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CAPILLARY ELECTROPHORESIS COMBINED WITH ELECTROSPRAY IONIZATION-MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY.

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SUMMARY

The combination of capillary electrophoresis, including capillary zone electrophoresis and capillary isotachophoresis, with electrospray ionization mass spectrometry provides a powerful analytical tool for the analysis of proteins and oligopeptides. The electrospray ionization process provides detection limits in the attomole to femtomole range. The multiple charging phenomena observed in the electrospray ionization mass spectra of large biomolecules also has two important advantageous effects. First, the mass range for analysis with conventional quadrupole mass spectrometers is extended by a factor equal to the extent of charging. This provides mass spectrometric resolution and precision of mass measurement equivalent or superior to mass spectrometric methods dedicated to the measurements at high *m/z*. Secondly, the efficiency of collisionally induced dissociation (for MS/MS) is markedly enhanced. This provides for an important flexibility and advantage in the qualitative investigation of analytes above the range of mass ordinarily accessible for singly charged analyte parent ions. The experimental results are described suggest the possible extension of tandem MS techniques for protein analysis.

INTRODUCTION

Electrospray ionization (ESI) is based upon the electrostatic nebulization of a solution of (typically) charged analyte ions under the influence of a large electrostatic field gradient. Droplets with diameters on the order of 1 μm bearing approximately 10^4 to 10^5 charges (the sign of which is determined by the imposed field) are formed at the rate of ~10^7 s^{-1}. If this process occurs in a flow of dry gas, at near atmospheric pressure, these charged droplets shrink by the evaporation of neutral solvent until the charge density of the droplet rises to a point where the charge repulsion can overcome the cohesive droplet forces. As this Rayleigh stability limit is approached, droplet instabilities can be magnified leading to a "Coulombic explosion". In the most widely accepted model, the smaller droplets continue evaporation and repeat the process until the droplet surface curvature is sufficiently high to permit the field assisted evaporation of charged solutes. This ion evaporation phenomena has been discussed thoroughly by Iribarne and Thomson (refs. 1-2), though others have argued persuasively for the importance of the production of desolvated ions from transient protrusions formed by electrohydrodynamic disintegration of the liquid surface under field stress (ref. 3). In this process one can postulate a jet of highly charged "nanodroplets" emitted from the larger droplet; such nanodroplets could be the direct precursors of ions ultimately detected. Such a model is more consistent with the break-up of larger droplets, which have been shown to emit a large fraction of their charge while expelling very little of their mass (ref. 4). Such a model is also consistent with our growing recognition of the inconsistencies of the field evaporation model. The only way in which the field evaporation rates of a wide variety of analyte ions can compete effectively is if the ions are highly solvated. In such a case the field evaporation of these species may become indistinguishable from the putative nanodroplet expulsion. Regardless of the mechanistic details, however, molecular ions (with an unknown extent of solvation) are produced from liquid solution under mild conditions by these processes which may also involve attachment of proton, alkali cation or ammonium ions (for positive ion formation).

Electrospray ionization was initially described some 20 years ago in the work of Dole and co-workers in attempted studies of the intact ions from synthetic and natural polymers of molecular weight in excess of 100,000 daltons (refs. 5-7). The method of mass analysis was based upon ion mobility, employing the so-called "plasma chromatograph", with detection by Faraday-cage current measurement. These experiments were handicapped by the resolution of this method and the inherent insensitivity of direct current measurement. However, in the present context it is impressive to note that an analysis of lysozyme was tentatively interpreted as arising from a multiple charging phenomena (refs. 8-9).

After a long period of latency these experiments were extended by Fenn and coworkers employing atmospheric pressure sampling of ions from the electrospray plume with a quadrupole mass spectrometer (refs. 10,11). These, and other experiments in progress contemporaneously in the Soviet Union (ref. 12,13), outlined the fundamental aspects of ESI for the charged species in solution and demonstrated its utility for the analysis of biomolecules of modest molecular weight and as a potential interface for the combination of a liquid chromatograph with a mass spectrometer (ref. 14). Potential access to much higher molecular weights was demonstrated by Fenn and coworkers through the multiple charging of large polymer molecules in ESI. For example, a net charge of +23 was observed on a polyethylene glycol oligomer of nominal molecular weight 17,500 daltons (ref. 15).

Subsequent studies, of galvanic significance to mass spectrometrists concerned with biochemical applications, demonstrated the multiple protonation of basic residues in the ESI mass spectra of oligopeptides and proteins. Fenn and coworkers showed the extension in the mass range for the analysis of multiply charged protein molecular ions up to 40,000 dalton analyzable using a quadrupole mass spectrometer of $\underline{m}/\underline{z}$ 1600 limit (refs. 16,17). These results were rapidly confirmed by work in other laboratories (refs. 18,19). The utility of this multiple charging phenomena has been demonstrated to extend to more than 130,000 dalton (ref. 20), employing quadrupole mass analysis of unexceptional mass/charge range, and to permit the measurement of relative mass in the range of 5-40 kDa with precision of better than 0.05% (refs. 19-21).

For mass spectrometry of smaller species bearing a single charge an extra dimension of selectivity and qualitative specificity has been obtained by tandem mass spectrometry. Our initial experiments indicated that the multiply charged molecular ions of oligopeptides and small proteins demonstrated an enhanced susceptibility to dissociation by collisions with a neutral gas (ref. 22). Subsequently, the collisional dissociation of multiply charged oligopeptide molecular ions has been demonstrated to enhance the formation of multiply charged product ions whose identity conforms to the well understood rules for the dissociation of singly charged oligopeptide ions and whose relative abundance is sensitive to the charge state of the parent (ref. 23). Further preliminary experiments have suggested the feasibility of tandem mass spectrometric measurements on small proteins (M_r > 10 kilodalton) to provide qualitatively informative results of potential utility for "fingerprinting" (ref. 24). The significance of these new capabilities for mass spectrometric investigations coupled with the extraordinary power of on-line capillary electrophoresis (CE) is the theme of the following discussion.

EXPERIMENTAL METHODS

The ESI interface developed for quadrupole mass spectrometers has been described in detail (refs. 25-27). In brief, the source consists of a 100 µm i.d. fused silica capillary that extends approximately 0.2 mm from a narrow tubular stainless steel electrode. A liquid sheath, typically methanol or acetonitrile, flowing in the annular space, establishes contact between the flowing analyte solution in the silica capillary and voltage of +4-6 kV (for positive ions) providing the electrospray field gradient. For direct infusion experiments analyte solutions at various concentrations in 1-5% acetic acid solution are delivered by linear displacement microsyringe pumps at 0.5-1 µL/min in conjunction with sheath flows of 1-3 µL/min. A flow of ~3 L/min of ~80°C nitrogen is provided between the ion sampling nozzle of the mass spectrometer and the ESI source to aid desolvation and minimize solvent cluster formation. In CE-MS studies the separation capillary, housed within a specially designed plexiglass isolation chamber, is connected to a second high voltage supply to generate the electrophoretic field gradient (100-500 V/cm). Methionine-human growth hormone was kindly supplied by Dr. I. Jardine of Finnigan Corporation, San Jose, CA. All other reference proteins and peptides were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification.

ESI-MS experiments were largely carried out on a prototype quadrupole mass spectrometer of m/z range 1700. Tandem MS experiments were preformed on a modified TAGA 6000E instrument (Sciex, Thornhill, Ontario, Canada) of m/z 1400 range. Of particular importance in the nozzle-skimmer interface, constructed at our laboratory, was the presence of an adjustable bias voltage on these elements: 0 - ±65 V on the skimmer and voltages of 0 - ±400 V on the nozzle according to the requirements of the experiment. Argon was employed as collision gas with thicknesses of 1×10^{14} cm^2.

