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COMBINED CAPILLARY ELECTROPHORESIS AND ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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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 kilodalton are demonstrated. The dynamic combinations of both capillary zone electrophoresis and capillary isotachophoresis with electrospray ionization are illustrated for mixtures of peptides and proteins. The potential extension of these methods for determination of the primary structure (sequence) of proteins 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 is discussed.

INTRODUCTION

The development of capillary electrophoresis (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[1], provide a basis for such further developments. The use of capillary zone electrophoresis (CZE) for separation of proteins[2], and recent demonstrations of restriction mapping of large deoxyribonucleotides[3], 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)[4], capillary isoelectric focusing (CIEF)[5], capillary electrokinetic chromatography (CEC)[6], and, most recently, capillary

polyacrylamide gel electrophoresis (CGE)[7]. As a result, there are concomitant and increasing demands upon detector sensitivity and information density.

Mass spectrometry is potentially an ideal detector for CE. At present, CE-MS interfacing methods are based upon either flowing (or dynamic) fast atom bombardment (FAB)[8-10] or electrospray ionization (ESI)[11-15]. 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 without degrading CZE separations[13]. These developments have allowed the first on-line combination of capillary isotachophoresis with MS (CITP-MS)[16], which provides an attractive compliment to CZE-MS where (among other situations) greater sample sizes are required. 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[17,18].

EXPERIMENTAL

The instrumentation developed at our laboratory has been described elsewhere in detail[11-13]. Figure 1 shows a schematic of the interface and

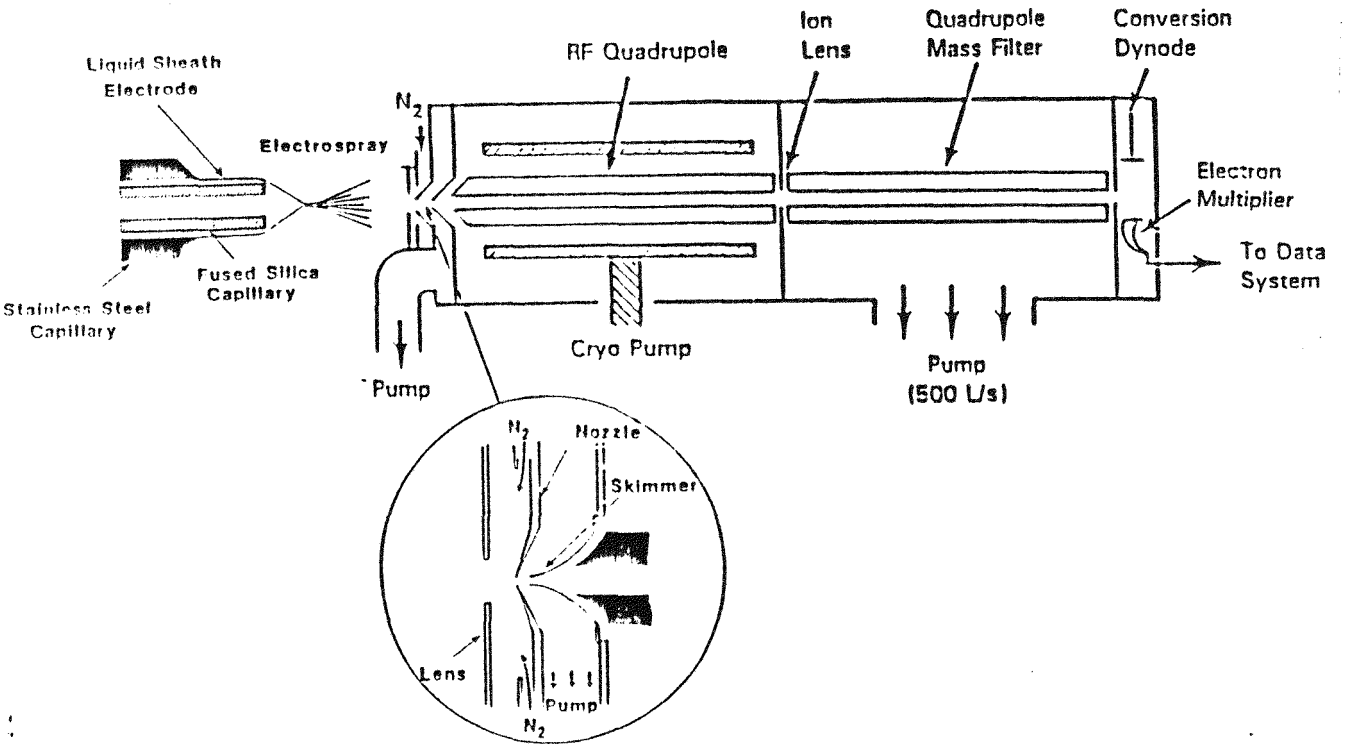

Figure 1. Schematic of the atmospheric pressure electrospray ionization interface and mass spectrometer. The electrospray capillary and sheath electrode assembly are scaled up by a factor of approximately 35.

mass spectrometer. The interface design employs a flowing liquid sheath interface which allows the composition and flow rate of the electrosprayed liquid to be controlled independently of the CZE buffer (which is desirable since high percentage aqueous and high ionic strength buffers useful for CZE are not generally compatible with ESI)[13]. The electrical contact is also established through the conductive liquid sheath (typically 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[13]. 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[11-13].

