a large number of synthetic peptides and proteins originating from many different laboratories that plasma desorption mass spectrometry provides an enormously useful, rapid, easy and definitive method for: assessing the correctness of structure, examining the homogeneity of the final product, identifying and determining the origin of unwanted side reaction products, and evaluating and optimizing details of the synthetic peptide chemistry. Our experience and findings have convinced me that the mass spectrometer should take its place in the peptide laboratories alongside the more established analytical tools like the amino acid analyzer and the sequenator.

ACKNOWLEDGEMENTS

This work was supported in part by the Division of Research Resources, NIH. I wish to thank Mrs. Tanuja Chaudhary for skilled assistance in obtaining the mass spectra, and Mrs. Gladys McMilleon for typing this manuscript.

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