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CAPILLARY ZONE ELECTROPHORESIS AND ISOTACHOPHORESIS–MASS SPECTROMETRY OF POLYPEPTIDES AND PROTEINS BASED UPON AN ELECTROSPRAY IONIZATION INTERFACE

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SUMMARY

The special capabilities of the capillary electrophoresis electrospray ionization–mass spectrometer interface for the analysis of peptides and proteins with molecular weights extending to in excess of 100 000 are reviewed. The dynamic combinations of both capillary zone electrophoresis and capillary isotachophoresis with electrospray ionization are illustrated for mixtures of peptides and proteins. Myoglobin and cytochrome *c* detection limits were *ca.* 100 fmol. The potential extension of these methods for determination of the primary structure (sequence) of polypeptides using tandem mass spectrometry is shown to be facilitated by the high charge state of ions produced by the electrospray interface. The relevance of these results for advances in analytical biochemistry are discussed.

INTRODUCTION

The versatility, resolving power, and speed of capillary electrophoresis (CE) is of rapidly growing interest for a wide range of applications, and particularly in those areas where micro-scale methods are desirable. As with other separation methods, "real world" applications generally provide situations where simple detection of a peak is insufficient, and the use of information-rich detection methods (such as Fourier transform infrared spectroscopy or mass spectrometry) is desired to identify components more reliably, to determine contributions to unresolved peaks and to survey complex mixtures. The characterization of many biological mixtures presents such challenges. For example, in the determination of the primary sequence of amino acid residues in polypeptides and proteins, several distinct steps are typically necessary. First, the substance must be obtained in a sufficiently purified state. Second, a chemical or enzymatic digests might be used to break down the substance into mixtures of smaller polypeptides. A third step typically involves separation (*e.g.*, by high-performance liquid chromatography) to obtain sufficient quantities of clean samples for each component of the digest. Finally, the primary sequence of polypeptides is (ideally) determined by automated Edman procedures[1] or alternatively by tandem mass spectrometry (MS–MS)[2–4], providing sufficient information to

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(hopefully) reconstruct the primary sequence of the entire substance. The procedures for such analyses continue to be advanced and refined (for example, reports of very high sensitivity analysis of Edman derivatives[5] and the use of on-line liquid chromatography with MS–MS sequencing of the constituents of enzymatic digests of proteins[6] have recently appeared). However, the cumulative task can require many days of effort and typically nanomole sample sizes (although low picomole amounts may be sufficient for certain steps). Even then, immediate success is not guaranteed.

The development of CE methods provides a basis for the efficient manipulation and separation of subpicomole quantities of polypeptides and proteins. Recent advances in microscale methods, such as the demonstration of the tryptic digestion of low picomole quantities of proteins using the immobilized enzyme in a small-diameter packed reactor column[7], provide a basis for such further developments. The use of capillary zone electrophoresis (CZE) for separation of proteins[8,9], and recent demonstrations of restriction mapping[10] of large deoxyribonucleotides, has propelled potential CE applications into the realm of conventional electrophoresis, while adding the attributes of speed, relatively simple on-line detection, automation, and reduced sample requirements (femtomole to picomole). A literal explosion of ancillary methods for sample manipulation, derivatization, and detection, as well as new methods of obtaining separation selectivity are being reported. Additionally, other CE formats are attracting increased interest, with the aim of exploiting the unique features of capillary isotachophoresis (CITP)[11,12], capillary isoelectric focusing (CIEF)[13,14], capillary electrokinetic chromatography (CEC)[15,16], and, most recently, capillary polyacrylamide gel electrophoresis (CGE)[17]. As a result, there are concomitant and increasing demands upon detector sensitivity and information density.

MS is potentially an ideal detection method for CE. At present, developments in CE–MS interfacing are based either flowing (or dynamic) fast atom bombardment (FAB)[18–20] or electrospray ionization (ESI)[21–25]. While the flowing FAB interface has the attraction of compatibility with conventional mass spectrometers, limitations include the use (and the necessity of methods for) the introduction of FAB matrix substances (*e.g.*, glycerol), the need to minimize any pressure gradient across the capillary (particularly for CZE) while avoiding long transfer lines, and the chemical noise and sensitivity constraints inherent in the ionization method.

The first CZE–MS combination was developed in our laboratory based upon the electrospray ionization interface[21] and has been shown to provide detection limits in the attomole range for certain compounds, while not contributing to loss of separation efficiency (over 600 000 theoretical plates have been obtained)[22]. More recently, we have demonstrated new interfacing methods that have greatly extended the utility of CZE–MS by allowing operation over an essentially unlimited range of flow-rates and buffer compositions[23]. The sheath flow electrode interface has been shown not to degrade CZE separations[23]. These developments have allowed the first on-line CITP–MS combination[26], which provides an attractive compliment to CZE–MS where (among other situations) greater sample sizes are required. Future combinations of CIEF and CGE with MS would also appear to be facilitated by the improved interfacing methods.

In this paper we describe the extension of CZE–MS and CITP–MS to polypeptides and proteins. The application of these methods to proteins constitutes one of the most challenging problems for MS, and our initial success in this area

portends dramatic future developments for biological applications. These developments have been augmented by the recognition and growing exploitation of the unique features of electrospray ionization, which include efficient ionization and the production of multiply charged ions from higher molecular weight compounds[27,28]. While ESI has the disadvantage of not being easily compatible with most existing MS instruments without extensive modification, the advantages of this approach appear significant; one can reasonably predict that appropriate instrumentation will become widely available within a few years. For this reason we also discuss several of the unique features of ESI–MS relevant to protein detection and polypeptide characterization. Finally, we discuss the potential for on-line separation *and sequencing* of polypeptide mixtures obtained from enzymatic digests using CITP–MS–MS (and ultimately CZE–MS–MS). This potential is illustrated by an initial triple quadrupole (*e.g.*, MS–MS) study of melittin (relative molecular mass, M_r = 2845). We also demonstrate methods for increasing the information content of ESI–MS spectra without resorting to the complexity and expense (and often lower sensitivity) of MS–MS instrumentation, an attractive feature for CE–MS combinations.

EXPERIMENTAL

The instrumentation developed at our laboratory has been described elsewhere in detail[21–23]. Fig. 1 shows a schematic of the interface and mass spectrometer. In earlier versions of the CZE–MS interface the electrical contact necessary to establish both the ESI and CZE electric fields was implemented using an electrodeposited metal contact at the end of the CZE capillary[21,22]. The more recent interface design employs a flowing liquid sheath which allows the composition and flow-rate of the electrosprayed liquid to be controlled independent of the CZE buffer (which is desirable since

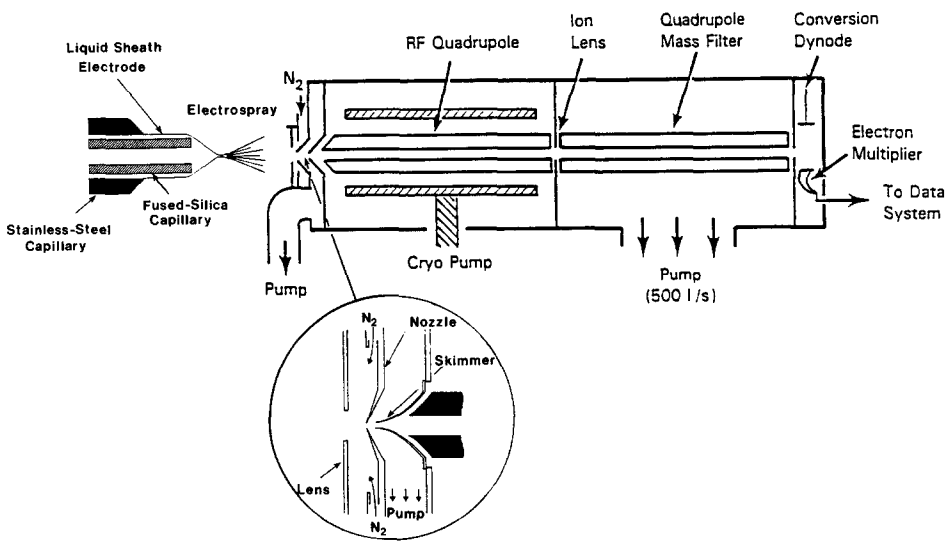

Fig. 1. Schematic of the atmospheric pressure electrospray ionization interface and mass spectrometer. The electrospray capillary and sheath electrode assembly are scaled-up by a factor of approximately 35.

high percentage aqueous and high ionic strength buffers useful for CZE are not generally compatible with ESI)[23]. The electrical contact is also established through the liquid sheath (typically composed of methanol, acetonitrile, acetone or isopropanol, although small fractions of water and acetic acid are generally added for protein analyses). With this arrangement no significant additional mixing volume (< 10 nl) is produced and analyte contact with metal surfaces is avoided. This interface provides greatly improved performance and flexibility and is adaptable to other forms of CE[23]. For direct ESI–MS experiments, syringe pumps control the flow of analyte solution and liquid sheath at ≈ 0.5 μl/min and 3 μl/min, respectively. CZE–ESI–MS experiments were conducted in untreated fused-silica capillaries using methods that have been described previously[21–23].