The electrospray ionization source consists of a 50 or 100 μm ID 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) of the quadrupole mass spectrometer. A 3 to 6 L/min countercurrent flow of warm (80°C) nitrogen gas is introduced between the nozzle and source to aid desolvation of the highly charged electrospray droplets and to minimize any solvent cluster formation during expansion into the vacuum chamber. 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 (Figure 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.

Biochemical samples were purchased from Sigma Chemical Co. (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

As originally reported by Fenn and coworkers, proteins can be effectively ionized by ESI yielding a distribution of charge states[17,18]. 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)[19].

A useful feature of our ESI interface is the ability to "heat" ions to any desired extent by manipulation of the nozzle-skimmer bias[21]. Solvent clustering with the analyte is substantially eliminated by a countercurrent flow of nitrogen and the high nozzle 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 (MW ~ 29,022), shown in Figure 2 (top).

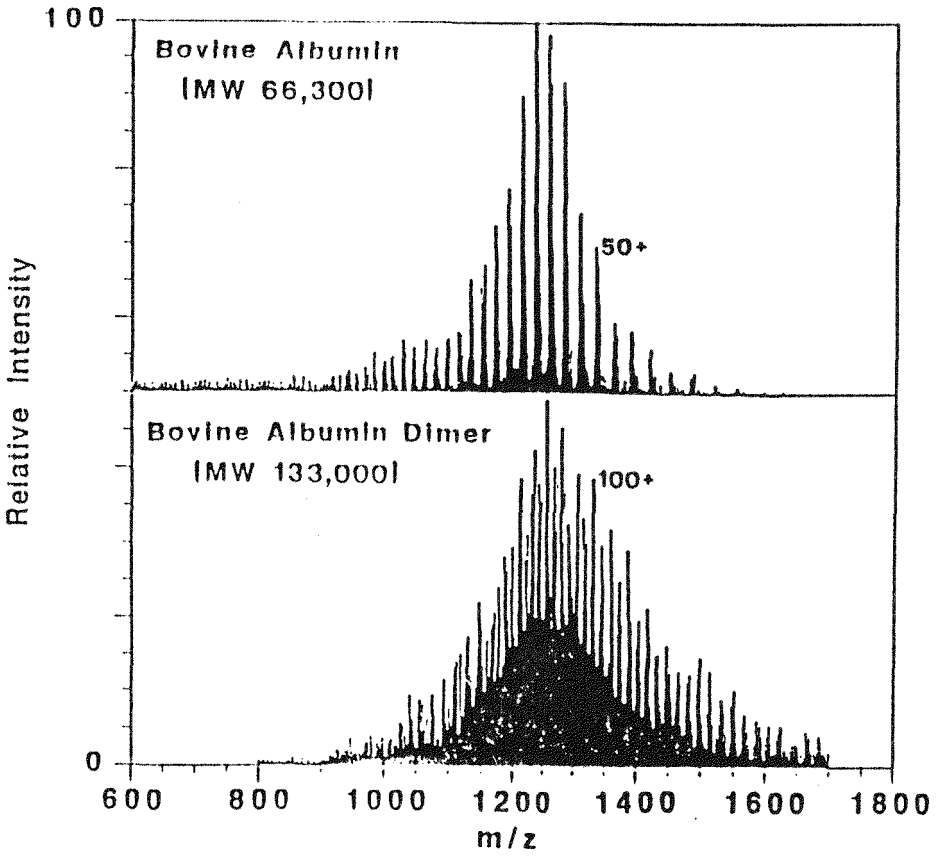

Figure 2. The top figure is the mass spectrum for bovine serum albumin with a molecular weight of ~ 66,000 (and containing ~ 100 basic amino acids) and the bottom spectrum is from the native dimer species of albumin, with a molecular weight of ~ 133,000 daltons is shown below with over 120 positive charges clearly resolved.

