Collisional Activation and Collision-Activated Dissociation of Large Multiply Charged Polypeptides and Proteins Produced by Electrospray Ionization

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Collisional activation (CA) and collision-activated dissociation (CAD) of multiply protonated molecular ions produced by electrospray ionization using an atmospheric pressure source are described. A TAGA 6000E triple-quadrupole mass spectrometer, in both unmodified and differentially pumped inlet arrangements, was used to investigate CA and CAD during transfer through the atmosphere–vacuum interface and subsequent CAD in the tandem instrument. Melittin, which has a molecular weight (M_r) of 2846, is efficiently dissociated in the interface at higher nozzle-skimmer voltages, yielding fragmentation that can be assigned to the various charge states. Selection of such product ions formed in the interface for subsequent tandem mass spectrometry allows confirmation of earlier sequence assignments and extends the utility of these methods. Various charge states of larger polypeptides, such as human parathyroid hormone (1-44) (M_r 5064), can be efficiently collisionally dissociated in the second (rf-only) quadrupole. However, for molecular ions of this size, the low-energy collisions used for CAD yield only partial sequence information. For large molecules such as horse heart myoglobin (M_r 16,951), the effects of nozzle-skimmer bias are explored, and it is shown that higher charge states (at $\leq m/z$ 1400) can be effectively dissociated in the interface. Initial results for both metastable (unimolecular) and CAD for myoglobin are reported. The potential and limitations of CAD for large biomolecular ions are discussed. The feasibility of fingerprinting for proteins is illustrated by the CAD spectra of cytochrome *c* from nine species. *(J Am Soc Mass Spectrom 1990, 1, 53–65)*

The production of multiply charged ions by electrospray ionization (ESI) at atmospheric pressure [1–8] presents an opportunity to study the unimolecular, collisional, and photodissociation of very large molecules. The ESI mechanism is based upon droplet evaporation in a high electric field [1–3], avoiding thermolysis while allowing rapid collisional loss of any excess (suprathermal) internal energy resulting from ion transfer to the gas phase. The ESI process can be extremely efficient (low attomole detection limits have been reported in conjunction with capillary electrophoresis [9]). Additionally, the multiply charged molecular ions, which can be formed from proteins or oligonucleotides, for example, can (once they are introduced into the low-pressure environment of the mass spectrometer) be efficiently transmitted and detected due to their relatively low *m/z* values. ESI/MS with instruments usable over a relatively low *m/z* range (<2000) have been shown to be attractive for molecular weight measurements of large biomolecules [3, 6, 7]. These methods also serve as the basis for a near-ideal interface for liquid chromatography [10] and a range of capillary electrophoresis formats (e.g., free solution zone electrophoresis and isotachophoresis) [9]. The potential of these methods is further enhanced by tandem mass spectrometry (MS/MS), which, ideally, permits efficient dissociation of the precursor molecular ion into product ions that are structurally informative or, at a minimum, sufficiently distinctive to be useful for fingerprinting.

The potential for MS/MS by effective collision-activated dissociation (CAD) of highly charged (protonated) molecular ions of polypeptides (and even relatively large proteins) was first suggested on the basis of studies involving molecular ions in the atmospheric pressure–vacuum interface of a single-quadrupole mass spectrometer [4]. In these experiments an increased nozzle-skimmer voltage bias was shown to result in effective attenuation of the more highly charged molecular ions (at lower *m/z*). This

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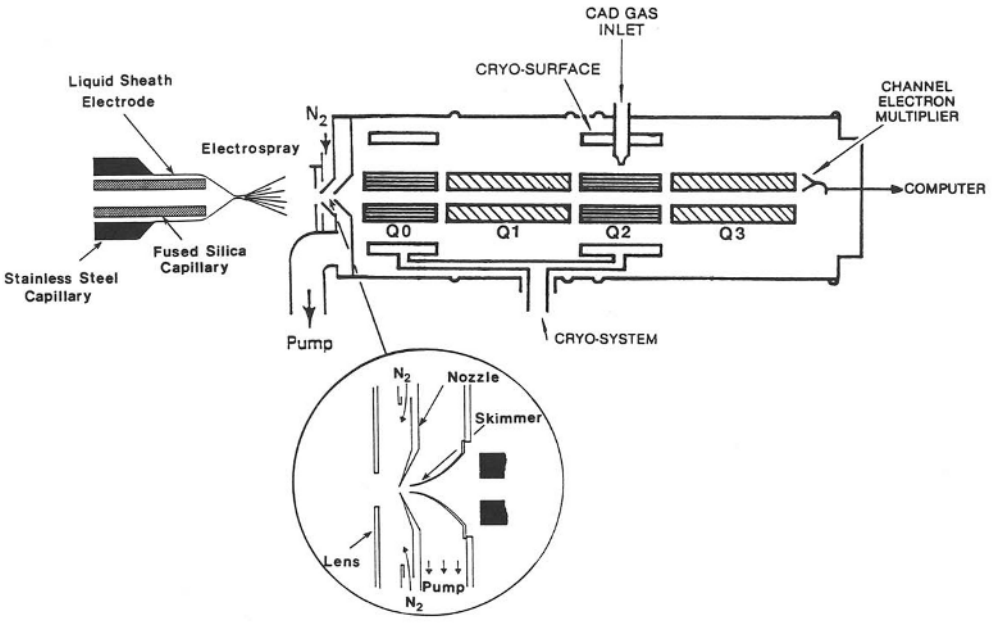