The electrospray ionization source consists of a 50 or 100 μm I.D. fused-silica capillary (which is generally the CZE capillary) that protrudes 0.2 to 0.4 mm from a cylindrical stainless-steel electrode. High voltage, generally +4 to 6 kV for positive ions or −5 kV for negative ions, is applied to this electrode. The ESI source (capillary) tip is mounted approximately 1.5 cm from the ion sampling nozzle of the ion sampling orifice (nozzle) inlet to the quadrupole mass spectrometer. A 3 to 6 l/min counter-current flow of warm (80°C) nitrogen gas is introduced between the nozzle and ESI source to aid desolvation of the highly charged electrospray droplets and to minimize any solvent cluster formation during expansion into the vacuum chamber. Analyte clustering is precluded by the mutual repulsion of highly charged ions and droplets (which, in contrast to the thermospray ionization, all have the same polarity). A lens placed in front of the sampling nozzle is used to help focus the ions (or electrosprayed droplets) to the sampling orifice. Ions enter through the 1-mm diameter orifice and are focused efficiently into a 2-mm diameter skimmer orifice directly in front of the radio frequency (RF) focusing quadrupole lens (Fig. 1). Typically, +350 V to +1000 V is applied to the focusing lens and +200 V to the nozzle (V_n), while the skimmer is at ground potential. A single-stage roots blower pumps the nozzle–skimmer region to 10^2 to 10^3 Pa. The cryopumped ion focusing region typically reaches pressures on the order to 10^{-4} Pa, while the analysis quadrupole housing is maintained at 10^{-5} Pa with a turbomolecular pump (500 l/s). The analysis quadrupole (Extrel, Pittsburgh, PA, U.S.A.) has an upper m/z limit of 1700.

For CITP–MS both 50 μm I.D. untreated and 100 μm I.D. DB-17 coated (bonded and cross-linked) fused-silica capillary columns were used. For most experiments the inlet of the capillary was biased to the desired potential by an electrode immersed in a buffer solution. The CITP-MS sheath flow-rate and cathode voltage[26] were adjusted to form a stable electrospray at the capillary terminus. The column was loaded with the leading electrolyte and the head of the capillary and the high-voltage electrode (the anode for cationic separations) were placed in the sample reservoir. The sample was loaded into the capillary by electromigration (although both hydrostatic and syringe injection procedures may also be used). When the desired amount of sample was loaded the high voltage is interrupted, the electrode and capillary inlet were then transferred to the trailing electrolyte reservoir, and the voltage was reapplied to begin the separation.

Biochemical samples were purchased from Sigma (St. Louis, MO, U.S.A.) except bovine apotransferrin (Calbiochem, San Diego, CA, U.S.A.) and were used without further purification. Sample solutions were prepared in distilled water with 1 to 5% glacial acetic acid.

RESULTS AND DISCUSSION

ESI–MS of proteins

As originally reported by Fenn and co-workers[27,28], proteins can be effectively ionized by ESI yielding a distribution of charge states. This is illustrated in Fig. 2 for an equimolar mixture of tuna and horse heart cytochrome *c* (M_r 12 029 and 12 360, respectively). Reasonable mass spectra can be obtained with picomole quantities of the protein and the molecular weight of unknown substances can be accurately calculated based upon any two peaks (assuming one knows that the peaks are related)[29,30]. Given a typical distribution, the calculation is straightforward and the accuracy of the determination (generally 0.005 to 0.02%) is enhanced by use of the multiple peaks[29]. Mixtures such as that shown in Fig. 2, or considerably more complex, can generally be deconvoluted to give the molecular weight of each component.

A useful feature of the ESI interface is the ability to "heat" ions to any desired extent by manipulation of the nozzle-skimmer bias[31]. Solvent clustering with the analyte is substantially eliminated by a countercurrent flow of nitrogen and the high potential (generally 100 to 250 V) relative to the skimmer, which leads to collisions in the nozzle–skimmer region that effectively detach weakly bound solvent molecules. As an example, with the nozzle at +200 V, over 40+ charges are clearly resolved in the ESI mass spectrum of bovine carbonic anhydrase (M_r = 29 022), shown in Fig. 3 (top). Decreasing the potential to +100 V yields peaks that are significantly broader and less intense, although the total ion current remains nearly constant; such changes can be largely attributed to incomplete desolvation of molecular ions at the lower voltage.

For smaller proteins a small amount of "tailing" is sometimes observed on the high *m/z* side of each multiply charged molecular ion peak. The "tailing" may be due to some molecules having incomplete desolvation and/or possible cation (*e.g.*, Na^+, K^+, etc.) adduct formation, but spectra for pure substances (*e.g.*, cytochrome *c* in Fig. 2)

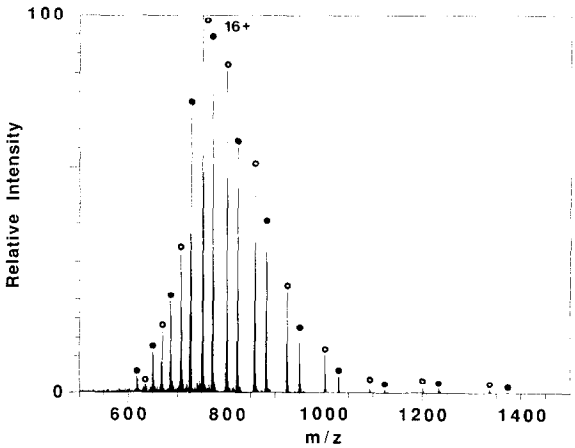

Fig. 2. Electrospray ionization mass spectrum obtained by direct infusion of an equimolar (0.1 m*M* in 5% aqueous acetic acid) mixture of tuna cytochrome *c* (○, M_r 12 029) and horse heart cytochrome *c* (●, M_r 12 360).

suggest that such contributions are minimal. Another contribution to the "tailing", which may be more likely in some circumstances, is actual (native) or induced heterogeneity of the protein sample. For larger proteins the contributions leading to increases in peak width become increasingly evident, as shown in Fig. 3.

Proteins consisting of non-covalently bound subunits yield ions indicative of the subunit M_r. Lactate dehydrogenase (Fig. 3) from rabbit muscle is an isozyme made of four subunits, each of $M_r \approx 35\,000$. The M_r of the subunit obtained from its mass spectrum is *ca.* 35 700. Similarly, a spectrum of creatine amidinohydrolase (*Pseudomonas* sp.) shows $M_r \approx 30\,700$, whereas the reported average M_r (Calbiochem Biochemicals) is 94 000. Also, creatine phosphokinase from rabbit muscle (a dimeric enzyme of 82 000) shows multiply charged ions from separate species of M_r 42 160. We have previously postulated that the production of high-charge states during ESI and the removal of the stabilizing effects of the solvent (with the potential loss of ternary structure) result in the mutual repulsion (separation) of protein subunits[29].

