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C-Terminal peptide identification by fast atom bombardment mass spectrometry

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A previously described technique [Rose, Simona, Offord, Prior, Otto & Thatcher (1983) Biochem. J. **215**, 273–277] permits the identification of the *C*-terminal peptide of a protein as the only peptide that does not incorporate any ^{18}O upon partial enzymic hydrolysis in ^{18}O-labelled water. Formation of chemical derivatives followed by combined g.l.c.–m.s. was used in this earlier work. We now describe the isolation from protein digests, by reversed-phase h.p.l.c., of labelled and unlabelled polypeptides and their direct analysis by fast atom bombardment mass spectrometry. Under the conditions used, the ^{18}O label is retained throughout the separation and analysis, thus permitting assignments of *C*-terminal peptides to be made. Enzyme-catalysed exchange of label into the terminal carboxy group was found to occur in some cases without hydrolysis of a peptide bond. This effect, which may be exploited to prepare labelled peptides, does not prevent application of the method (two separate digests must then be used). We have applied our method to the analysis of enzymic partial hydrolysates of glucagon, insulin and of several proteins produced by expression of recombinant DNA.

INTRODUCTION

C-Terminal analysis using enzymic methods has been reviewed by Jones (1986), and a procedure using tritium labelling has been reviewed by Spiess (1986). In a previous article (Rose *et al.*, 1983*a*), we described a technique that permits the identification of the *C*-terminal peptide of a protein. The technique involves incorporation of ^{18}O into all α-carboxy groups liberated during enzyme-catalysed partial hydrolysis of the protein, followed by m.s. The *C*-terminal peptide is the only one that does not incorporate any ^{18}O. In this earlier work, the partial hydrolysate was lyophilized, then subjected, under anhydrous conditions, to reactions designed to render the peptide components volatile. Combined g.l.c.–m.s. was used to separate and identify the peptide derivatives.

We have successfully employed the above technique on many occasions (Blumberg *et al.*, 1984; Liang *et al.*, 1985; Wingfield *et al.*, 1986; Schrimsher *et al.*, 1987; Wingfield *et al.*, 1987). Nonetheless, since relatively short peptides must be produced for reasons of volatility, it can sometimes be difficult to separate by g.l.c. the derivatives of the many peptides forming a partial hydrolysate of a protein. It would be advantageous, for some samples, to be able to examine peptides of too great a molecular mass for analysis by g.l.c.–m.s. A technique which has become known as fast atom bombardment mass spectrometry (f.a.b.-m.s.) is well suited to this task (Barber *et al.*, 1981; Williams *et al.*, 1982). When nanomolar amounts of peptide are available, some sequence information may sometimes be obtained, otherwise molecular mass information only is obtained (Williams *et al.*, 1982). By combining enzymic digestion and peptide analysis by f.a.b.-m.s., it is possible to obtain *C*-terminal sequence information (e.g. Morris *et al.*, 1983; Self & Parente, 1983).

We now describe the isolation from protein digests, by reversed-phase h.p.l.c., of labelled and unlabelled polypeptides and their direct analysis by f.a.b.-m.s.

MATERIALS AND METHODS

All reagents and solvents were of analytical grade or better unless otherwise stated. Glucagon (crystalline) was from Eli Lilly, Indianapolis, IN, U.S.A., insulin (Monocomponent grade) was from Novo Industri, Bagsvaerd, Copenhagen, Denmark; *Staphylococcus aureus* protease V8, and bovine trypsin (treated with tosylphenylalanylchloromethane) were from Cooper Biomedical; soybean trypsin inhibitor (type I S) was from Sigma, St. Louis, MO, U.S.A., and recombinant-derived proteins were from Biogen S.A.

Insulin was freed from zinc as previously described (Rose *et al.*, 1983*b*). Digestion of insulin with V8 protease, in labelled and unlabelled water, was performed as follows. To 1.2 mg of zinc-free insulin dissolved in 80 μl of 2% (w/v) ammonium bicarbonate, was added 120 μl of 95 atom % $H_2{}^{18}O$ (120 μl unlabelled water in the control digestion), followed by 24 μl of V8 (1 mg/ml in unlabelled water). After 4 h at 37 °C, portions were dried overnight in a vacuum centrifuge (SpeedVac; Savant Instruments Farmingdale, NY, U.S.A.). These portions were taken up in either 50 mM-ammonium bicarbonate (pH 8.1), 50 mM-ammonium acetate (pH 6.75), 0.1% trifluoroacetic acid (pH approx. 2), or 50 mM-ammonium acetate brought to pH 5 with pure acetic acid. They were then examined by f.a.b.-m.s. after incubation at 22 °C for various times.