ESI PROCESSES

Figure 1 shows the ESI mass spectrum for methionine-human growth hormone (M_r 22,255) and the typical distribution of multiply charged ions. For unknown substances, molecular weights may be accurately calculated based on any two peaks in such a spectrum, assuming that these are from the same substance and related by a

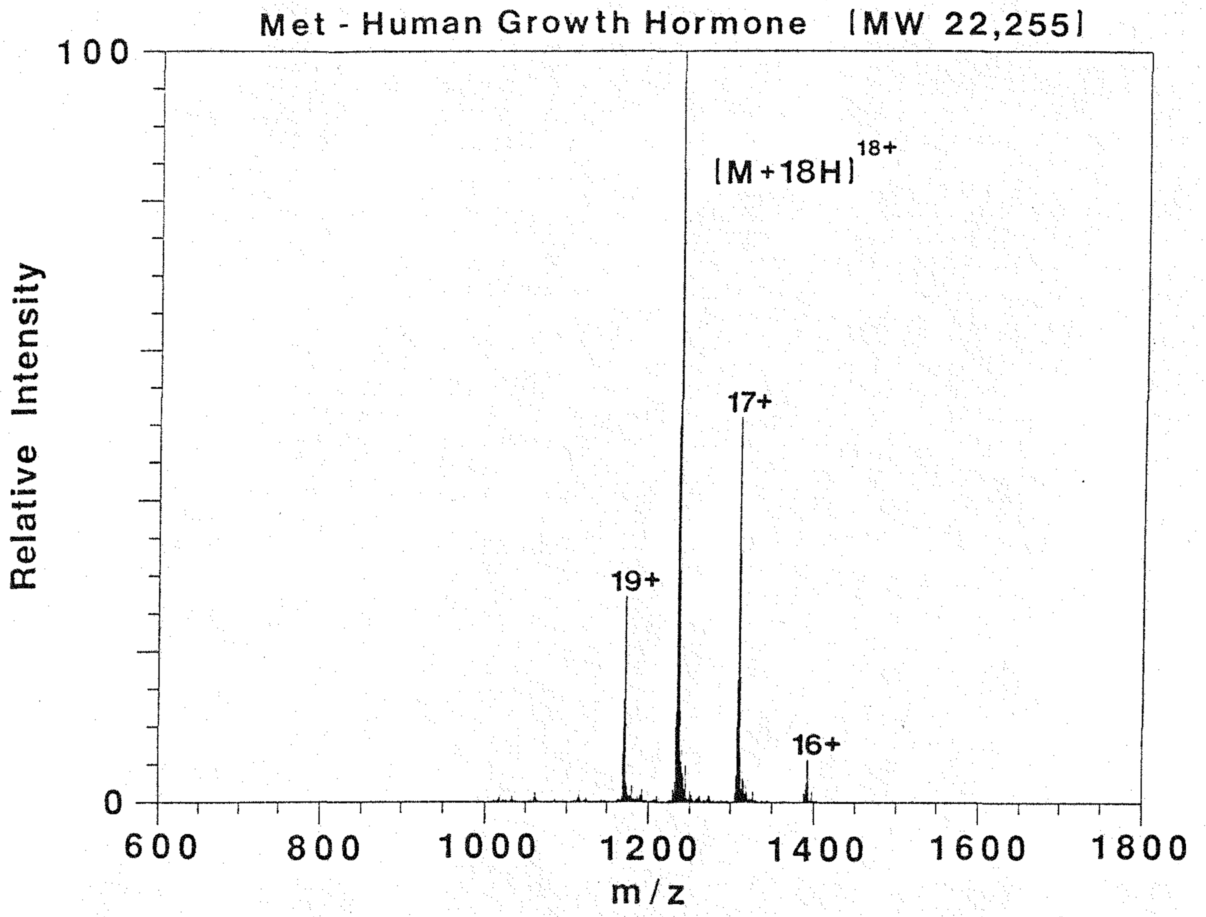

Figure 1. Electrospray ionization mass spectrum of methionine-human growth hormone (M_r 22,255) (ref. 28).

known increment of charge (e.g., due to protonation, or other modes of cationization for positive ions) (refs. 19-21).

In positive ion electrospray for most proteins in the range of a few hundred to approximately 40,000 daltons, a relatively good correlation is observed between the number of sites for protonation (i.e., basic residues plus the N-terminus) and the maximum charging in ESI-MS (ref. 20). However, for proteins containing at least one disulfide bridge we observe a significant decrease in the extent of charging in ESI (ref. 29). This effect is illustrated in Figure 2 for the case of hen egg lysozyme (M_r 14306), a protein containing four intramolecular cystine disulfide linkages and 19 chargeable residues.

The lower panel shows the alteration of the charge state obtained after partial reduction of these intramolecular bonds by treatment with 2.5% aqueous dithiothreitol (DDT). It is our

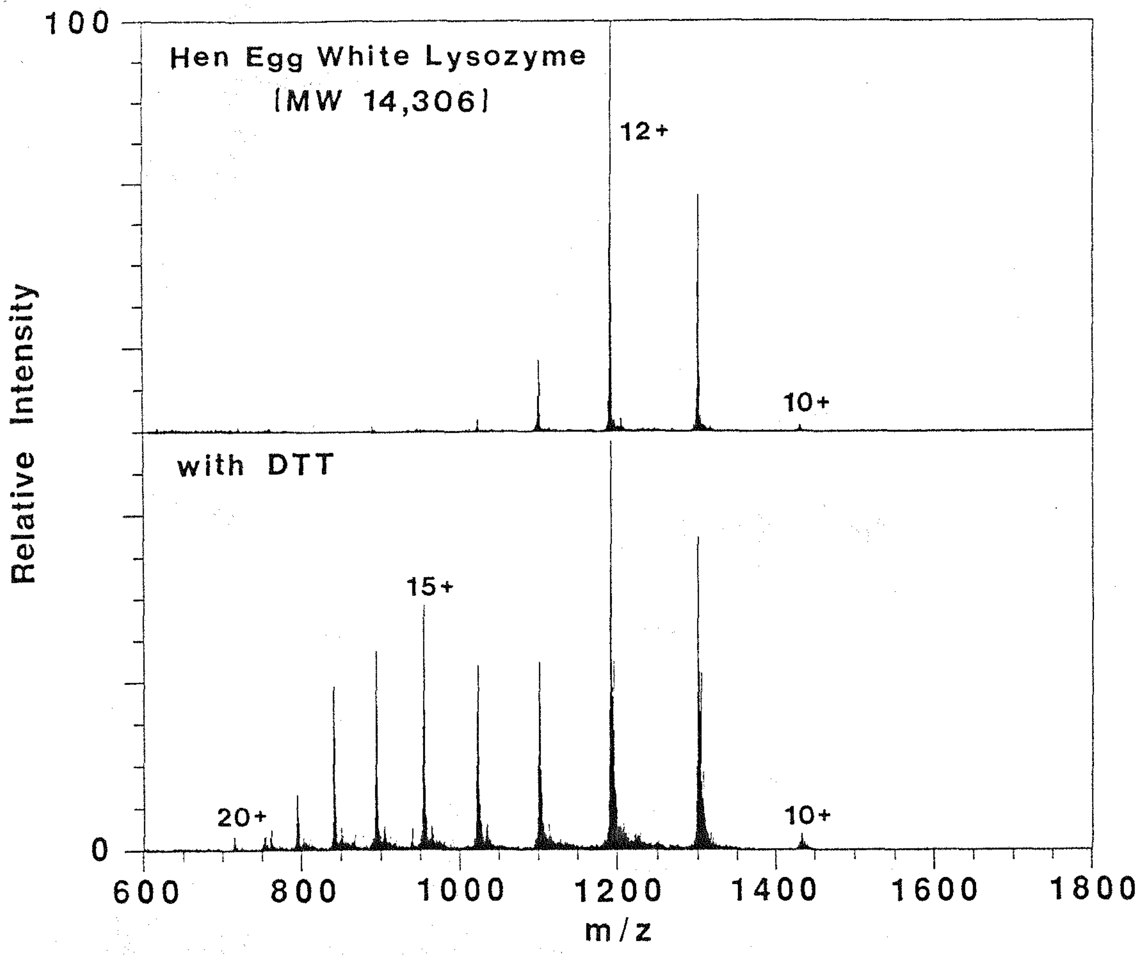

Figure 2 (a) ESI mass spectrum of hen egg lysozyme in 5% aqueous acetic acid and (b) upon addition of DTT (ref. 29).