ESI mass spectra were readily obtained from higher-M_r proteins; for example, intact molecular ions were obtained in good yield for ovalbumin from chicken egg ($M_r \approx 43\,300$) (Fig. 3), bovine albumin ($M_r \approx 66\,300$) (Fig. 4), bovine apotransferrin ($M_r \approx 77\,013$), and turkey egg conalbumin ($M_r \approx 77\,500$)[29]. The spectrum for apotransferrin shows the peaks are a series of doublets that indicates the presence of another species with a molecular weight of 76 736 ± 30. Bovine transferrin is known to be heterogeneous, with two bands observed in electrophoresis[32]. A sample of conalbumin from chicken egg (ovotransferrin) yielded a mass spectrum identical to the turkey egg sample, both indicating multiple charging up to the 73+ ion. Fig. 4 gives the spectrum for a bovine albumin "native" dimer ($M_r \approx 133\,000$). The highest

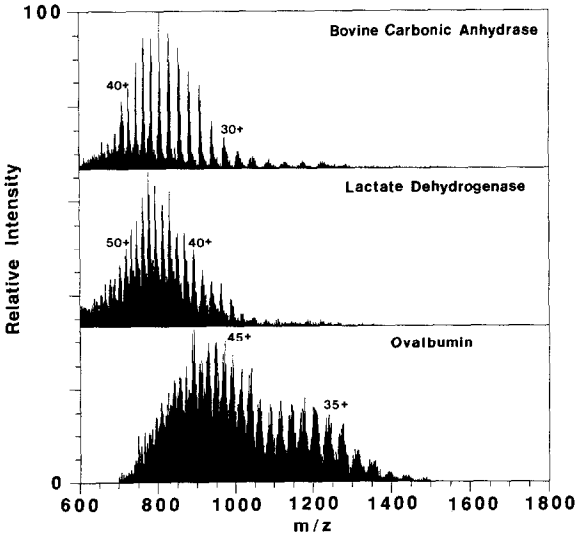

Fig. 3. Electrospray ionization mass spectra for three proteins with $M_r > 25\,000$. Bovine carbonic anhydrase (top, $M_r \approx 29\,000$) yields relatively abundant molecular ions species with charge states between +30 and +40. Lactate dehydrogenase (middle, M_r 35 700 × 4) from rabbit muscle, a protein of M_r 140 000, is composed of four equal subunits. Ovalbumin (bottom, M_r 43 300) from chicken egg yields molecular ions with over 50 positive charges.

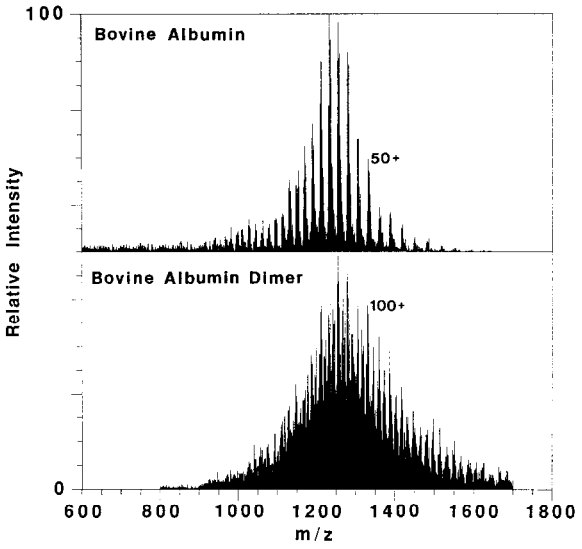

Fig. 4. The top figure is the mass spectrum for bovine serum albumin with M_r 66 300 (and containing *ca.* 100 basic amino acids) and the bottom spectrum is from the native dimer species of albumin, with $M_r \approx 133\ 000$ is shown below with over 120 positive charges clearly resolved[29].

charged species clearly resolved is the 120+ multiply protonated albumin dimer. The large width of the molecular ion envelopes suggests that either such large molecules can retain solvent in substantial quantity (unlike smaller proteins) or that substantial heterogeneity exists.

Fig. 5 shows that a plot of the most intense charge state observed *vs.* molecular weight for a representative set of proteins displays a crude linear relationship. Also shown are lines roughly defining the MS observational window (*m/z* 500 to *m/z* 2000).

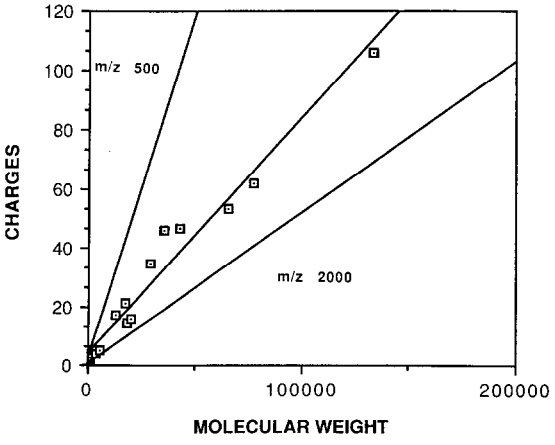

Fig. 5. Plot of most abundant molecular ion charge state *vs.* molecular weight for a representative set of proteins studied by ESI–MS.

Proteins observed must obviously fall within this observational window. However, with our current instrumentation we have yet to observe evidence for large proteins with charge state distributions for which the maximum intensity for a charge state is above m/z 1700, and electrostatic restrictions may exist on the desorption rates for higher m/z ions. The maximum charge state for proteins seems to be generally predicted by the number of readily protonated sites. In fact, a good linear correlation is also observed for polypeptides and smaller proteins between the maximum number of charges and the number of basic amino acid residues (*e.g.*, arginine, lysine, histidine, etc.), *i.e.*, the probable protonation sites. For example, angiotensin I (M_r 1296) contains one arginine and two histidine residues in its sequence (along with an N-terminal amino group), consistent with the $(M + 4H)^{4+}$ ion being the maximum charged species observed experimentally. The $(M + 5H)^{5+}$ and $(M + 6H)^{6+}$ are the most abundant and most highly charged species, respectively, produced by ESI for melittin from bee venom, a peptide for which protonation sites include the five basic amino acids and the terminal NH_2 group[31]. Similarly, horse heart cytochrome *c* (M_r 12 360) has 24 basic amino acids while up to the $(M + 21H)^{21+}$ species is observed[29]. (The role of other contributing moieties, such as the iron-porphyrin complex bound to the cytochrome *c* molecule, is uncertain). It is also noteworthy that a common feature of the few proteins where we have not been successful in obtaining ESI spectra with our instrument is the relatively small number of basic amino acid residues (*i.e.*, where only high m/z ions might be predicted).

One can speculate on the ultimate M_r limitations of the ESI–MS method. Each charge state has a minimum peak width determined by the ^{13}C and other isotopic contributions[33]. Peak widths for multiply charged proteins actually decrease with increasing molecular weight because the increased isotopic envelope width (at half height) is less than the increase in charge state necessary for a constant m/z. The peak width, among other factors, defines how closely adjacent charge states can be located and still be resolved. For example, the polystyrene oligomer $[C_4H_9(C_8H_8)_nH]$ at n = 100 (average $M_r \approx$ 10 470), has a calculated full width at half the peak height (FWHH) for the 10+ multiply protonated ion of ≈ 0.7 m/z units[33]. For polystyrene of n = 1000, FWHH for the 100+ ion is approximately 0.22 m/z units. M_r limitations will also depend on instrument resolution, signal-to-noise, and compound purity, and (when ions are formed in the m/z 1000 range) can be estimated to be on the order of a few million due to the isotopic distribution. While the capability of producing molecular ions in the $M_r = 10^6$ range has not been demonstrated, practical limitations may exist due to sensitivity constraints. Sensitivity is predicted to drop with increasing M_r due to the increased number of charge states (and their charges) through which the signal (and ion current) is dispersed.

CZE–MS of proteins

Proteins pose a tremendous challenge for CZE and are currently the subject of intense development efforts. One objective is to avoid interaction of the protein with the capillary surface so that the high efficiency possible by CZE can be realized. Previous attempts at CZE of small proteins (with UV detection)[34] have often resulted in broad, tailing peaks due to adsorption of proteins on active sites of the negatively charged capillary wall. One approach for circumventing this problem is to conduct separations in buffered systems with the pH above the protein isoelectric point (p*I*) to

allow both the proteins and the capillary wall to be negatively charged and mutually repulsive. Such an approach can greatly improve resolution[35]. Initial studies with positive ion ESI–MS of peptides and proteins in buffer solutions above their p*I* resulted in over an order of magnitude loss in sensitivity compared to more acidic conditions, reflecting lower efficiencies for analyte protonation. However, by using a liquid sheath consisting of methanol–water–glacial acetic acid (80:10:10), initial attempts at CZE–MS of peptides in a pH 11 sodium phosphate solution allowed polypeptides such as bradykinin, angiotensin I and porcine insulin to be resolved. For larger proteins with p*I* < 11, denaturation often occurs under the high pH conditions[35], which limits the general application of this approach.