Abbreviation used: f.a.b.-m.s., fast atom bombardment mass spectrometry.

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Tryptic digestion was performed by adding, per mg of protein (glucagon, or reduced and carboxymethylated recombinant-derived protein), 30 μl of unlabelled water, 10 μl of 1 M-ammonium bicarbonate solution in unlabelled water, 50 μl of 95 atom % $H_2{}^{18}O$ (Isotech, Centerville, OH, U.S.A.) and 10 μl of trypsin (1 mg/ml in unlabelled water). After incubation at 37 °C for 3.5 h, digestion was arrested by adding 10 μl of trypsin inhibitor (1 mg/ml in unlabelled water; the batch of inhibitor used was stated to inhibit 1.8 times its weight of trypsin).

H.p.l.c. was performed with a Waters system (Wisp 710B injector, M6000A and M45 pumps, model 680 gradient programmer, model 440 absorbance detector, extended wavelength module at 214 nm). The column was a μBondapak C_{18} radial-compression cartridge in a Z-module (Waters Chromatography Division, Milford, MA, U.S.A.) operated at a flow rate of 1 ml/min. Two solvent systems were used. System 1: solvent A was 0.1% trifluoroacetic acid (h.p.l.c. grade, Pierce, Rockford, IL 61105, U.S.A.) in h.p.l.c.-grade water (MilliQ system); solvent B was acetonitrile (h.p.l.c. grade, Merck, Darmstadt, West Germany). System 2: solvent A was 50 mM-ammonium acetate in h.p.l.c.-grade water; solvent B was 50 mM-ammonium acetate in a mixture (1:1, v/v) of h.p.l.c.-grade acetonitrile and water. The pH of solvent A of system 2 was 6.75 (measured at the glass electrode) and was not adjusted. Digests injected with solvent system 1 were acidified with glacial acetic acid just before injection. Digests injected with solvent system 2 were injected as solutions in ammonium bicarbonate. Any fine suspension present in the sample was removed before injection by passage through an HV4 membrane (Millipore A.G., CH-8302 Kloten, Switzerland). The column was equilibrated with solvent A, the sample injected, and, after 5 min, at 100% solvent A, a linear gradient (generally 1%/min for 100 min) of solvent B was applied until 100% B (corresponding to 50% acetonitrile for solvent system 2) was reached. After 5 min at 100% B, the column was re-equilibrated with 100% A. The effluent was monitored at 214 nm, having backed-off the strong absorbance due to trifluoroacetate or acetate, and fractions absorbing at this wavelength were collected manually. When the trifluoroacetic acid system was used, evaporation of solvent in the vacuum centrifuge was achieved in one step. When the ammonium acetate system was used, some of the ammonium acetate remained after a first evaporation in the vacuum centrifuge, and such samples were redissolved in 10 mM-ammonium bicarbonate and lyophilized in the vacuum centrifuge before analysis by m.s.

F.a.b.-m.s. was performed as previously described (Savoy *et al.*, 1987). For analyses in positive-ion mode, the stainless steel target was loaded with 1 μl of a mixture of glycerol and 1-thioglycerol (2:1, v/v) followed in some experiments by 0.5 μl of acetic acid. Sample (1 μl) was then applied to the loaded target, either directly or after being dried and taken up in 0.1% trifluoroacetic acid.

A simple computer program was written to generate the exact masses of all possible fragments of glucagon up to the mass of the intact polypeptide. The resulting table was interrogated by computer to provide all possible sequences corresponding to a given mass.