tentative conclusion that this alteration in charging is the result of a relaxation in the higher order structure of protein in the solution and/or gas phase permitting more facile protonation of the basic sites of the molecule.

Our routine experience suggests that high quality ESI mass spectra are readily obtained with picomole quantities of most proteins. In favorable cases mass spectral data may be obtained on substantially less material. Figure 3 illustrates such a result for the now standard case of horse heart cytochrome c. During this direct infusion experiment of a solution of 200 pg/µL (16 fmoles/µL) of the protein, a total of 23 fmoles was required to record this spectrum. Substantial improvements in limits of detection may be realized by reduction in background chemical noise, which is obvious in this spectrum. Further improvements may also be gained by optimization of the ion sampling arrangements and improved focusing and transmission of the resulting ion beam. In the context of the combination with capillary electrophoresis, the present results are encouraging for techniques which permit the analysis of only dilute solutions (e.g., capillary zone electrophoresis), and challenge our skills in devising methods of sample introduction and separation which permit very efficient utilization of limited sample (e.g., capillary isotachophoresis).

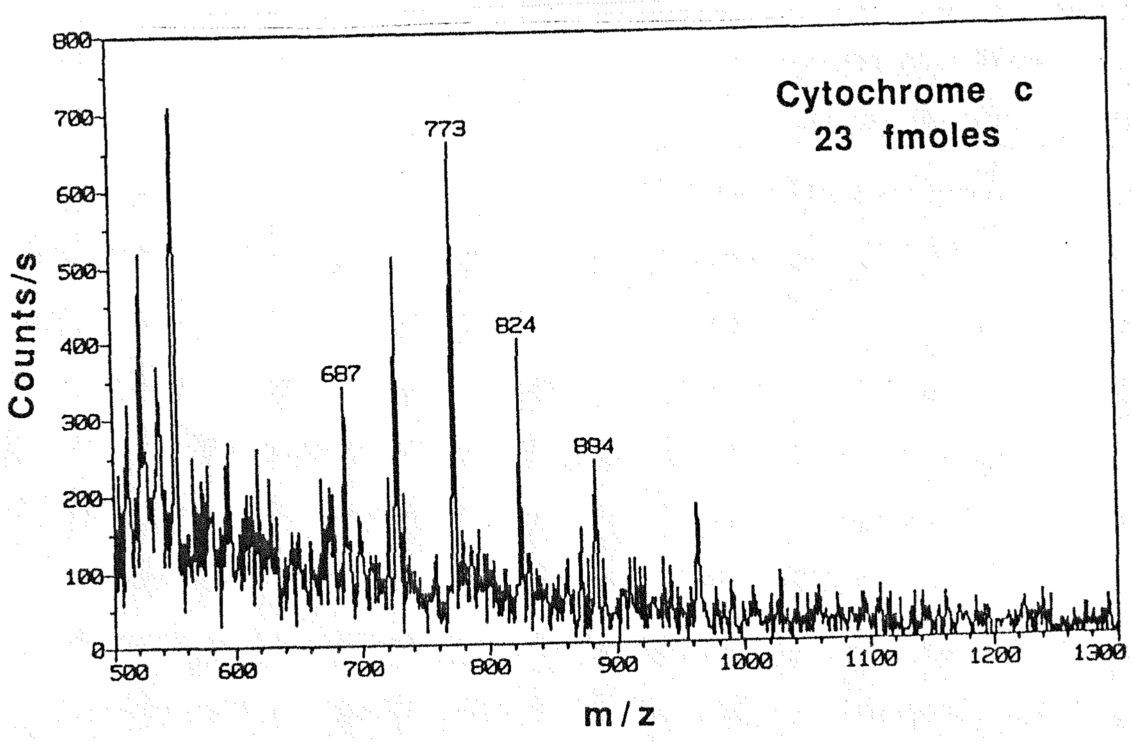

Figure 3 ESI mass spectrum obtained on 23 fmoles of horse heart cytochrome c.

CAPILLARY ZONE ELECTROPHORESIS AND CAPILLARY ISOTACHOPHORESIS COMBINED WITH ESI-MS.

Our developmental efforts have, from the outset, focused on the combination of capillary electrophoresis and ESI-MS. The history of analytical advances in mass spectrometry has highlighted the special importance of the combination of separation methods having high selectivity and resolving power in conjunction with the high sensitivity and specificity of mass spectrometric detection. "Real world" samples are invariably mixtures, often very complex mixtures. Any useful analytical method must accommodate contributions from the sample matrix, interfering substances, etc. The dynamic combination of capillary electrophoresis, a separation method of high efficiency and flexibility, with ESI-MS is thus particularly advantageous. Electrophoresis forms a family of related techniques including polyacrylamide gel electrophoresis, isotachophoresis, isoelectric focusing, gel electrofocusing and two dimensional electrophoresis for very complex mixtures (i.e., extraordinarily high "peak capacity"). These methods are of fundamental importance in biochemical analysis where different techniques are applied according to the type of sample and the information required. Electrophoresis may be conducted in narrow tubes (10 - 250 μm i.d.) with important advantages in efficiency, speed of analysis, providing unique advantages where the manipulation of very small samples is mandatory. A suite of operational modes highly complementary to "conventional" electrophoretic methods are available in the capillary format including capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (CITP).

We have previously described the on-line combination of CZE with ESI-MS (refs. 25,26), the first report of any electrophoretic separation technique in dynamic (i.e., "on-line") combination with mass spectrometry. This development was based upon the recognition that both ends of the CZE capillary did not have to be immersed in buffer reservoirs, as conventionally practiced. The capillary terminus is adapted as an ESI source that accepts the low (from 0-1 μL/min) electroosmotic flow and allows ion production at atmospheric pressure from the electrostatically induced nebulization process.