Sperm whale and horse myoglobin (M_r 17 199 and 16 950, respectively) were chosen for our initial experiments. The successful separation of myoglobins by CZE was initially reported by Lauer and McManigill[35] using conventional on-column UV detection and later by Rose and Jorgenson[36] using post-capillary fluorescence detection. Aqueous samples were prepared at a concentration of 0.1 m*M*/component. An aqueous 20 m*M* Tris solution adjusted to pH 8.25 with an HCl–KCl solution was used as the CZE buffer. Electromigration for 10 s at 15 kV resulted in injection of *ca.* 1.7 pmol/component. The separation was conducted at 30 kV (17 μA current) in a 100 cm × 50 μm I.D. untreated fused-silica capillary. ESI–MS detection utilized multiple ion monitoring methods of the major charge states expected for each myoglobin species. Since ESI–MS detection of proteins generally requires an acidic solution, an methanol–water (80:20) solution was augmented by addition of 5% acetic acid for the sheath liquid. This capability highlights one of the unique features of the sheath flow interface: buffer conditions otherwise inappropriate for ESI–MS can be used and modified "post-column" through the sheath liquid. Fig. 6 shows the single ion electropherograms obtained for three multiply protonated molecular ions for each myoglobin as well as the reconstructed ion electropherogram. The results show nearly baseline resolution between the myoglobins; the number of theoretical plates (*N*) based upon the peak width at half height was about 30 000. The results suggest detection limits in the 100-fmol range. Improved detection limits could likely be obtained by a simple strategy involving summation of the signals from all reasonably intense protein charge states.

Improved total plate counts can be obtained by decreasing sample size or increasing capillary length (to some extent), as shown in Fig. 7, to minimize local heating (probably a major factor in the loss of separation efficiency for the myoglobins). Protein molar concentrations which can be tolerated in CZE are generally lower than for other analytes due to their greater number of charges and the resulting effect on local solution conductivity. In this case a mixture of leucine enkephalin and horse myoglobin (0.1 m*M* each) were separated under identical conditions in a 125 cm × 50 μm I.D. untreated fused-silica capillary. The injection volume was ≈ 10 nl corresponding to ≈ 1 pmol/component and the separation yielded *ca.* 125 000 theoretical plates for both peaks.

In an attempt to obtain improved detection limits, mass spectrometer resolution was decreased to about 300 and the nozzle–skimmer bias was lowered. While the latter step increased ESI–MS response for the myoglobins, substantial peak tailing was observed in the mass spectrum due to incomplete desolvation. Electromigration (5 s at

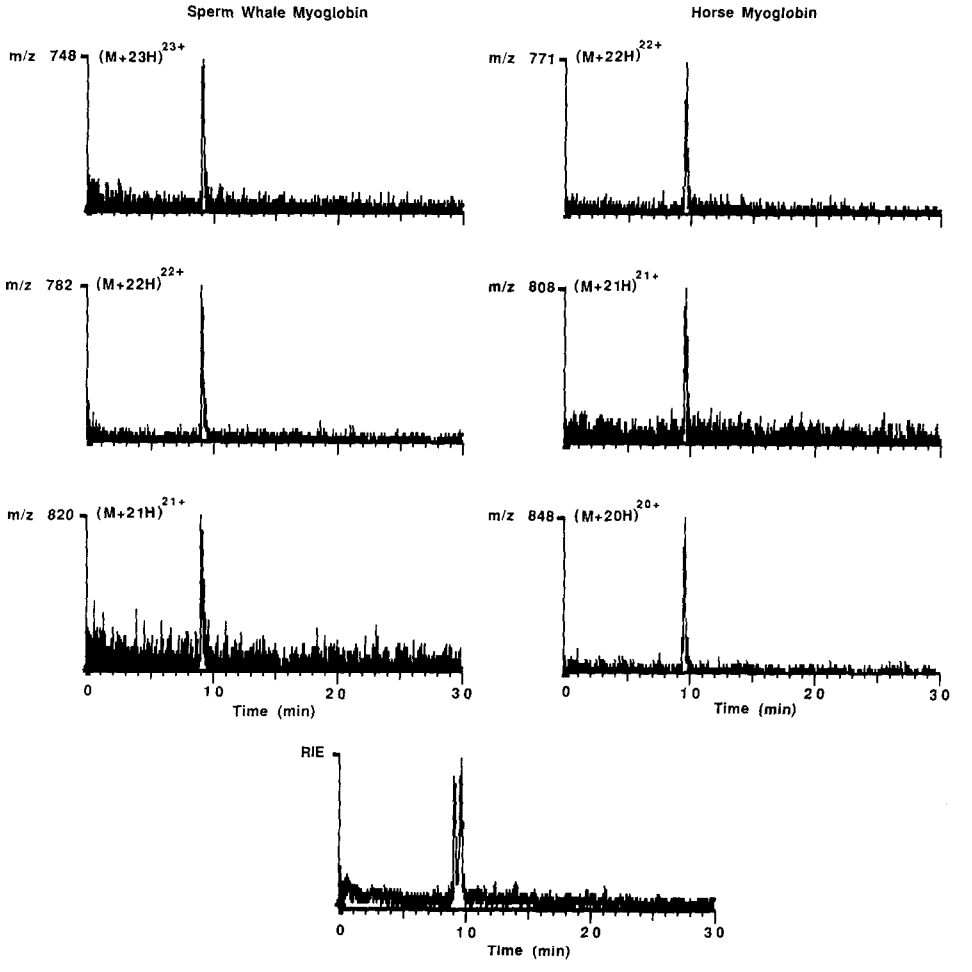

Fig. 6. CZE–ESI–MS selected ion monitoring separation of a sperm whale myoglobin (M_r 17 199) and horse myoglobin (M_r 16 950) mixture obtained using a pH 8.25, Tris–HCl buffer and a 100 cm × 50 μm fused-silica capillary.

20 kV) was used to inject < 1 pmol/component on a 100 cm × 50 μm I.D. capillary for CZE separation using a pH 8.25 Tris buffer at 30 kV. The resulting CZE–MS separation is shown in Fig. 8. The myoglobins are clearly resolved and separated from the four enkephalins, which apparently have a similar charge under the conditions chosen and co-elute. It is interesting that while detection limits for the myoglobins are somewhat improved (especially horse myoglobin), detection limits for the enkephalins are relatively poor compared with our experience for low-M_r analytes. While this may be partially due to the particular buffer system chosen, which appears to degrade ionization efficiency for the enkephalins, this result may also be partially attributed to increased "chemical noise" resulting from the low MS resolution. These results suggest that attempts to improve detection limits by degrading mass spectrometer performance, while useful in certain well defined situations, should be approached with caution.

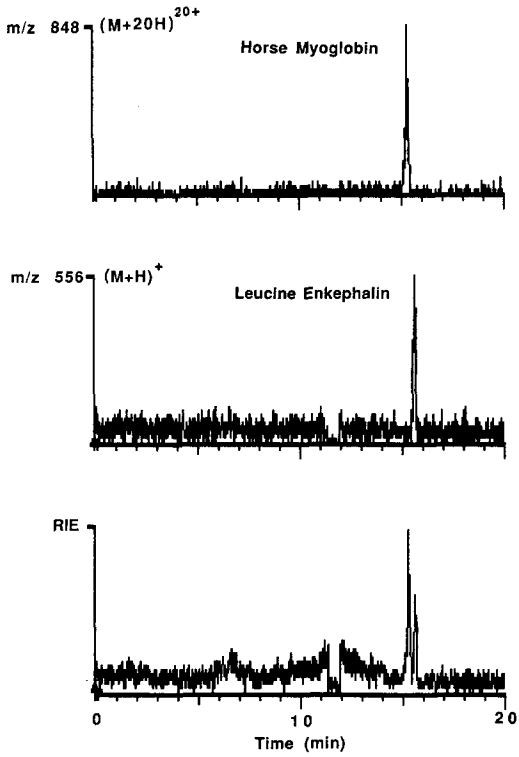

Fig. 7. CZE–ESI–MS separation of horse myoglobin (M_r 16 950) and leucine enkephalin (Tyr-Gly-Gly-Phe-Leu, M_r 555) at pH 8.25 in a 125 cm × 50 μm fused-silica capillary, at 30 kV (17 μA).