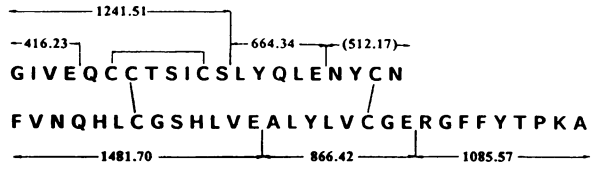

Fig. 1. Scheme showing relative molecular masses of insulin fragments produced by action of V8

The sequence of porcine insulin (A and B chains) is shown in the one-letter code. Fragments produced by cleavage with *Staphylococcus aureus* protease V8 are indicated, together with their relative molecular masses as uncharged molecules. The fragment containing residues A1–12 is only visible in negative-ion mode. Cys residues are calculated as the reduced form (–SH), except for residues A18–A21, which are found still linked by a disulphide bond to B14–21 (neutral mass 1376.59). To calculate the mass of positively charged fragments, the mass of a proton (1 Da) was added to the neutral mass values shown in the Figure.

RESULTS AND DISCUSSION

Preliminary experiments (insulin)

Digestion of insulin with *Staphylococcus aureus* protease V8 produces fragments of a convenient size for analysis by f.a.b.-m.s. (Fig. 1). It is interesting to note that extensive cleavage occurs between residues A12 and A13; preparations of V8 protease have been found previously to cleave *C*-terminally to Ser residues (Evenberg *et al.*, 1977).

Examination by f.a.b.-m.s. of digests performed in ^{18}O-labelled water showed that the *C*-terminal peptide of the B-chain, responsible for the signal at *m/z* 1086, had, as expected, not incorporated ^{18}O label (spectrum not shown: see below for post-h.p.l.c. molecular ion regions). Following the arguments developed in our previous paper (Rose *et al.*, 1983*a*), this peptide must be *C*-terminal. We were surprised to find evidence of the incorporation into most fragments of more than a single O atom from the digestion medium (about 50 atom % ^{18}O). Incubation of the labelled digest at pH 8.1, 6.75, 5 and approx. 2 (see the Materials and methods section), at 22 °C for 2 h, showed effectively complete loss of label from A13–17 at all pH values, whereas loss of label from A1–4 was pH dependent (Fig. 2). Loss was not noticeable at pH 2, was slight at pH 8.1, was more noticeable at pH 6.75, and was extensive at pH 5.

Excessive incorporation and loss of label was unexpected, since we had found no evidence of such phenomena in our original study with trysin and chymotrypsin (Rose *et al.*, 1983*a*). We shall see below that the phenomenon can and does occur with trypsin also.

To determine whether the excessive incorporation and its subsequent loss was catalysed by the enzyme still present in the digests, we separated the component peptides of a control (unlabelled) digest by h.p.l.c. using solvent system 1 (trifluoroacetic acid). Peptides A1–4 and A13–17 were incubated at 22 °C (pH 8.1 and 6.75) in solutions containing 40 atom % ^{18}O in the presence and absence of V8 protease. Under these conditions, f.a.b.-m.s. showed that neither peptide incorporated label in the absence of enzyme (results not shown), even after 24 h. In the presence of enzyme, after 24 h, there was

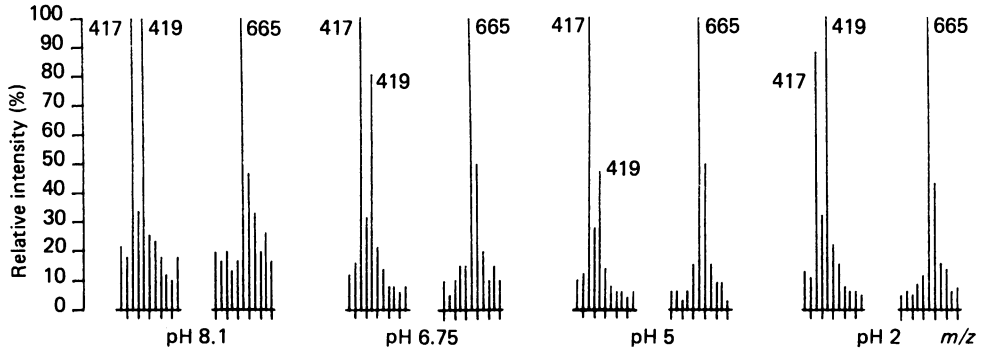

Fig. 2. pH dependence of loss of label from A1–4 and A13–17

See the text for details. Divisions of the mass scale are 2 Da. These spectra were obtained in positive-ion mode. Labelled fragment A13–17 (*m/z* 667 and 669) is replaced by *m/z* 665 (unlabelled form) at all pH values, while labelled fragment A1–4 (*m/z* 419) shows a pH-dependent loss of ^{18}O to give the corresponding unlabelled fragment (*m/z* 417).