For larger proteins the contributions from protein heterogeneity become increasingly evident, as shown in Figure 2. Proteins consisting of noncovalently bound subunits yield ions indicative of the subunit MW. Lactate dehydrogenase from rabbit muscle is an isozyme made up of four subunits, each of MW of approximately 35,000 Da. The MW of the subunit obtained from its

mass spectrum is ~ 35,700 Da. Similarly, a spectrum of creatine amidinohydrolase (*Pseudomonas* sp.) shows a MW of approximately 30,700 Da, whereas the reported average MW (Calbiochem Biochemicals) is 94,000 Da. Also, creatine phosphokinase from rabbit muscle (a dimeric enzyme of 82 kDa) shows multiply charged ions from separate 42,160 Da species. 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 loss of secondary and ternary structure) result in the mutual repulsion (separation) of protein subunits[19].

ESI spectra were readily obtained from higher MW proteins; for example, intact molecular ions were obtained in good yield for ovalbumin from chicken egg (MW ~ 43,300) (Figure 2), bovine albumin (MW ~ 66,393), bovine apotransferrin (MW ~ 77,013), and turkey egg conalbumin (MW ~ 77,500)[19]. The spectrum for apotransferrin shows the peaks are a series of doublets that indicate the presence of another species with a molecular weight of 76,736 ± 30 Da. Bovine transferrin is known to be heterogeneous, with 2 bands observed in electrophoresis. 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. The highest molecular weight protein examined to date is the bovine albumin "native" dimer (MW ~ 133,000). The highest charged species clearly resolved is the 120+ multiply protonated albumin dimer. The maximum charge state for proteins seems to be generally predicted by the number of readily protonated sites. A good linear correlation is 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. It is also noteworthy that a common feature of the few proteins where we have not been successful in obtaining ESI spectra is the relatively small number of basic amino acid residues (i.e., where only high m/z ions might be predicted). Molecular weight limitations of ESI-MS 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 Da due to the isotopic distribution.

Initial CZE-MS studies of proteins have been conducted. Since ESI-MS detection of proteins requires a mildly acidic solution, an 80/20 methanol/water 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.

Reasonable plate counts can be obtained, as shown in Figure 3, for a mixture of leucine enkephalin and horse myoglobin (0.1 mM each) separated in a

125 cm x 50 µm ID untreated fused silica capillary. The injection volume was ~ 10 nL corresponding to ~ 1 pmole/component and the separation yielded ~ 125,000 theoretical plates for both peaks. The present results represent the first CZE-MS application to proteins. While detection limits (~ 100 femtomole) are presently only marginal for many purposes, significant improvements may be anticipated with further instrumental advances related to more efficient detection of ions generated by the electrospray process.