Recently, McCormick[37] has demonstrated CZE separations of proteins under conditions of low pH and using specially modified capillary surfaces. Separations at low pH are attractive since differences in electrophoretic mobilities for many proteins will be substantially larger than at high pH. In addition, concerns related to the potential loss of sensitivity by post-column manipulation of pH (as required for high pH separations) would not apply. Our initial studies have utilized a relatively low pH (2.5) 50 m*M* phosphate buffer in an attempt to minimize surface interactions with the untreated 75 cm × 50 μm I.D. fused-silica capillary. The low pH results in an increase in separation time due to the lower electroosmotic flow-rate, even though a shorter column was used than at higher pH. A four-component sample was injected (by electromigration) amounting to *ca.* 0.9 pmol/component of horse heart and *Candida krusei* (yeast) cytochrome *c*, and sperm whale and horse myoglobin. The CZE–MS single ion electropherograms for this mixture are shown in Fig. 9 for the most intense multiply protonated molecular ion for each protein. While detection limits are similar to separations obtained at higher pH, separation quality is significantly degraded. In particular, the myoglobins yielded much poorer separations. Improved separation quality, perhaps obtained by an appropriate capillary surface treatment, should result in improved detection limits. It should be noted, however, that even considering the loss in separation efficiency, detection limits are not significantly changed compared to

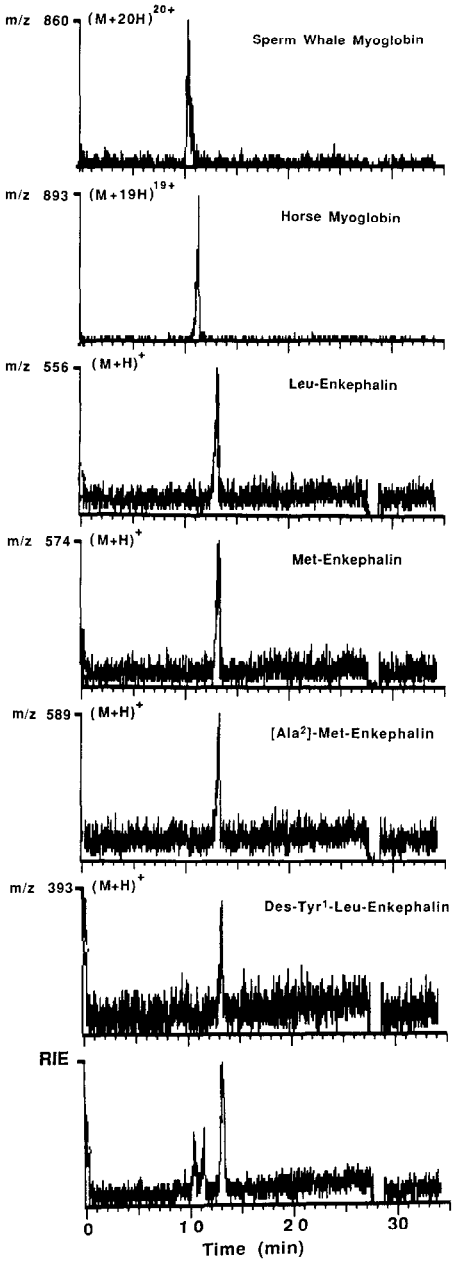

Fig. 8. CZE–MS separation of a mixture of (0.1 m*M* each) whale myoglobin (M_r 17 199), horse myoglobin (M_r 16 950), des-Tyr^1-Leu-enkephalin (M_r 392), Leu-enkephalin (M_r 555), Met-enkephalin (M_r 573), and [Ala^2]-Met enkephalin (M_r 588) obtained in a 20 m*M* Tris buffer (at pH 8.25) in a 100 cm × 50 μm fused-silica capillary at 30 kV (17 μA).

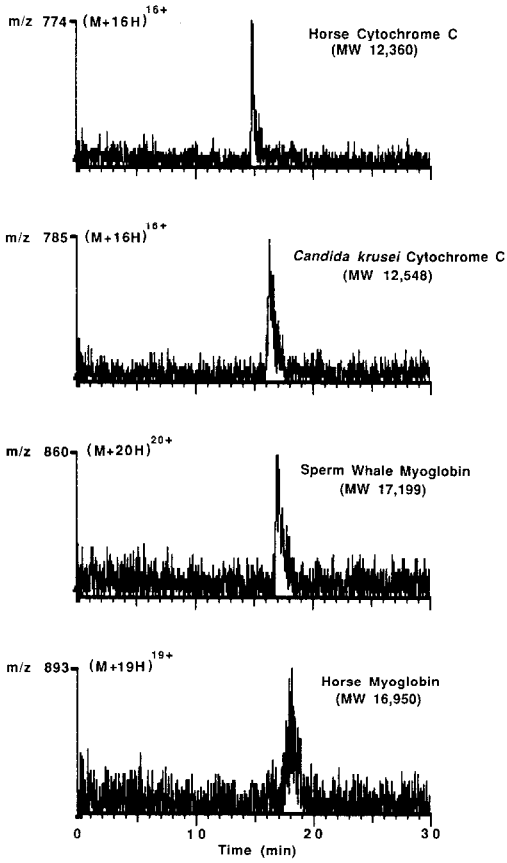

Fig. 9. Separation at "low" pH of mixture of horse heart cytochrome *c* (M_r 12 360), *Candida krusei* cytochrome *c* (M_r 12 548), whale myoglobin (M_r 17 199) and horse myoglobin (M_r 16 950) in a 50 m*M* H_3PO_4–NaH_2PO_4 buffer at pH 2.5 in a 75 cm × 50 μm I.D. column at 25 kV (20 μA).

separations at higher pH. This indicates that the post-column manipulation of solution pH (in previous studies) does not greatly degrade detection efficiency. Additional results of CZE–MS studies of proteins will be presented elsewhere[38].

The present results represent the first CZE–MS application to proteins. While detection limits (≈ 100 fmol) are presently only marginal for many purposes, significant improvements may be anticipated with further instrumental advances. For many applications for proteins, however, the currently available detection limits preclude CZE–MS. In such cases, capillary isotachophoresis may provide a potential alternative approach.

CITP–MS of polypeptides and proteins

In CITP all analytes move through the separating medium at the same final velocity[11,12]. Isotachophoresis, though utilizing similar equipment and principles as zone electrophoresis, can accommodate larger samples and result in an *increase* in the concentration of the material being separated. The steady state concentration of the

analyte ion is largely determined by the lead ion concentration. Accordingly, if the analyte is much more dilute than the lead ion concentration, the analyte will be *concentrated* as it separates into its own band. In an ideal and fully developed separation, the concentration of each band is equivalent and the relative abundance of the bands is proportional to the length of the band[11,12]. Thus, CITP offers an approach for higher sample loading than CZE (and increased molar sensitivity), and can yield increased concentration of analytes in the separated bands. Application of these methods can be much more complex depending upon the sample and details of the electrolyte systems[11,12].

We have recently reported the first on-line CITP–MS in which the separation of phosphonium salts, amines and amino acids were demonstrated[26]. Generally, CITP separations are conducted in coated capillaries to eliminate electroosmotic flow. Since electroosmotic flow-rates are dependent upon buffer composition (and particularly buffer pH), which can vary from band to band, a substantial loss of separation quality can result when separations are conducted in untreated capillaries. This loss of separation efficiency arises in part from mixing at the band boundaries where discontinuities in electroosmotic flow-rates exist[39]. However, our initial study showed that in situations where large variations in electroosmotic flow do not exist, excellent separations can be obtained using untreated capillaries[26].

Our initial attempts at CITP–MS of polypeptides involved use of untreated 50 μm I.D. fused-silica capillaries so that electroosmotic flow would assist migration of the dynamically separating bands to the electrospray region at the capillary terminus. The leading electrolyte was a 10 m*M* ammonium acetate solution titrated to pH 4.9 with acetic acid. The trailing electrolyte was 10 m*M* alanine titrated to pH 7.1 with BaOH. The sheath liquid was a methanol–water–acetic acid (90:9:1) mixture. The sample consisted of a 0.1 m*M*/component mixture of angiotensin I (M_r 1296) and bradykinin (M_r 1060) in distilled water. About 10 cm (200 nl) of a 100-cm column was loaded with sample by pressurization of the sample reservoir into a column previously filled with the leading electrolyte. The capillary was then inserted in the trailing electrolyte reservoir and a constant 32 kV was applied throughout the separation. Fig. 10 shows the selected ion isotachopherograms for the doubly protonated molecular ion of bradykinin (*m/z* 531), the triply protonated molecular ion of angiotensin I (*m/z* 433), and trailing electrolyte (*m/z* 90). These results show that incomplete separation was obtained, which we partly attribute to the limited time allowed for the separation to develop.