Figure 1. Schematic illustration of the ESI differentially pumped interface to the tandem quadrupole mass spectrometer.

was found to produce an increased signal intensity across the mass spectrum, tentatively attributed to CAD products from the various charge states. Subsequently, a tandem triple-quadrupole mass spectrometer was used to study various charge states of the polypeptide melittin (M_r 2846) [11]. It was shown that fragmentation was efficient and readily related to the primary amino acid sequence. In fact, CAD product ions were obtained that permitted the assignment of most of the polypeptide sequence. More recently we reported the first CAD mass spectra for a protein (horse heart cytochrome *c*, M_r 12,360) and showed that efficient dissociation was obtained for such high molecular weight biomolecules in appropriately high charge states [8].

In this paper we discuss the importance of several experimental parameters relevant to the CAD of large multiply charged biomolecules. The potential utility of CAD in the atmosphere-vacuum interface is illustrated for melittin, which undergoes efficient dissociation in this region, allowing CAD of selected fragment ions in the triple-quadrupole mass spectrometer (i.e., effectively MS/MS/MS or MS^3). Sequence information obtainable for larger polypeptides is illustrated by the CAD spectrum for human parathyroid hormone (1-44) (M_r 5064). We show the effects of subsequent metastable dissociation and CAD spectra following manipulation of internal energy by collisional activation (CA) (i.e., heating) in the interface of horse heart myoglobin (M_r = 16,951). We briefly consider the potential utility of such methods for the "fingerprinting" of proteins, illustrated by CAD of molecular ions of various cytochrome *c* proteins. Finally, we discuss the potential for protein "mapping" and the possible generation of sequence-specific information with ESI/MS.

Experimental

The tandem (triple-quadrupole) mass spectrometer used in this study, a TAGA 6000E (Sciex, Thornhill, Ontario, Canada), was modified with an electrospray ionization interface developed at our laboratory. The interface is similar to an earlier single-quadrupole instrument described in detail elsewhere [7, 9, 12]. Two instrumental configurations of the atmosphere-vacuum interface were used in this research: a simple 120-μm aperture originally supplied by Sciex and a second design developed at our laboratory that uses a larger inlet aperture and a second region of differential pumping. Figure 1 shows a schematic illustration of the modified interface and mass spectrometer. In this arrangement, ions enter the vacuum system through the 1-mm-diameter orifice (nozzle) and are efficiently sampled by a 1-mm-diameter skimmer directly in front of the radio-frequency (rf) focusing quadrupole lens (Q0). A single-stage roots blower pumps the nozzle-skimmer region to $\sim$2 torr. The cryopumped mass spectrometer chamber is maintained at pressures on the order of 10^{-6}-10^{-5} torr. Typically, +600-700 V is applied to the focusing lens and +65-350 V to the nozzle (V_n), while the skimmer is at +65 V for this study.

The ESI source employs a flowing liquid sheath interface that allows the analyte flow rate and total flow rate of the electrosprayed liquid to be controlled independently [12]. The electrospray ionization source consists of a 50- or 100-μm i.d. fused silica capillary that protrudes 0.2-0.4 mm from a cylindrical stainless steel electrode. The electrical contact is also established through the liquid sheath (typically methanol). High voltage, generally +4-5 kV for positive ions or −4 kV for negative ions, is applied to the sheath electrode. Syringe pumps control the flow of analyte solution and liquid sheath at 0.5 μL/min and 3 μL/min, respectively. The ESI source (capillary) tip is mounted approximately 1.5 cm from the ion-sampling nozzle or the ion-sampling orifice of the quadrupole mass spectrometer. A 3-L/min flow of nitrogen gas is generally used between the nozzle and ESI source to aid desolvation of the highly charged droplets.