In earlier versions of the CZE/ESI interface the electrospray dispersion and ionization was accomplished from an electrodeposited metal contact established at the end of the CZE capillary (refs. 25,26). Subsequently, we developed a liquid sheath electrode interface from which the solvent composition and flow rate of the electrosprayed liquid may be controlled independent of the CZE buffer (which is desirable since high percentage aqueous and high ionic strength buffers useful in CZE are not well tolerated by ESI) (ref. 27). The electrical contact is established through the conductive sheath, no additional mixing volumes (< 10 nL) are produced, no significant positive or negative hydrostatic pressure is produced at the outlet of the electrophoretic capillary (i.e., no induced flow) at normal flow rates of the sheath liquid (1-3 μL/min), and analyte contact with metal surfaces is avoided. This interface provides greatly improved performance and is adaptable to other forms of CE. Because CE relies on analyte charge in solution

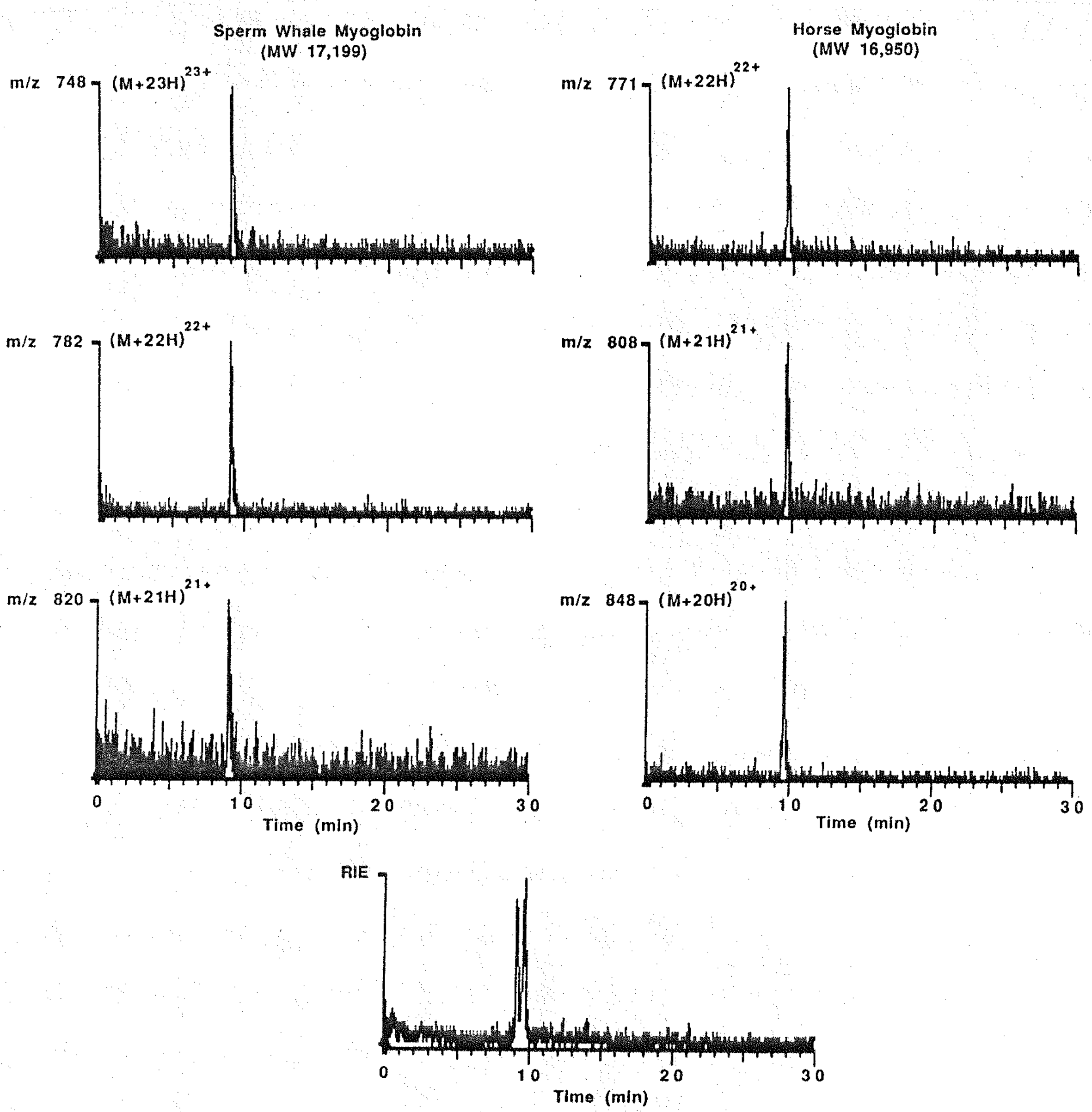

Figure 4. CZE/ESI-MS selected ion monitoring separation of sperm whale and horse myoglobins (ref. 28).

separating medium at the same velocity (ref. 31). This method, though utilizing the same equipment and principles as zone electrophoresis, can accommodate larger samples and result in an increase in the concentration of the material being separated. For anion separations the column is initially filled with a "leading" electrolyte that contains anions with a mobility higher than that of any of the anions in the sample mixture (and, cations having a useful buffering capacity). The solutes are then injected on the head of the column as a second band. The head of the column is placed in a trailing electrolyte solution which contains the anions with an effective mobility lower than that of any anion in the and the ESI process appears to function most effectively for ionic species, the CE/ESI-MS combination is highly complementary. We have reported the analysis by CZE/MS of a mixture of quaternary ammonium compounds (ref. 25) obtaining over 330,000 plates, an order of magnitude better than obtainable by LC in similar time. In this case sample sizes were ~300 femtomoles; however detection limits of $\leq$ 10 attomoles are obtainable using single ion detection. Thus, the CZE/MS approach offers previously unobtainable separation efficiencies (for the combination with mass spectrometry) as well as detection limits that can greatly surpass existing methods.

As initially demonstrated by Lauer and McManigill (ref. 30), high efficiency CZE separations of protein mixtures can be obtained with unmodified fused silica capillaries by using buffer systems at a pH above the isoelectric points of the analytes, thus allowing the peptides and proteins to be negatively charged. This improved efficiency results from decreased absorption on capillary surfaces due to the mutual repulsion of the negatively charged protein and fused silica surface. Unfortunately, the detection of anions in this separation mode is handicapped by reduced production, or outright absence of multiply charged negative ions for peptides and proteins by ESI from high pH solutions. However, one can still produce and detect multiply charged positive ions from peptide and protein samples that are negatively charged in solution (but with somewhat lower efficiencies that at lower pH) by the addition of an aqueous acetic acid solution to the methanol sheath electrode. This charge state inversion is illustrated in Figure 4 for the pH 8.3 separation obtained using a pH 8.25, TRIS-HCl buffer and a 100 cm x 50 um untreated fused silica capillary of sperm whale skeletal muscle myoglobin (M_r 17,199; pI 8.1) and horse heart myoglobin (M_r 16,950; pI 7.4) with detection by ESI-MS in positive ion mode. Ion signal for each analyte is distributed among its several charge states, three of which are monitored in this experiment. In positive ion operation we have noted that ionization efficiencies are decreased at higher buffer concentrations. The dilution provided by the sheath electrode interface does not eliminate such effects. However, differences between the requirements for separation and for optimum electrospray ionization may be minimized.

In isotachophoresis, or displacement electrophoresis, all analytes separate into bands in order of decreasing electrophoretic mobility, and upon complete development, progress through the sample mixture. On application of an electric field the anions in the lead electrolyte will attempt to "pull away from" the solute anions. This results in a gap where the conductivity is dropping (the solute solvent has no significant conductivity) and the electric field rising. Simplistically this increased field will "pull" the analyte anions along until they "catch up" to the anions in the lead electrolyte, with the highest mobility analyte ion arriving first. This "pulling along" effect will continue as each solute band stack in decreasing order of mobility until finally the trailing electrolyte anion is reached. Eventually a steady state will be reached in which each solute is isolated in its own band and all bands are moving at the same velocity.

The steady state concentration of the analyte anion is determined by the lead anion concentration and the relative mobilities of the solute, lead anion and common cation (ref. 32). Accordingly, if the analyte is much more dilute than the lead anion concentration (which is the case in many analytical separations), the analyte will be <u>concentrated</u> as it separates into its own band. In a fully developed separation the concentration of each band is similar and the relative abundance for each component is proportional to the length of the band. Thus, capillary isotachophoresis offers the potential for higher sample loading (and increased molar sensitivity), high resolution separations and actual concentration (in many cases) of separated sample bands.