Since separations obtained under the above conditions were generally unsatisfactory, alternative conditions allowing longer separation times were explored. Coated capillaries were used to eliminate electroosmotic flow and to (hopefully) minimize sample interactions with the capillary surface. A 2.5-m length of 100 μm I.D. DB-17 coated bonded and cross-linked capillary (as prepared for gas or supercritical fluid chromatography) was used. The absence of electroosmotic flow was verified in separate CZE experiments. Samples were loaded by pressurization of the sample reservoir. The leading and trailing electrolyte buffers were identical to those described above, but the sheath electrode was 100% methanol. A 64-cm length of the capillary was loaded with the sample ($\approx$ 5 μl) and a CITP voltage of 58 kV was applied across the capillary for 15 min. At this point the electrospray was turned "on" (6 kV, dropping the CITP voltage to 52 kV) and a small positive pressure ($\approx$ 3 p.s.i.) was

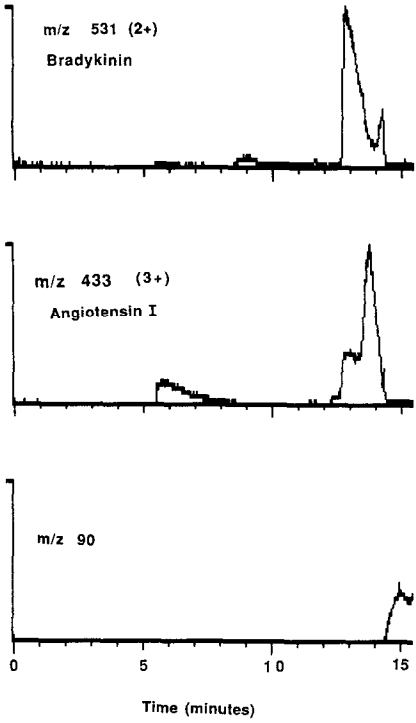

Fig. 10. CITP–MS separation of bradykinin and angiotensin I in an untreated fused-silica capillary (1 m × 50 μm I.D., 32 kV).

applied to the (high-voltage) trailing electrolyte reservoir. The pressure drop resulted in laminar flow and elution of two well resolved bands for bradykinin and angiotensin I in 12 to 15 min, for a total separation time of 30 min. Fig. 11 shows single ion isotachopherograms for the doubly and triply protonated molecular ions of both bradykinin and angiotensin I. Application of voltage in conjunction with such a pressure driven elution process appears generally useful in minimizing loss of resolution (from laminar flow) by exploiting the focusing nature of the CITP process.

An advantage of CITP–MS compared to CZE–MS is the ability to address larger sample volumes and concentrations. This allows greatly improved mass spectra to be obtained and provides a realistic basis for on-line MS–MS methods (as discussed later), particularly for cases where CZE–MS is presently precluded due to sensitivity constraints (*i.e.*, for larger proteins). Fig. 12 shows mass spectra obtained for bradykinin and angiotensin I during the CITP–MS separation shown in Fig. 11. While small contributions of each component are observed in the bands ascribed to the major components, such contributions are generally only observed from adjacent bands and can be easily (and quantitatively) subtracted.

We have previously shown[26] that CITP–MS methods provide a potentially powerful basis for "targeted" analyses, where samples of known electrophoretic mobility are trapped and concentrated between two bands (or the leading and trailing electrolytes) selected to bracket the ion mobility of interest. Other components which

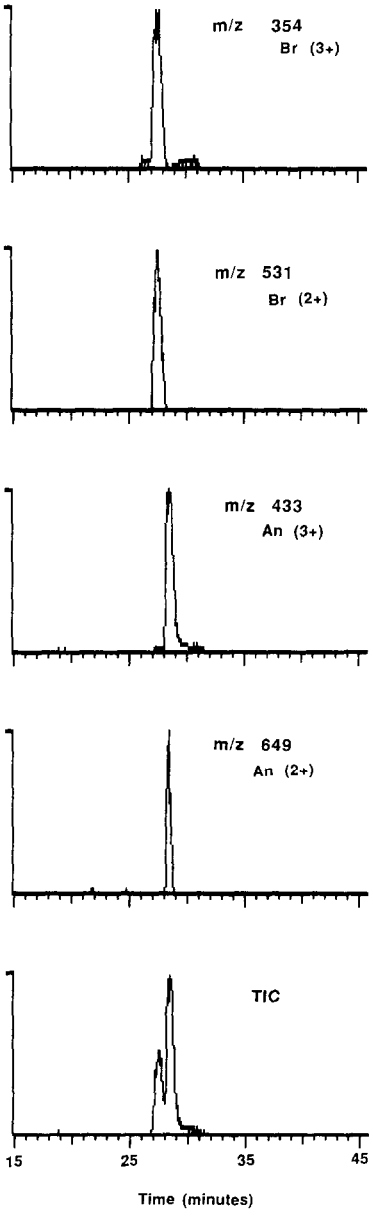

Fig. 11. CITP–MS analysis of an equimolar mixture of bradykinin and angiotensin I (2.5 m × 100 μm I.D. DB-17, 55 kV). Selected electrospray ion current isotachopherograms for bradykinin $(M+3H)^{3+}$ (*m/z* 354) and $(M+2H)^{2+}$ (*m/z* 531) and angiotensin I $(M+3H)^{3+}$ (*m/z* 433) and $(M+2H)^{2+}$ (*m/z* 649).

are not "captured" will move into either the leading or trailing electrolyte bands. Similarly, low-concentration sample components and impurities will tend to focus at the boundaries between major component bands. An example of this is shown in Fig. 13, which gives a mass spectrum for the boundary between angiotensin I and

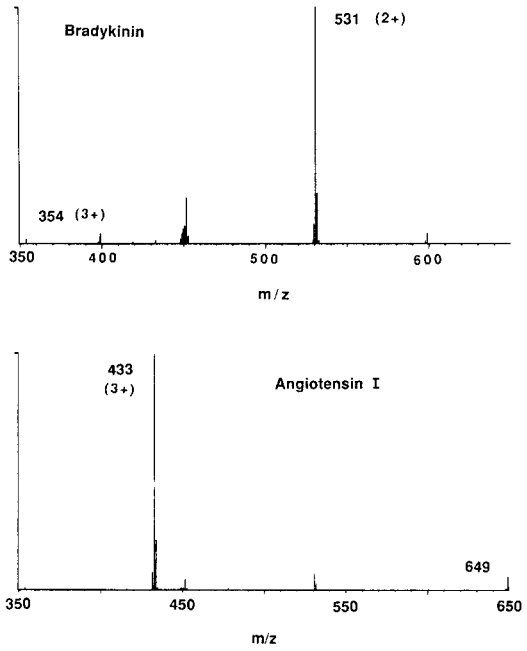

Fig. 12. ESI mass spectra of bradykinin and angiotensin I obtained during CITP–MS analysis.

bradykinin for the previous separation. Many additional peaks, absent from Fig. 12, are evident and may be attributed to impurities focused at the boundary between the major bands. This phenomenon, noted earlier[26] is likely the reason for the low MS background ("chemical noise") previously observed using this method.

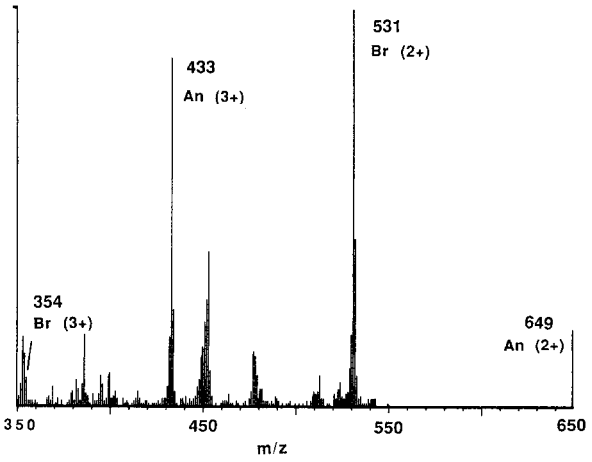

Fig. 13. Mass spectrum obtained from a CITP–MS separation at the boundary between the angiotensin I and bradykinin bands (see Fig. 11).

An initial investigation of CITP–MS methods for proteins has been conducted using the DB-17 coated capillary and electrolyte system described above. A 0.1-m*M*/component solution containing horse heart cytochrome *c* and horse myoglobin was injected and separated using conditions identical to that given above (with the exception of a sheath liquid of methanol–water–acetic acid, 80:19:1). As shown in Fig. 14, only partial separation of the cytochrome *c* and myoglobin components was obtained. A partial mass spectrum for cytochrome *c* (obtained at 20 min) is shown in Fig. 15, clearly demonstrating that sufficient information for accurate M_r determination can be obtained from such analyses. Studies aimed at determining conditions for improved CITP–MS (and CITP–MS–MS) separations of proteins and enzymatic digests of proteins are in progress.