Mass spectra were typically obtained with sample concentrations of ~ 50 pmol/μL (aqueous solution with 5% glacial acetic acid). Efforts were made to maximize primary ion currents (rather than to minimize sample consumption) to aid CAD studies. The poor transmission efficiencies of the mass selection quadrupoles (Q1 and Q3) at $m/z \approx 1000$ required operation at a resolution of ~ 400 in both stages. Thus, assignments of m/z at higher values have an uncertainty that may be as large as ± 1 for lower intensity CAD spectra (such as those obtained for proteins). Collision energies in Q2 with the differentially pumped inlet arrangement are calculated on the basis of the voltage difference between the skimmer and Q2. The mass spectra typically represent an average of one to five scans requiring 0.5-2 min each; the slower scans were generally required with the original TAGA 6000E interface due to lower ion currents. The argon target gas thickness in Q2 was 1×10^{14} molecules/cm^2 unless otherwise stated. The instrument has an upper m/z limit of 1400. All samples were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Results and Discussion

Electrospray Ionization Desolvation at Atmospheric Pressure

Considerable doubt remains concerning the detailed mechanism of ESI, particularly related to the identity of the charged species desorbed, evaporated, or "ejected" from the surface of the larger highly charged and rapidly evaporating droplets [2, 3, 13]. Are the charged species naked molecular ions, small "nanodroplets" that have (in effect) randomly sampled the droplet surface, or some intermediate? This question is difficult to resolve using mass spectrometry because only the end result is observed and only after transport through the atmosphere-vacuum interface. However, an advantage of the ESI interface is that the dry nitrogen environment will minimize the condensation effects caused by cooling when gas expands into the vacuum. Indeed, condensation of nitrogen upon small molecular ions is not evident, and such condensation must be considered even less likely for large molecular ions, which are less efficiently cooled in a supersonic expansion. This suggests that any perturbation of the atmospheric pressure ion population in the interface will most likely be due to desolvation rather than condensation.

Conditions that avoid desolvation in the atmosphere-vacuum interface can be obtained by setting the nozzle-skimmer bias, Δ(N-S), to 0 V. However, this results in a significant loss of sensitivity, partlv because of poorer ion transmission. As shown in Figure 2 for bradykinin (M_r 1060), significant association of other solvent components (tentatively ascribed to acetic acid) is observed under such conditions, which reduces molecular ion intensities. At more typical ESI interface conditions, Δ(N-S) = 100 V, no such adduct association is observed because of low-energy collisions. Further evidence that these adduct ions exist in the ESI region at atmospheric pressure is obtained by comparing the relatively low resolution spectra in Figure 2, obtained at nitrogen bath gas temperatures of 120 °C and 25 °C (top and middle, respectively). The decreased bath gas temperature results in the formation of substantially greater amounts of adducts and a decrease in absolute abundance for the $(M+2H)^{2+}$ and $(M+3H)^{3+}$ species. It should be noted that mass spectra can be obtained even without a nitrogen gas flow, but only at greatly reduced sensitivity and with even greater solvent adduction, as shown in Figure 2 (bottom). (We attribute the latter observation to the poor

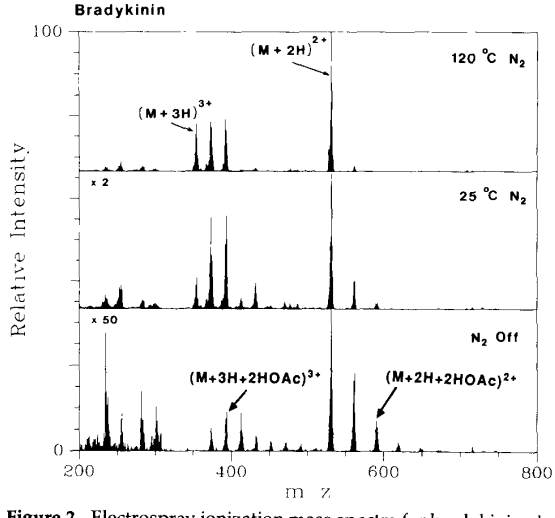

Figure 2. Electrospray ionization mass spectra for bradykinin obtained at zero nozzle-skimmer bias, where the effects of both gas flow and temperature at atmospheric pressure become evident. Peaks at m/z above the molecular ions are tentatively ascribed to acetic acid adducts.