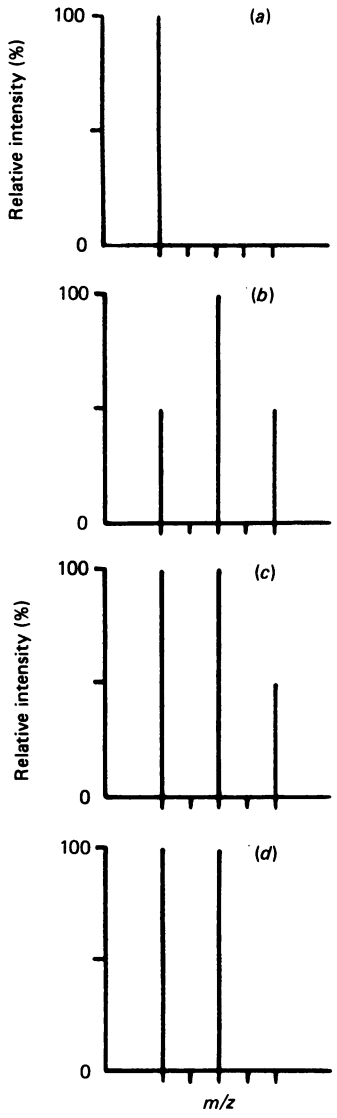

Fig. 3. Theoretical mass spectra

See the text for assumptions made. Divisions of the mass scale are 1 Da. (*a*) No label; (*b*) complete exchange of two atoms with a medium of 50% abundance of heavy isotope; (*c*) partial exchange of two atoms [this spectrum is a mixture of one part spectrum (*a*) and two parts spectrum (*b*)]; (*d*) complete incorporation of one atom from the medium of 50% abundance of heavy isotope. Partial incorporation of one atom from the medium is not considered since it cannot occur. The difference between partial (and complete) exchange of two atoms, and complete incorporation of one atom, is evident.

noticeable incorporation of label into peptide A1–4 at pH 8.1 and full exchange of both O atoms at pH 6.75. In the presence of enzyme, after 20–25 min, there was noticeable incorporation of label into peptide A13–17 at pH 8.1 and full exchange of both O atoms at pH 6.75. After 120 min at pH 8.1 in the presence of enzyme, incorporation into peptide A13–17 was sufficient to produce a pair of signals of almost equal intensity.

It is clear from the above results that label may be incorporated into peptides not only during cleavage of a polypeptide chain but also during incubation with the enzyme (V8) when it recognizes the *C*-terminal residue of the peptides in question. We shall refer to this process as enzyme-catalysed exchange. No such exchange was observed into peptide B22–30, which has as *C*-terminal residue of Ala, which is not recognized by V8. The rate of enzyme-catalysed exchange is very dependent on the structure of the peptide substrate and on the pH of the medium, the order of increasing rate being pH 5 > 6.75 > 8.1 > 2. It is interesting that the pH optimum for exchange is thus found to be between pH 2 and 6.75, and probably fairly close to pH 5. This pH area includes the p*K* of carboxy groups and of the second pH optimum of proteolytic activity of V8 protease.

We have reported the occurrence of the phenomenon of enzyme-catalysed exchange under very particular conditions: in the presence of high concentrations of butane-1,4-diol, trypsin catalyses the incorporation of ^{18}O into the *C*-terminal (Lys-B29) carboxy group of des-Ala-B30-insulin, before catalysing the coupling of an amino acid ester (Rose *et al.*, 1984). It now appears that enzyme-catalysed exchange into peptides is a more general phenomenon. Experimentally, what distinguishes enzyme-catalysed exchange from enzyme-catalysed incorporation is that both O atoms can be exchanged by the former process whilst only one is incorporated by the latter. While non-incorporation may still be taken as evidence of a peptide being *C*-terminal (provided, of course, that loss of label is avoided), the observation of