ESI with MS–MS detection

An important aim of our CZE–MS and CITP–MS efforts is to develop methods that will yield primary structural information (*i.e.*, sequence) for polypeptides and small proteins. The ESI method affords unique opportunities in this regard since ionization efficiencies are high and good results can be obtained, even for large proteins. The fact that ESI mass spectra generally consist of only intact multiply charged molecular ions is sometimes cited as a disadvantage of this method since it is claimed that structural information cannot be obtained. However, as we have shown recently, effective dissociation of molecular ions can be induced in the nozzle–skimmer region of the ESI interface[31].

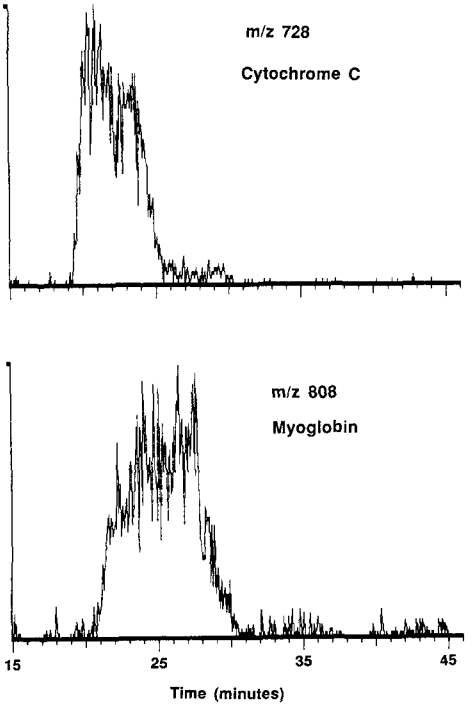

Fig. 14. CITP–MS separation of cytochrome *c* and horse myoglobin (2.5 m × 100 μm I.D. DB-17, 55 kV).

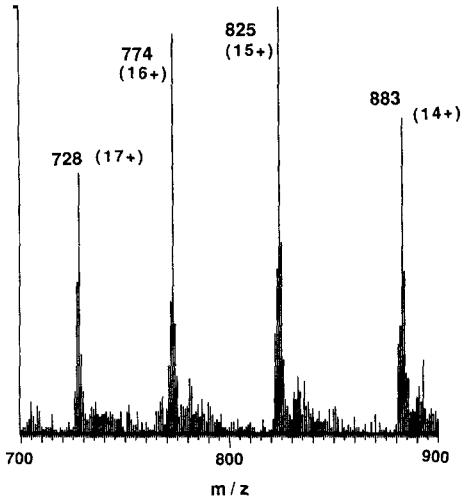

Fig. 15. Mass spectrum obtained for cytochrome *c* during CITP–MS separation (see Fig. 14).

The potential of this approach is illustrated for the case of melittin (M_r 2845), for which the conventional ESI spectrum is given in Fig. 16. By increasing the nozzle–skimmer bias from the usual range (100 to 200 V) to 250 V significant fragmentation is observed, as shown in Fig. 17. Nearly all the peaks in this spectrum can be ascribed to important (sequence specific) fragmentation of the polypeptide chain, as shown using accepted abbreviations[40] to denote fragmentation site (augmented by a superscript to give the ion charge state). The advantage of this

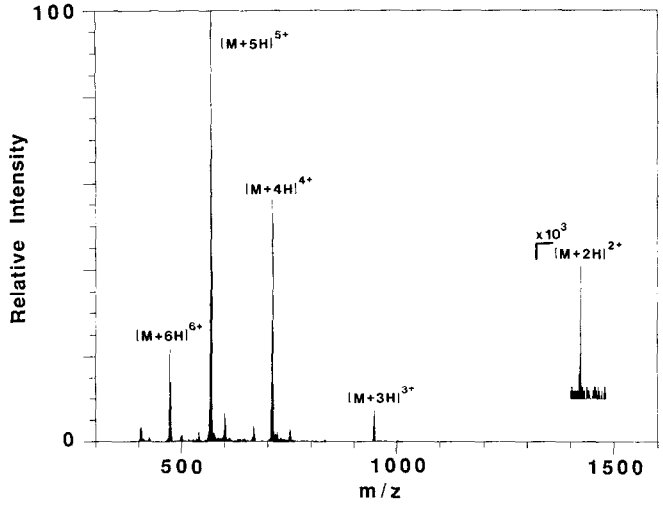

Fig. 16. Positive ion ESI mass spectrum of melittin (M_r 2845.5), an amphiphilic bee polypeptide.

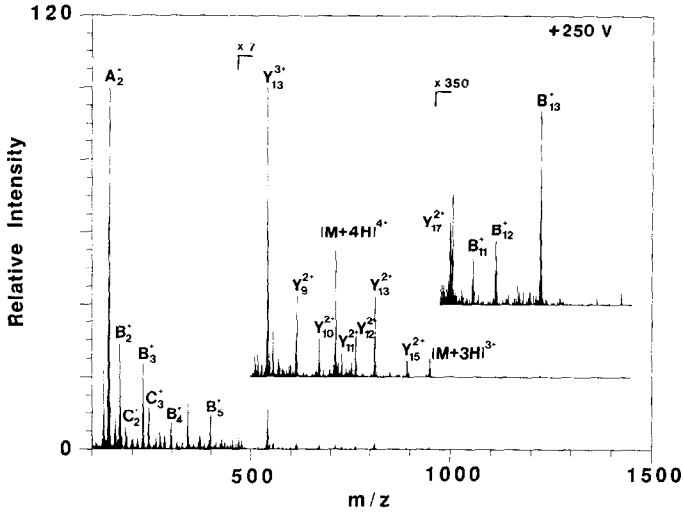

Fig. 17. Collisional induced fragmentation spectrum of melittin in the region between the nozzle and skimmer of the atmospheric pressure electrospray interface, promoted by the bias voltage of the skimmer of 250 V. Increasing the collision energy effectively breaks apart the molecule, yielding singly and multiply charged sequence ions, with fragmentation occurring from both ends of the molecule.

approach is that structural information for relatively pure substances can be obtained using a relatively simple single quadrupole mass spectrometer. A disadvantage of this approach, however, is that the multiplicity of charge states can result in mass spectra for which interpretation becomes difficult if significant additional structural information is not already available.

A powerful alternative approach is to apply tandem mass spectrometry to collisionally dissociate molecular ions for several of the major charge states. Such an example, obtained using a tandem (triple) quadrupole instrument (*e.g.*, MS–MS) is shown in Fig. 18 which gives the MS–MS mass spectra for the +3 to +6 multiply protonated molecular ions of melittin. As evident, extensive fragmentation (singly and multiply charged daughter ions) is observed for each charge state. An analysis of these spectra, given elsewhere[41], has shown that fragmentation representative of nearly the complete sequence can be obtained. Thus, the production of multiply charged molecular ions by electrospray ionization not only allows for molecular weight determination of large polypeptides with instruments of limited m/z range, but a potential method for obtaining sequence information when combined with MS–MS techniques. The extension of these methods to small proteins is in progress[42].

The combination of MS–MS with CZE and CITP provides a powerful new analytical tool, unsuspected only a few years ago. For example, CZE separations of enzymatic digests have been demonstrated in many laboratories (initial CZE–MS results based upon a similar electrospray interface have been presented by Henion[43]). The combination with improved detection sensitivity or CITP methods should soon allow on-line tandem mass spectrometry of such mixtures, providing a potential basis for faster, more sensitive, and (perhaps) more widely applicable sequencing of proteins. Many similar opportunities and challenges await for application of these methods.