CITP is an attractive complement to CZE, and is ideally suited for combination with mass spectrometry. We have demonstrated the feasibility of the isotachophoretic separation of quaternary phosphonium and ammonium salts, amino acids, and catacholamines (ref. 33) and reference peptides (ref. 28) with detection by ESI-MS. Detection limits of approximately 10^{-11} M have been demonstrated for quaternary phosphonium salts and substantial improvements appears feasible. Sample sizes that can be addressed by CITP are much greater (>100 fold) than those addressed by CZE. CITP results in concentration of analyte bands, which is in contrast to the inherent dilution obtained in CZE. Samples eluting in CITP are ideally flat-topped bands (the length of the analyte band provides information regarding analyte concentration), well suited to MS as the scan speed of the mass spectrometer need not be challenged by the dynamic nature of "sharp" peaks. Most importantly, however, is that CITP provides a relatively pure analyte band to the ESI source, without the large concentration of a supporting electrolyte demanded by CZE. Thus, CITP/MS has the potential of allowing much greater sensitivities (and analyte ion currents) than may be possible by CZE/MS.

CITP/ESI-MS is also complementary to CE/ESI-MS in many ways. The CZE column is easily overloaded, whereas CITP tolerates very high loading. Thus, larger injection volumes (0.1-1.0 µL) of dilute sample solutions may be analyzed. Electromigration injection allows effective sample volumes to be much larger still due to enrichment during migration into the capillary from low ionic strength samples. CITP is well suited to low concentration samples where the amount of solution is relatively large, whereas CZE is ideal for the analysis of minute quantities of solution.

Figure 5 shows the total ionization current and uv absorbance

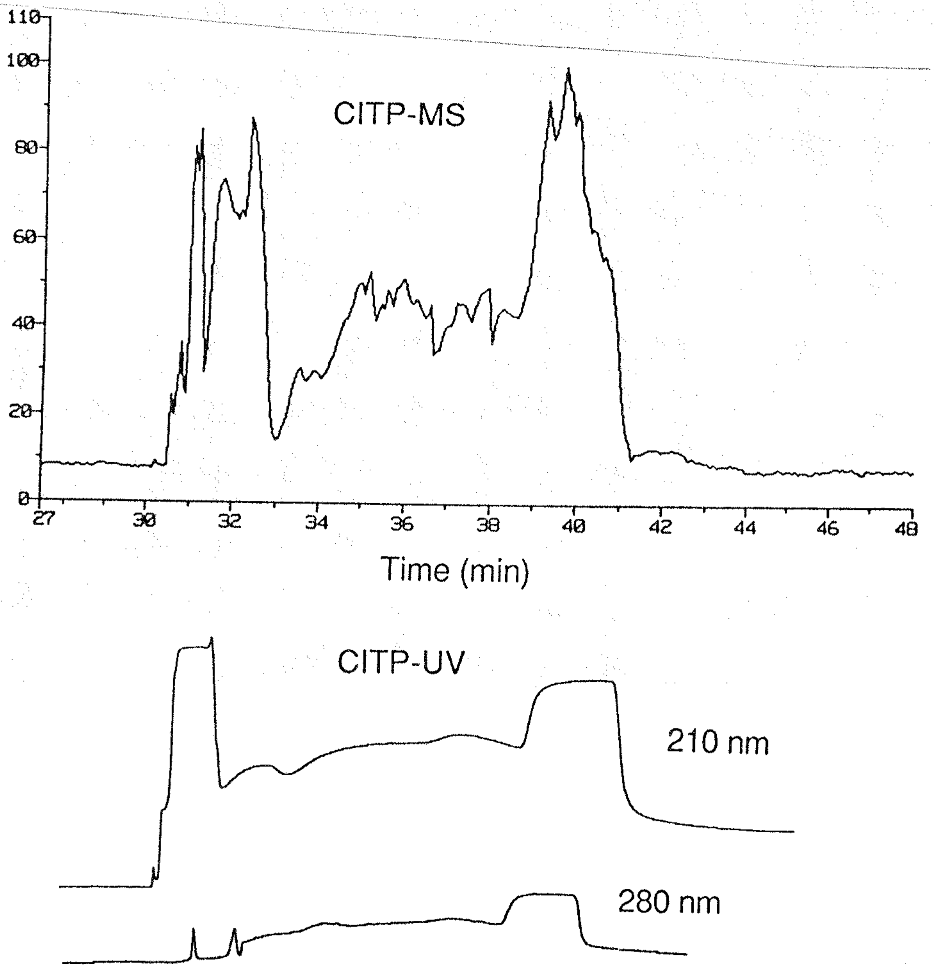

Figure 5. Electrospray total ion current isotachopherogram (upper) and corresponding uv absorbance (210 and 280 nm) traces (lower) for the CITP/MS analysis of a tryptic digest of glucagon. 2 m x 100 micron column, leading buffer 0.01 M NH_4OAc, pH 4.9; sample concentration 0.0001 M, 25% column volume loading; trailing buffer 0.01 M acetic acid, pH 3.3.

(210 and 280 nm) isotachopherograms for the CITP/ESI-MS analysis of a mixture of peptides derived from the digestion with trypsin of glucagon (M_r 3483). The times shown on the axis are marked from the beginning of the separation. Separation development in CITP may be accomplished off-line and, as shown here, the actual time for CITP/MS analysis is determined by the application of a hydrostatic head to the separation capillary with elution of the focused bands (here at 27 minutes). The traces show typical bands and step changes at boundaries between bands. The centers of sample bands show no obvious structure. Strong absorbance at 280 nm indicates the presence of tryptophan in the final band eluting. The TIC follows the general features of the uv traces with better characterized bands at the beginning and the end of the separation with an unexplained structureless region in the middle. Figure 6 further illustrates CITP/ESI-MS for this analysis. Selected ion isotachopherograms are shown for the tryptic products

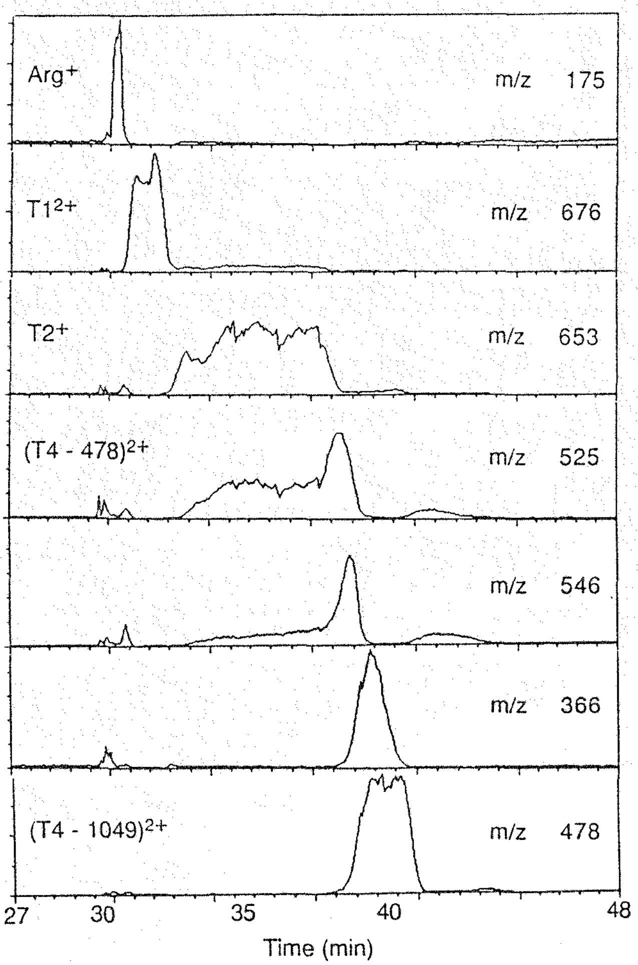

Figure 6. Selected electrospray ion current for the isotachopherograms analysis of an enzymatic digestion with trypsin (and also contaminating chymotrypsin) of glucagon.

arginine (Arg^+, m/z 175), $T1^{2+}$ (m/z 676) and $T2^+$ (m/z 653). Also detected are additional peptide fragments that arise from the action of contaminating chymotrypsin on the predicted tryptic fragments $(T4-478)^{2+}$ and $(T4-1049)^+$ and on intact glucagon (m/z 546 and 366).