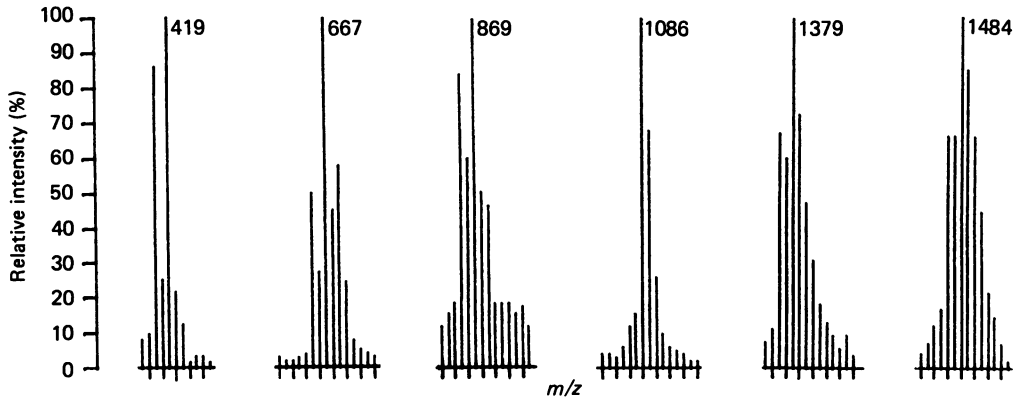

Fig. 4. Molecular ion regions of insulin V8 peptides isolated by h.p.l.c. from a digest performed in labelled water

The divisions of the mas scale are 2 Da. The peptides were produced, isolated and then analysed by f.a.b.-m.s. in positive-ion mode as described in the Materials and methods section. See Fig. 1 for assignments. For plotting purposes, masses have been rounded down when the mass defect is less than 0.75 (i.e. 1086.6 is plotted as 1086).

apparent incorporation (presence of doublets or triplets of signals with a spacing of 2 Da) needs more careful measurement and interpretation. In cases where relative intensities can be measured precisely, it is possible to distinguish the exchange (even partial) of two labelled atoms (both labelled at say 50% isotopic abundance) from the incorporation of a single O atom labelled at 50% abundance. Ignoring contributions from ^{13}C, and complications due to relative ionization strengths of various species (in particular of M^+ and $M + H^+$ in the case of positive-ion f.a.b.-m.s.; Fujita *et al.*, 1985), theoretical mass specra expected from these two extreme cases are shown in Fig. 3. It is evident from this Figure that spectra involving complete (Fig. 3*b*) or partial (Fig. 3*c*) exchange of two O atoms with atoms from the medium, differ from a spectrum (Fig. 3*d*) involving complete incorporation of one O atom from the medium. In real mass spectra, however, particularly ones obtained at high gain from peptides of high molecular mass, it is not always easy to distinguish between partial exchange of two O atoms and full incorporation of one. Further complications arise if one considers the possibility of the existence of peptides of molecular masses differing by 2 Da: here again a non-doublet must be due to a *C*-terminal peptide, but a doublet may wrongly be interpreted as being due to a single non-*C*-terminal peptide.

From the above arguments, we see that there is no problem in the interpretation of non-doublets as being due to *C*-terminal peptides, but that multiplets must be treated with caution. In order to reduce the possibility of ambiguity in the assignments of mutliplets, one may perform two separate digestions with enzymes of different specificity and examine both digests by f.a.b.-m.s.

Separation of labelled peptides by h.p.l.c. in solvent system 2 (insulin)

Just as with the g.l.c.–m.s. method described previously (Rose *et al.*, 1983*a*), it is advantageous to separate the peptide components before analysis by m.s. Not only is there less chance of confusion in the interpretation of multiplets, but also, probably for reasons of relative surface activity while on the probe (Clench *et al.*, 1985), the mass spectrometric signals from some peptides do not stand out well when other peptides are present.