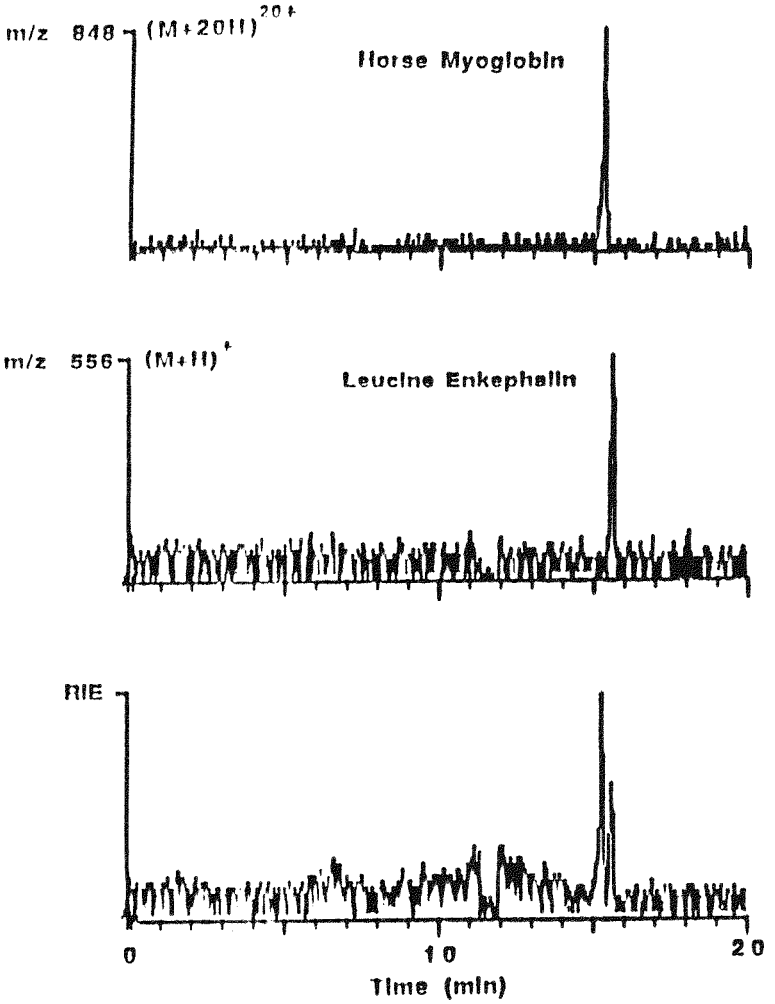

Figure 3. CZE/ESI-MS separation of horse myoglobin (M_r 16,950) and leu-enkephalin (Tyr-Gly-Gly-Phe-Leu, M_r 555) at pH 8.25 in a 125-cm x 50 µm fused silica, at 30 kV (17 uA). Separation efficiency was ~125,000 theoretical plates for each component.

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[21]. A powerful 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) for the +3 to +6 multiply protonated molecular ions of melittin has yielded. As evident, extensive fragmentation (singly and multiply charged daughter ions) is observed for each charge state. An analysis of these spectra, given elsewhere[22], has shown that nearly complete sequence information can be obtained.

Finally, the extension of MS/MS methods to proteins is illustrated in Figure 4 which shows the CID spectrum obtained for the $(M+20H)^{20+}$ molecular ion of horse heart myoglobin. We show elsewhere that reasonably efficient CID can be obtained for such species[23], and that CID information can be obtained which is useful for "finger printing" purposes[24]. Methods for deriving useful sequence information from such mass spectra remains a future goal.

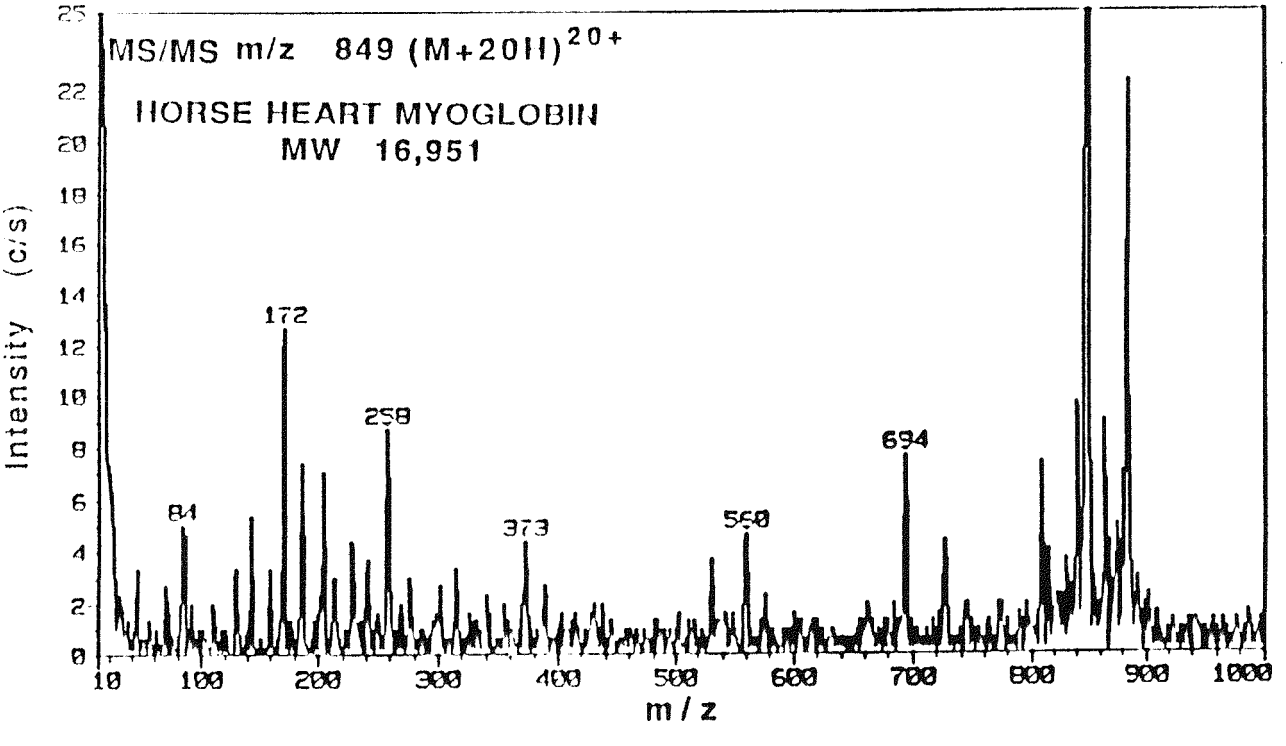

Figure 4. MS/MS spectrum obtained for the 20+ protonated molecular ion of horse heart myoglobin.

CREDIT

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