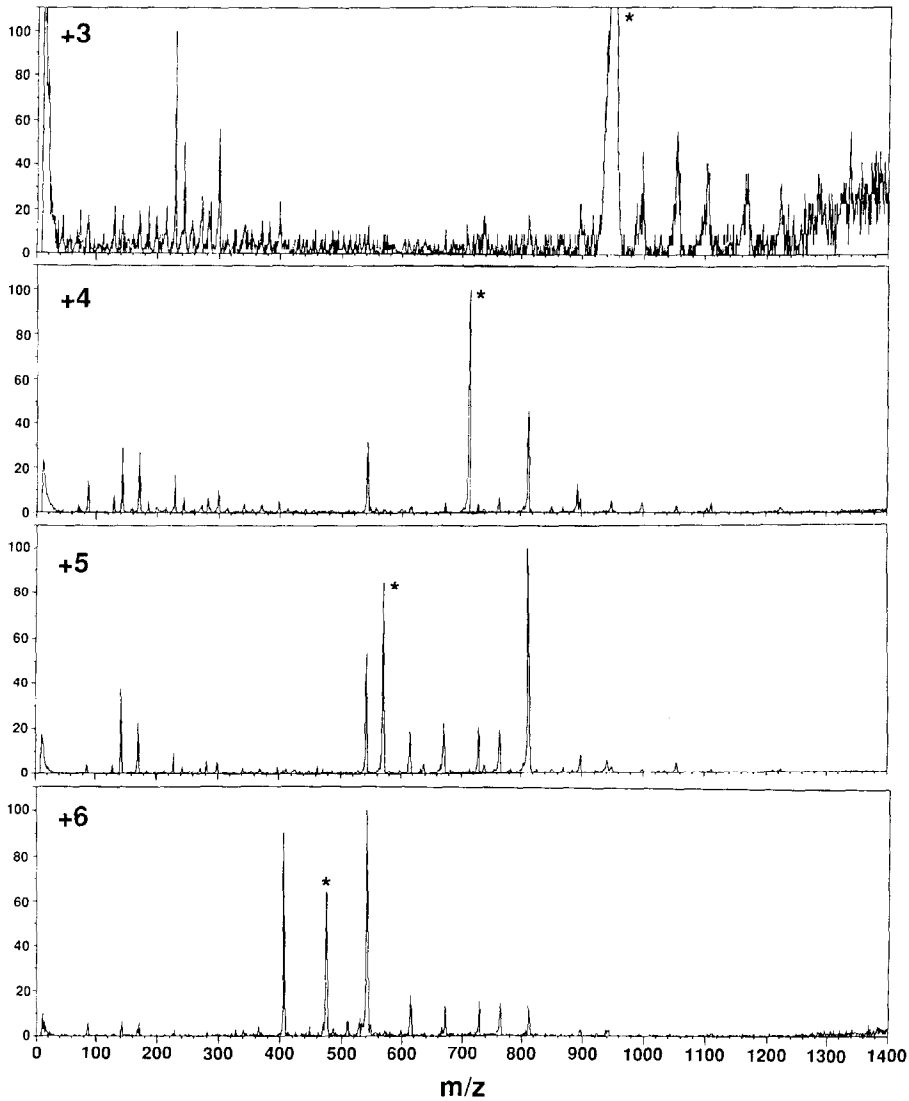

Fig. 18. MS–MS spectra of multiply charged protonated molecular ions of melittin (denoted by *). The singly and multiply charged ions, obtained by MS–MS are the same as fragment ions shown in Fig. 17, generated by collision processes in the electrospray interface region, but are much more readily interpreted. MS–MS of higher charged parent ions (4+, 5+ and 6+) yields higher charged daughter ions, and the information obtained can be combined to yield most of the peptide sequence[41].

ACKNOWLEDGEMENTS

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REFERENCES

1 R. M. Hewick, M. W. Hunkapiller, L. E. Hood and W. J. Dryer, *J. Biol. Chem.*, 256 (1981) 7990.
2 K. Biemann, *Anal. Chem.*, 58 (1986) 1288A.
3 K. Biemann and S. A. Martin, *Mass Spectrom. Rev.*, 6 (1987) 1.
4 K. Biemann and H. A. Scoble, *Science (Washington, D.C.)*, 237 (1987) 992.
5 Y.-F. Cheng and N. J. Dovichi, *Science (Washington, D.C.)*, 242 (1988) 562.
6 M. Kanai and P. Rudewicz, *37th ASMS Conference on Mass Spectrometry and Allied Topics, Miami Beach, FL, May 21–26, 1989*, p. 953.
7 K. A. Cobb, J. Liu, and M. Novotny, presented at *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlanta, GA, March 6–10, 1989*, abstract No. 1422.
8 S. W. Compton and R. G. Brownlee, *BioTechniques*, 6 (1988) 432.
9 M. J. Gordon, X. Huang, S. L. Pentoney, Jr. and R. N. Zare, *Science (Washington, D.C.)*, 242 (1988) 224.
10 A. S. Cohen, D. Najarian, J. A. Smith and B. L. Karger, *J. Chromatogr.*, 458 (1988) 323.
11 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis—Theory, Instrumentation and Applications (Journal of Chromatography Library*, Vol. 6), Elsevier, Amsterdam, 1976.
12 F. M. Everaerts and Th. P. E. M. Verheggen, in J. W. Jorgensen and M. Phillips (Editors), *New Directions in Electrophoretic Methods*, American Chemical Society, Washington, DC, 1987, p. 199.
13 S. Hjertén and M.-D. Zhu, *J. Chromatogr.*, 346 (1985) 265.
14 S. Hjertén, K. Elenbring, F. Kilár and J.-L. Liao, *J. Chromatogr.*, 403 (1987) 47.
15 S. Terabe, K. Otsuka, I. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
16 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
17 A. Guttman, A. Paulus, A. S. Cohen and B. L. Karger, in C. Schafer-Nielsen (Editor), *Electrophoresis '88*, VCH, Copenhagen, 1988, p. 151.
18 R. D. Minard, D. Chin-Fatt, P. Curry, Jr. and A. G. Ewing, presented at the *36th ASMS Conference on Mass Spectrometry and Allied Topics, San Francisco, CA, June 5–10, 1988*, p. 950.
19 M. A. Moseley, L. J. Detering, K. B. Tomer and J. W. Jorgenson, presented at the *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlanta, GA, March 6–10, 1989*, Abstract No. 713.
20 W. T. Moore, M. Martin, B. Dague, R. M. Caprioli, K. J. Wilson and S. E. Moring, presented at the *37th ASMS Conference on Mass Spectrometry and Allied Topics, Miami Beach, FL, May 21–26, 1989.*
21 J. A. Olivares, N. T. Nguyen, C. R. Yonker and R. D. Smith, *Anal. Chem.*, 59 (1987) 1230.
22 R. D. Smith, J. A. Olivares, N. T. Nguyen and H. R. Udseth, *Anal. Chem.*, 60 (1988) 436.
23 R. D. Smith, C. J. Barinaga and H. R. Udseth, *Anal. Chem.*, 60 (1988) 1948.
24 E. D. Lee, W. Mück, J. D. Henion and T. R. Covey, *Anal. Chem.*, in press.
25 E. D. Lee, W. Mück, J. D. Henion and T. R. Covey, *J. Chromatogr.*, 458 (1988) 313.
26 H. R. Udseth, J. A. Loo and R. D. Smith, *Anal. Chem.*, 61 (1989) 228.
27 C. K. Meng, M. Mann and J. B. Fenn, *Z. Phys. D*, 10 (1988) 361.
28 M. Mann, C. K. Meng and J. B. Fenn, *Anal. Chem.*, 61 (1989) 1702.
29 J. A. Loo, H. R. Udseth and R. D. Smith, *Anal. Biochem.*, 179 (1989) 404.
30 T. R. Covey, R. F. Bonner, B. I. Shushan and J. Henion, *Rapid Commun. Mass Spectrom.*, 2 (1988) 249.
31 J. A. Loo, H. R. Udseth and R. D. Smith, *Rapid Commun. Mass Spectrom.*, 2 (1988) 207.
32 N. E. Richardson, N. Buttress, A. Feinstein, A. Stratil and R. L. Spooner, *Biochem. J.*, 135 (1973) 87.
33 J. Yergey, D. Heller, G. Hansen, R. J. Cotter and C. Fenselau, *Anal. Chem.*, 55 (1983) 353.
34 J. W. Jorgenson and K. D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266.
35 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
36 D. J. Rose, Jr. and J. W. Jorgenson, *J. Chromatogr.*, 447 (1988) 117.
37 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
38 J. A. Loo, H. K. Jones, H. R. Udseth and R. D. Smith, *J. Microcolumn Sep.*, in press.
39 F. M. Everaerts, A. A. A. M. van de Goor, Th. P. E. M. Verhaggen and J. L. Beckers, *J. High Resolut. Chromatogr.*, 12 (1988) 28.
40 P. Roepstroff and J. Fohlman, *Biomed. Mass Spectrom.*, 11 (1984) 601.
41 C. J. Barinaga, C. G. Edmonds, H. R. Udseth and R. D. Smith, *Rapid Commun. Mass Spectrom.*, 3 (1989) 160.
42 R. D. Smith, C. J. Barinaga and H. R. Udseth, *J. Phys. Chem.*, 93 (1989) 5019.
43 J. D. Henion, presented at the *1st International Symposium on High Performance Capillary Electrophoresis, Boston, MA, April 10–12, 1989.*