Of course, any system selected for the peptide separation step must avoid loss of ^{18}O label, otherwise peptides having lost their label would be wrongly assigned to *C*-terminal positions. Since our preliminary experiments described above showed that there was no exchange over 24 h at 22 °C in 50 mM-ammonium acetate pH 6.75, in the absence of enzyme, we selected this solvent system for h.p.l.c. We rejected ammonium bicarbonate solutions because their relatively high pH makes them unsuitable for extended use with silica-based columns. We rejected the commonly used trifluoroacetic acid system because of its low pH, which increases the possibility of acid-catalysed loss of label: loss of ^{18}O from aspartic acid is reported to have a half-life of 6.5 h at pH 1.5 at 20 °C, but be immeasurably slow (> 3 days) at pH 7.4, even at 37 °C (Murphy & Clay, 1979: note the error in Table 2 of their paper, where the units of

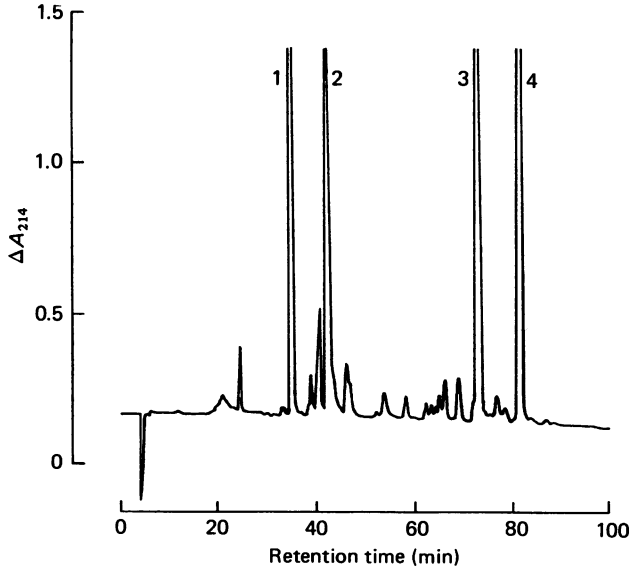

Fig. 5. H.p.l.c. elution profile of a tryptic digest of glucagon performed in labelled water

Conditions of digestion and chromatography are described in the Materials and methods section. Fractions 1–4 were collected for analysis.

reaction half-lives are stated to be days, but, from the rate constants and the text, should clearly be hours). Although much less commonly used than strong acid systems, ammonium acetate has been in use for h.p.l.c.

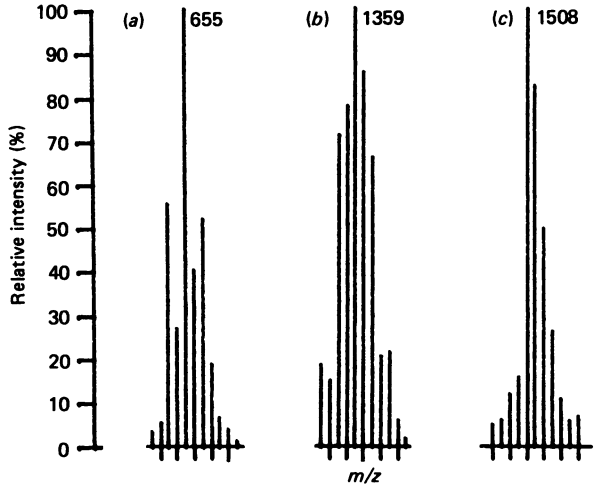

Fig. 6. Molecular ion regions of tryptic peptides of glucagon

Positive-ion mass spectra of fractions isolated according to Fig. 5. Divisions of the mass scale are 2 Da. (*a*) Fraction 1, residues 13–17; (*b*) fraction 2, residues 1–12; (*c*) fraction 4, residues 18–29. Together, these fractions cover the entire sequence of glucagon. Only the *C*-terminal fragment, fraction 4, is not labelled with ^{18}O. As in Fig. 4, for plotting purposes, masses have been rounded down when the mass defect is less than 0.75.

for a number of years. It is less volatile than ammonium bicarbonate, but may be removed by repeated lyophilization without special agents such as P_2O_5 in the trap.

Although we had established that no exchange occurs, in the absence of enzyme, even after 24 h at 22 °C in 50 mM-ammonium acetate at pH 6.75, we wished to test whether or not loss of label occurred upon h.p.l.c. (which involves higher pressures, interaction of the peptide with the stationary phase, and the presence of organic solvent).