The relatively long and stable period of elution of separated bands in CITP facilitates MS/MS experiments requiring longer integration, signal averaging or more concentrated samples than provided by CZE. Such an analysis is illustrated in Figure 7 for $T1^{2+}$ (m/z 653) and $(T4-1049)^+$ (m/z 478). Each spectrum is an average of several scans acquired during the elution of the band show expected B and Y [according to standard nomenclature (ref. 34)] peptide fragment ions consistent with the assignments of the peaks.

TANDEM MASS SPECTROMETRY WITH ESI

The effective collision induced dissociation (CID) of multiply protonated molecular ions of polypeptides and small proteins was first demonstrated in studies by ESI during sampling through the atmospheric pressure/vacuum interface. In this interface a voltage bias between the nozzle and skimmer (Δ(N-S)) was shown to be effective in attenuation of the more highly charged molecular ions (at lower m/z), leading to an increase in signal intensity across the spectrum which was attributed to CID products in various charge states (ref. 22). This effect is illustrated on the small polypeptide, substance P (M_r 1347) shown in Figure 8 at low and high nozzle-skimmer bias voltages. In the upper panel of this figure, at a Δ(N-S) of +150 volts (with skimmer at ground potential), the 3+ protonated molecular ion predominates with a significant 2+ and a very small 1+ molecular ion contributions. Some triply charged fragment ions, are also seen due to losses from the C-terminus. Raising the Δ(N-S) to 250V (lower pane) results in a substantial reduction in the 3+ molecular ion abundance, with a dramatic increase in the fragment ions, singly and doubly charged, arising from most of the possible B and Y cleavage sites in the sequence (according to standard nomenclature of ref. 34, with addition of superscript for charge state).

A significant disadvantage of such an approach arises with the increasing complexity of such spectra with increasing molecular weight and charge state. The multiplicity of charge states of product ions results in mass spectra for which interpretation

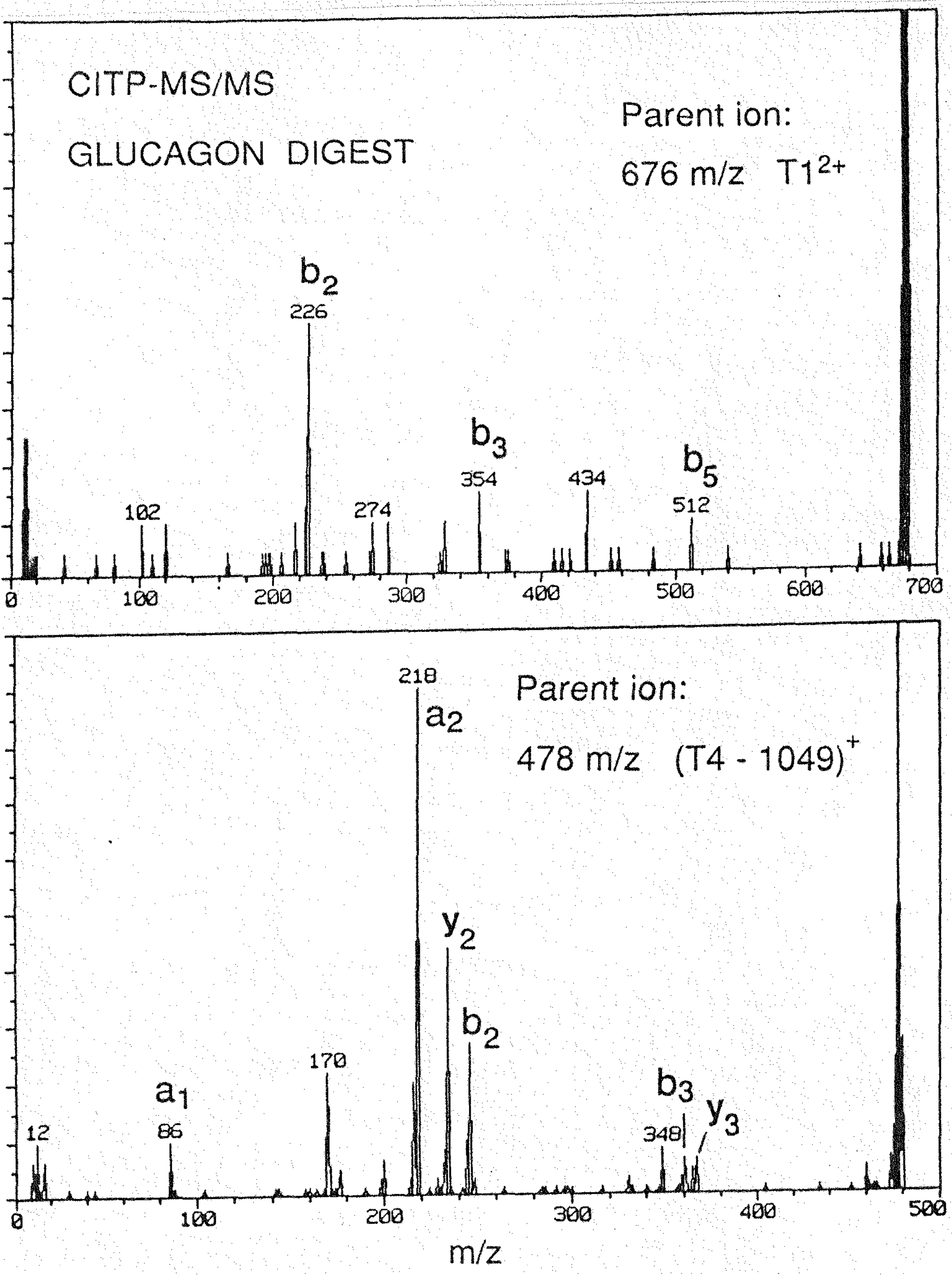

Figure 7. Daughter ion spectra of glucagon T1^{2+} tryptic fragment and the tryptic/chymotryptic artifact, (T4-1049)$^+$ from a CITP separation.

becomes increasingly difficult. In subsequent CID studies by tandem mass spectrometry, product ion spectra of the $\underline{m}/\underline{z}$ separated charge states using a quadrupole mass spectrometer have been obtained of the multiply charged product ions, and interpretable according to the sequence specific cleavage of the polypeptide chain (ref. 23). For the simple example of substance P, product ion spectra for the 2+ and 3+ molecular ions are shown in Figure 9. In this case and for other more complex oligopeptides examined