The V8 digest of insulin, performed in labelled water, was separated by h.p.l.c. in solvent system 2 and the peptide components analysed by f.a.b.-m.s. Fig. 4 shows the molecular ion regions of the various peptides found. In this Figure, the *C*-terminal fragment of the B-chain is evident (*m/z* 1086; no label, as expected). The *C*-terminal fragment of the A-chain is evident (A18–21 linked via a disulphide bond to B14–21; see Fig. 1). Since the incorporation of label into this disulphide-linked peptide is seen to be associated with B14–21 (*m/z* 867–871), we may conclude that the *C*-terminal peptide of the A-chain is devoid of label, as expected. The non-*C*-terminal fragments, which have incorporated label, did not lose the label during h.p.l.c.

Application of the f.a.b.-m.s.-based method (glucagon)

Having established with insulin the conditions for isolation and analysis of labelled peptides, we chromatographed a tryptic digest of glucagon on solvent system 2 (Fig. 5) and examined the resulting peptides by f.a.b.-m.s. Fig. 6 shows the molecular ion regions of fractions 1 (residues 13–17), 2 (residues 1–12) and 4 (residues 18–29). These three fractions cover the whole sequence of

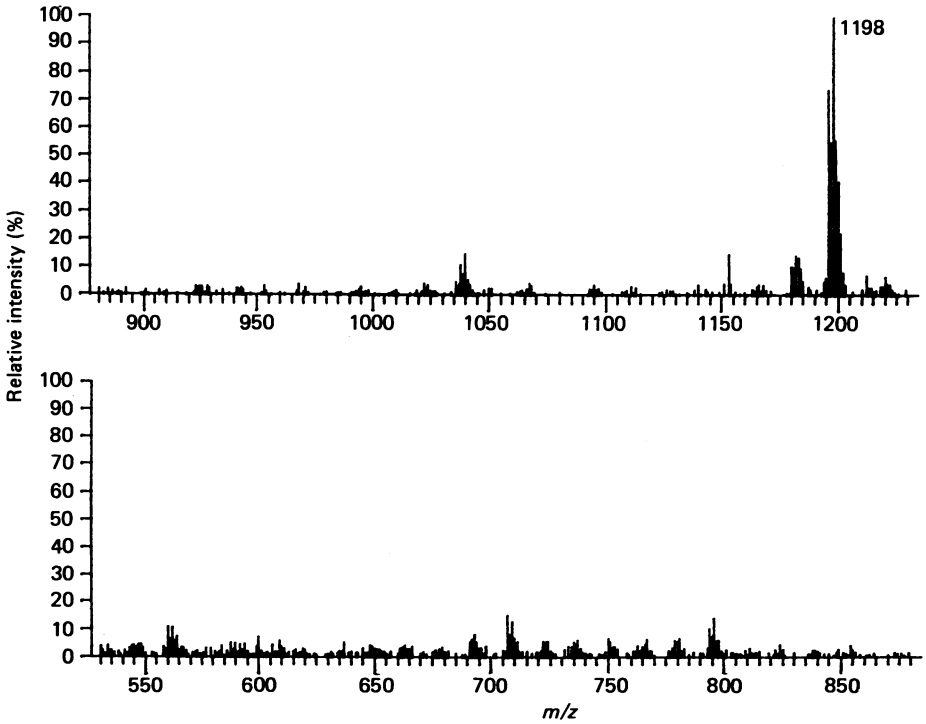

Fig. 7. Positive-ion mass spectrum of *N*-terminal peptide of Il-1-α

An intense protonated molecular ion region around *m/z* 1196–1200 shows that labelling with more than one O atom from the medium has occurred. As in Fig. 4, for plotting purposes, masses have been rounded down when the mass defect is less than 0.75. Sequence ions containing the *C*-terminal region (labelled with ^{18}O) are evident at *m/z* 560–562 (L S N V K), 707–709 (F L S N V K), 794–796 (S F L S N V K) and 1038–1040 (P F S F L S N V K).

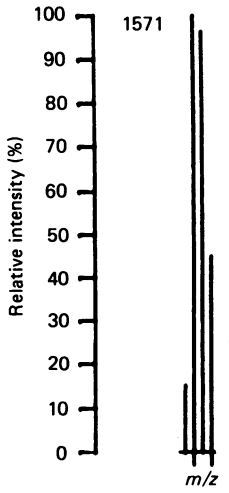

Fig. 8. Positive-ion mass spectrum of the *C*-terminal peptide of IL-2

The molecular ion region of the peptide corresponding to residues 121–133 of the protein is shown. Divisions of the mass scale are 2 Da. For plotting purposes, masses have been rounded down when the mass defect is less than 0.8. Incorporation of ^{18}O has not occurred, so this peptide must be *C*-terminal, in agreement with results of g.l.c.–m.s. analysis.

glucagon. Comparison of the masses found with a tabulation of all glucagon fragments showed no possible ambiguity: all other possible fragments differed in mass from those actually found by at least 5 Da. Fragments 1–12 and 13–17 (m/z 1357 and 653 in positive-ion mode) are seen to have incorporated label to the extent of about two atoms of 50% abundance ^{18}O, whereas the *C*-terminal peptide (m/z 1508 in positive-ion mode) has not incorporated label. Fraction 3, representing residues 19–29 (m/z 1350 in negative-ion mode), is formed by partial tryptic cleavage at Arg-18 and was found by negative-ion f.a.b.-m.s. not to have incorporated label, as expected (results not shown).

Application of the f.a.b.-m.s.-based method (recombinant-derived proteins)

We have separated by h.p.l.c. in solvent system 2, the components of tryptic digests performed in ^{18}O-labelled water of three recombinant-derived proteins: IL-1-α, IL-2 and methionine aminopeptidase. Each peptide component was studied by f.a.b.-m.s. All of the peptides arising from non-*C*-terminal regions were found to be labelled with at least one atom from the digestion medium (which was about 50 atom % ^{18}O), and a majority of these non-*C*-terminal peptides were found to have incorporated more than one O atom from the medium. None of the *C*-terminal peptides were found to have incorporated any label.

By way of example, Figs. 7 and 8 show, respectively, the mass spectra of the *N*-terminal tryptic peptide of IL-1-α and the *C*-terminal tryptic peptide of IL2. Calculation (confirmed by the f.a.b.-m.s. experiment) shows that there are no other tryptic peptides of similar mass expected from the known sequences of recombinant-derived IL-1-α (March *et al.*, 1985; Wingfield *et al.*, 1987) and IL-2 (Liang *et al.*, 1985). The finding (Fig. 8) of a *C*-terminal peptide of mass corresponding to that of residues 121–133 of IL-2, is in agreement with the result of g.l.c.–m.s. analysis (Liang *et al.*, 1985) which determined residues 131–133 to be *C*-terminal.

An advantage of the g.l.c.–m.s. method is that clear sequence information, not just molecular mass information, is obtained (compare Fig. 2*c* of Liang *et al.*, 1985 with Fig. 8). However, the relative intensity of the M^+ region of permethylated peptides is rather low and it is in this region that the labelling manifests itself (or not). Although the M^+ region of permethylated peptides may be enhanced by using chemical ionization, the clear molecular ion region obtained under f.a.b.-m.s. conditions is an advantage. Having used f.a.b.-m.s. to identify an h.p.l.c. fraction as containing a *C*-terminal peptide (i.e. unlabelled), the amino acid composition and sequence of the peptide could be determined by non-mass spectrometric techniques.

Fig. 7 is particularly interesting since we see that the ^{18}O-labelling is retained by certain fragments of this peptide which are produced within the mass spectrometer (so-called 'sequence ions'). Retention of label upon mass spectrometric fragmentation permits a distinction to be made between fragments containing the labelled carboxy group and those which do not: such a distinction can assist peptide identification and sequence assignment in f.a.b.-m.s. experiments.

CONCLUSIONS

We have demonstrated that a combination of h.p.l.c., positive- and negative-ion f.a.b.-m.s., with the use of ^{18}O-labelling, provides a useful means of identifying the *C*-terminal peptide in a digest of a protein. The advantages over a previous method based on g.l.c.–m.s. (Rose *et al.*, 1983*a*) are considerable in that much larger peptides may be analysed and thus enzymes of great specificity (e.g. trypsin, V8) may be generally employed, and the method opens the way to quantitative experiments based on spiking with labelled standards.

The observation that enzyme-catalysed exchange is a fairly general phenomenon, even in fully aqueous solution, may be worth exploiting for the preparation of labelled peptides for purposes such as ^{17}O n.m.r. experiments.

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