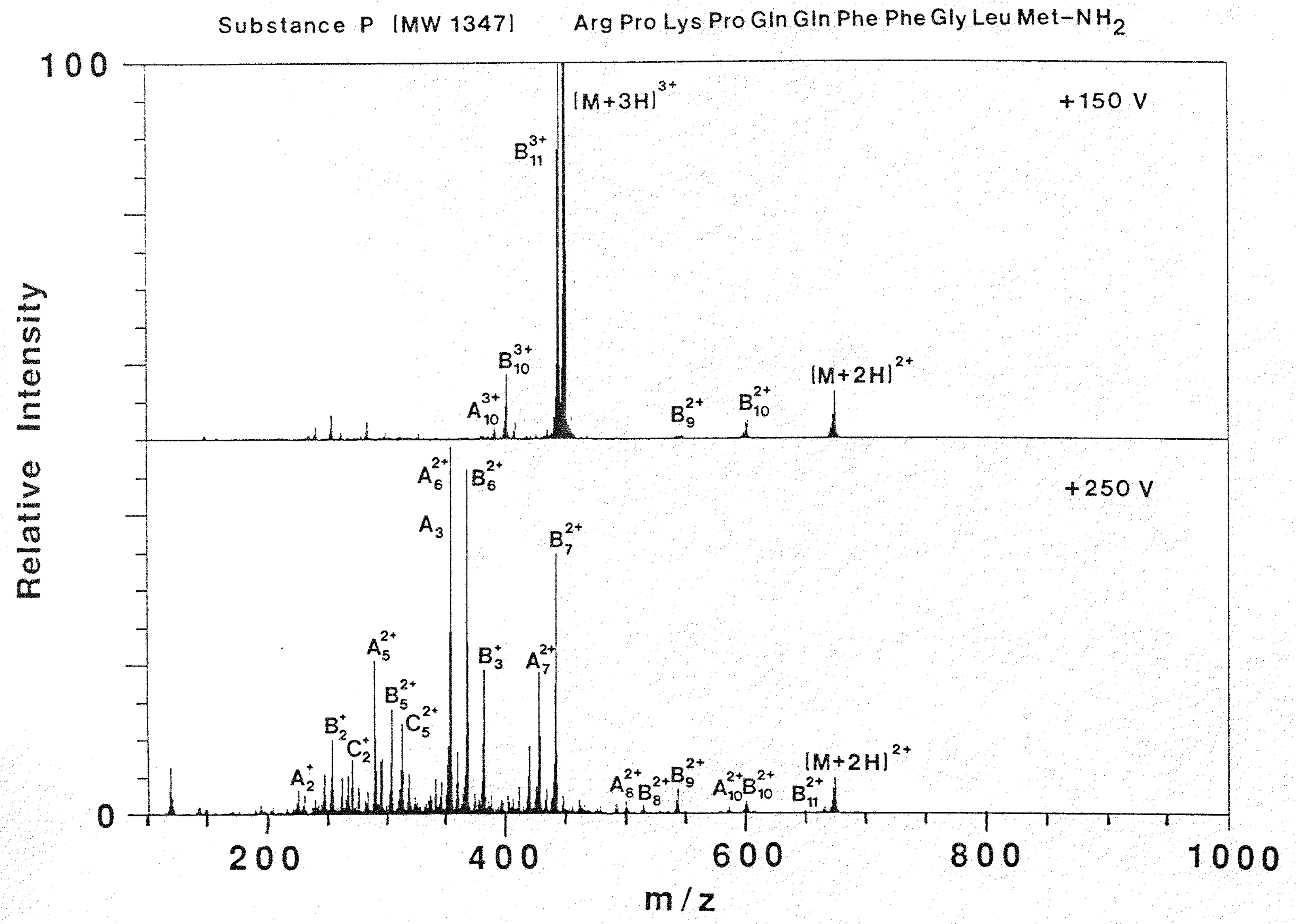

Figure 8. ESI mass spectrum of substance P (M_r 1347) at Δ(N-S) +150V (upper) and +250V (lower) (ref. 28).

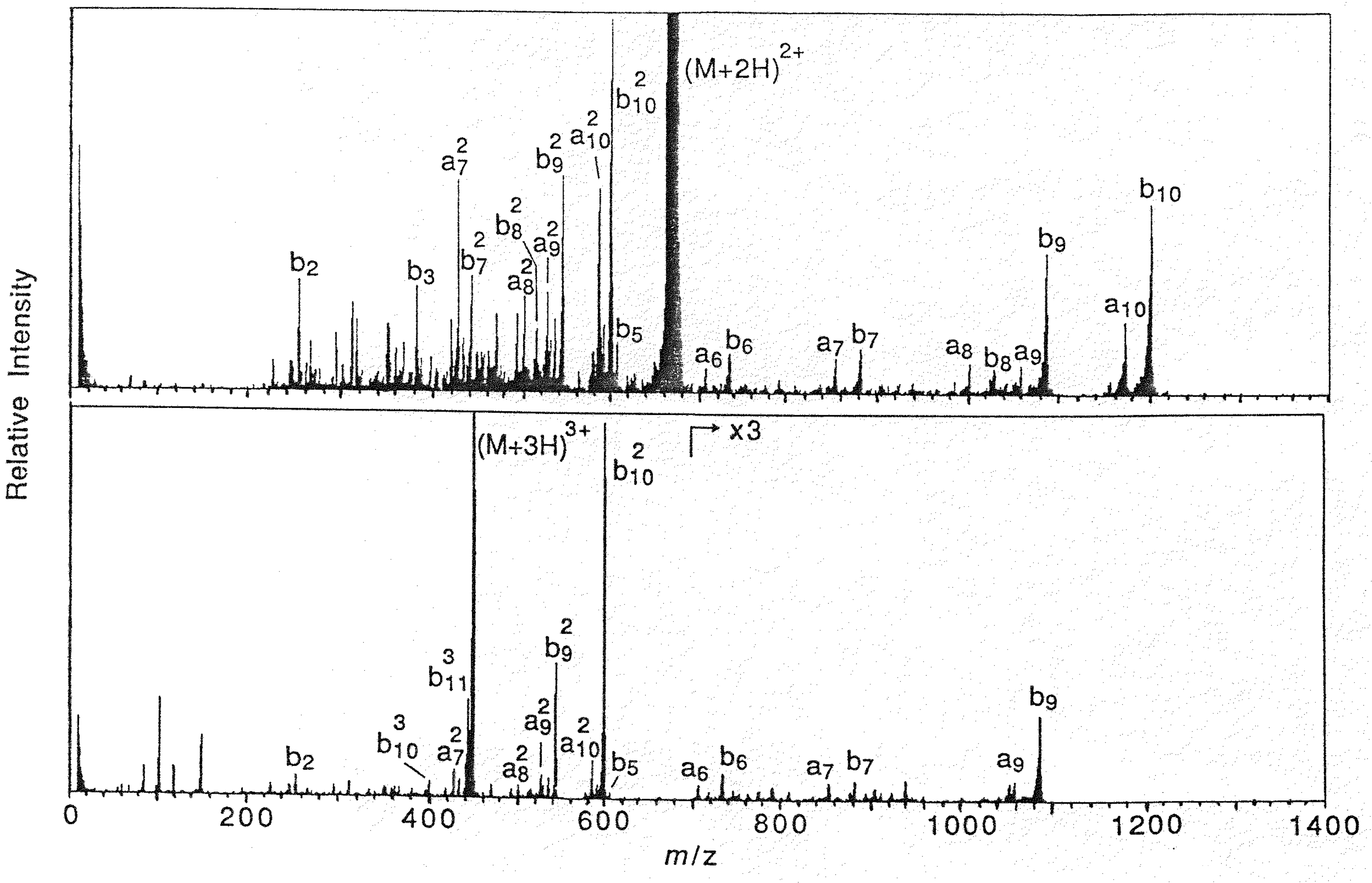

Figure 9. The CID spectra of the 2+ and 3+ protonated molecular ions of substance P.

(refs. 23,35), the various fragment charge states were principally attributed to b and y modes (here designated by the standard nomenclature as modified by Biemann (ref. 36) with superscripts to indicate charge state) of cleavage and showing distinct shifts in the identity and relative abundance of product ions for the parent ions examined. A comparison among such product ion spectra provides sequence data with overlapping and confirmatory assignments.

Studies have demonstrated the special flexibility for collisional activation (CA) by frequent low energy collisions in the atmosphere/vacuum interface, where energy may be controlled by the voltage bias between the nozzle and skimmer (Δ(N-S)) and/or between the skimmer and the initial rf-only focusing quadrupole (Δ(S-Q0) of the tandem mass spectrometer. This collisional heating affords dissociation while minimizing loss of ion current due to scattering (increased Δ(N-S) typically improves ion transmission by enhancing focusing in this region) and is an effective route for the generation of CID product ions in the interface which are available for subsequent interrogation by tandem mass spectrometry (ref. 35). We have examined in detail the CID product ion spectra of oligopeptides of increasing molecular weight ranging from the present simple case of substance P to ubiquitin (M_r 8500). A detailed discussion of these will appear elsewhere (ref. 37). These investigations suggest that the combination of ESI-MS with capillary zone electrophoresis and capillary isotachophoresis will offer significant advantages in the rapid and sensitive determination of primary sequence for oligopeptides beyond the range presently accessible for singly charged molecular ions.

In common with oligopeptides, multiply charged molecular ions of small proteins of M_r greater than 10 kilodalton bearing approximately proportional increases in charge are formed by electrospray ionization and can also be collisionally activated and dissociated with our tandem quadrupole instrument (refs. 22,24). As discussed above, CA (or "heating" for quasithermal excitation) can be used to increase the efficiency of CID processes by elevation of the internal energy of the molecular ion and so minimize the subsequent CID collisional energy input required after mass selection for dissociation in tandem mass spectrometry (refs.35,38). Figure 10 shows an example of such a CA/MS/CID/MS experiment for the 15+ protonated molecular ion of horse heart cytochrome c. In the mass region above m/z 500 and partially surrounding the parent molecule (m/z 825) distinct product ions are observed. Also observed at lower m/z are small fragments putatively arising from fragmentation at the ends of the molecule (and presumably from other CID fragments due to the multiple collision conditions). In the same experimental regime, but in the absence of the gase use for CID, we also observed "metastable" contributions (fragments arising from unimolecular processes), which are greatly augmented by collisional heating in the interface. A comparison of product ion mass spectra among a series of different cytochrome c compounds (independently distinguishable by measurements of M_r, 12,040-12,700) demonstrates similarities and differences of unmistakably qualitative significance (ref. 35). A detailed interpretation of these correlations is not presently possible due to the relatively low resolution available in these quadrupole experiments and the failure of these to distinguish among the multiple charge states possible for these daughter ions. However, the empirical "fingerprinting" of proteins is demonstrated. We postulate that, as the detailed examination of smaller more tractable analytes permits the development of rules for the fragmentation of large multiply charged oligopeptide ions,

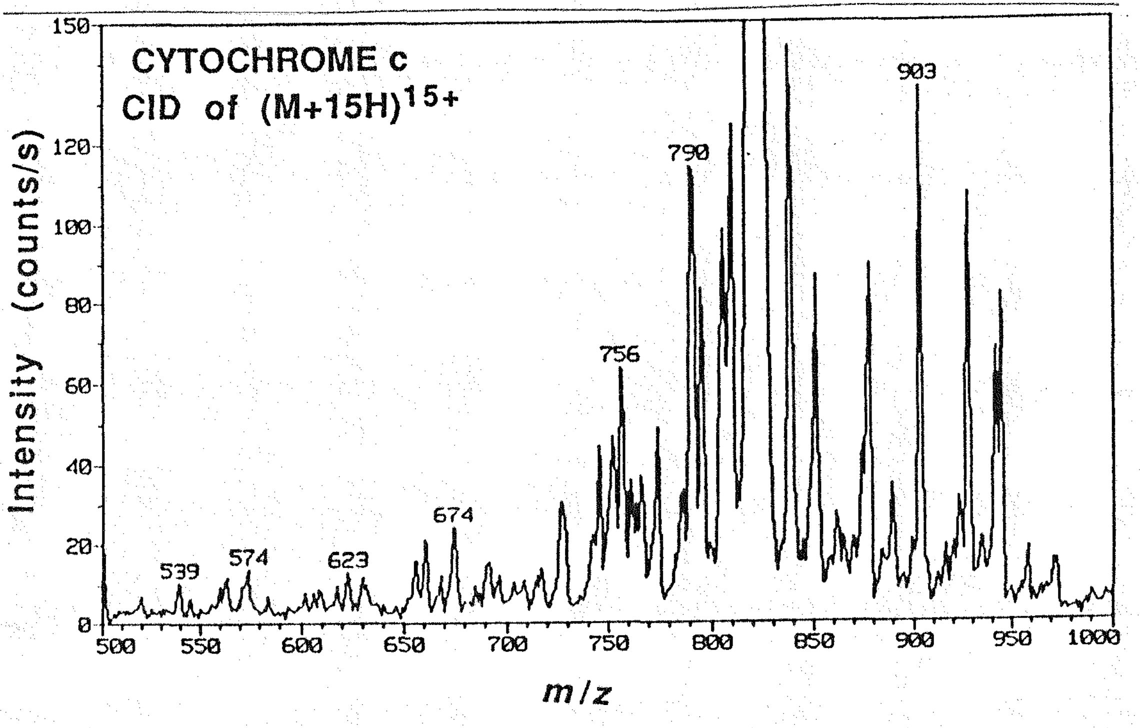

Figure 10. CID mass spectrum of the collisionally activated 15+ protonated molecular ion of horse heart cytochrome c.

multiply charged oligopeptide ions, rapid and sensitive "mapping" experiments might be accomplished along the lines that are presently employed for specific proteolytic cleavage followed by HPLC, fast atom bombardment or plasma desorption mass spectrometry. It requires an imaginative leap to envision, even with dramatic advances in instrumentation and our understanding of the complex ion chemistry, the sequencing of a molecule such as cytochrome c which requires the ordering of more than one hundred amino acids. However, such futuristic possibilities are the stuff of technological and scientific progress.

CONCLUSIONS AND FUTURE PROSPECTS

Mass spectrometry is making increasing contributions to the study of the relation of structure and function in biological systems. The mass spectrometric measurement, the determination of mass via the measurement of the ratio of mass to charge, is qualitatively very important but (except for very small species) insufficient for full characterization given a range of possible charge states. Qualitative investigations of complex systems proceed in combined studies with other techniques, e.g., nuclear magnetic resonance, crystallography, etc. Of these mass spectrometry is frequently the initial experiment by virtue of its relatively high sensitivity. The engine of progress in biochemical mass spectrometry is driven by advances in techniques for ion detection, mass analysis and ion production and sampling. This latter category, where efficiencies are generally poorer than 10^{-3}, also contains the largest opportunity for future advances in ESI-MS, where important advantages for the analysis of labile and involatile molecules exist, as with introduction of FAB and LSIMS methods, developments may be rapid and dramatic. In this dual context, ESI-MS provides exciting prospects for the molecular analysis of biopolymers. Significant, intact and functional pieces of biochemical machinery may be examined, with enhanced facility, by mass spectrometry.

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