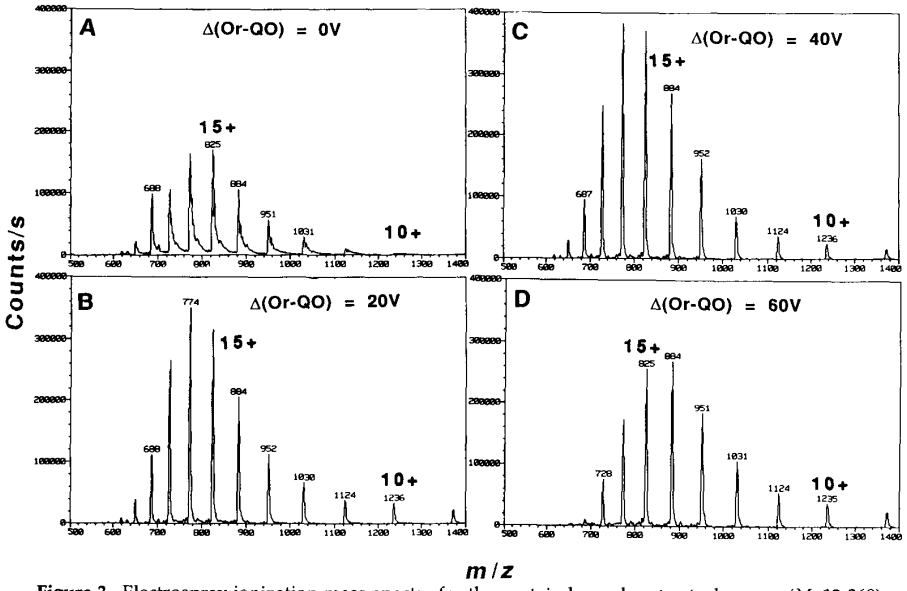

Figure 3. Electrospray ionization mass spectra for the protein horse heart cytochrome *c* (M_r 12,360) obtained with the original TAGA 6000E ion-sampling interface at various values of the inlet orifice-Q0 bias, Δ(Or-Q0). The major peaks represent a series of protonated molecular ion charge states. Each peak includes the unresolved isotopic contributions to each molecular ion charge state.

efficiency of desolvation by convection of room air into the open ESI source.) Interestingly, a greater extent of solvation is observed for the higher charge state (3+) of bradykinin. This may be due to the greater relative stability of such adducts, that is, higher charge states may be more effectively stabilized than lower charge states by the greater solvent association.

Residual association of solvent (or other neutral species) at typical ESI source conditions is likely ubiquitous. However, such effects are generally masked by empirical optimization of atmosphere-vacuum interface conditions. We note that almost all previous ESI studies used a substantial electric field in the interface region. The utility of applying electric fields in this region for atmospheric pressure ionization was noted previously [14].

Figure 3 gives ESI mass spectra of horse heart cytochrome *c*, a protein of M_r 12,360. The spectra were obtained with the unmodified TAGA 6000E ion-sampling orifice, with which the electric field in the atmosphere-vacuum interface results only from the voltage difference between the orifice and the rf-only quadrupole lens, Δ(Or-Q0). As shown in Figure 3a, when Δ(Or-Q0) = 0 V, the cytochrome *c* molecular ion peaks exhibit substantial tailing to higher *m/z*, which is attributed to unresolved adduction or solvent association. Under typical operating conditions, with Δ(Or-Q0) of typically +5-40 V chosen to optimize desolvated molecular ion intensity, solvent association is essentially eliminated, and peak width is determined by instrumental performance (relatively poor in this case). These results explain the poorer performance of an earlier interface design in which the nozzle-skimmer bias was constrained to 0 V [12]. In this earlier work, good spectra were obtained by using a relatively large voltage (up to 90 V) between the skimmer and the rf quadrupole lens. As discussed in the following section, CA can also occur in this region. Such conditions, however, result in greatly reduced ion transmission. The reduced transmission may be due to the greater kinetic energy spread that will result from accelerating ions from a region in which gas density varies greatly. These experiments have implications for instrumentation design. They also alert us to potential complications for understanding the ESI process based on mass spectra of precursor ions formed at atmospheric pressure.

Collisional Activation of Melittin in the Interface Region

As we have described recently [13], manipulation of the nozzle-skimmer bias, Δ(N-S), also allows for CA of ESI-generated molecular ions formed at atmospheric pressure. As pressure drops through the interface, ions can be accelerated to higher velocities, allowing numerous low-energy collisions. Figure 4a gives the ESI mass spectrum for melittin, a polypeptide with 26 amino acid residues, obtained at Δ(N-S) = 85 V, which is dominated by the +3 to +6 multiply protonated molecular ions, as reported previously [4, 6, 11]. Under these conditions, sufficient collisional

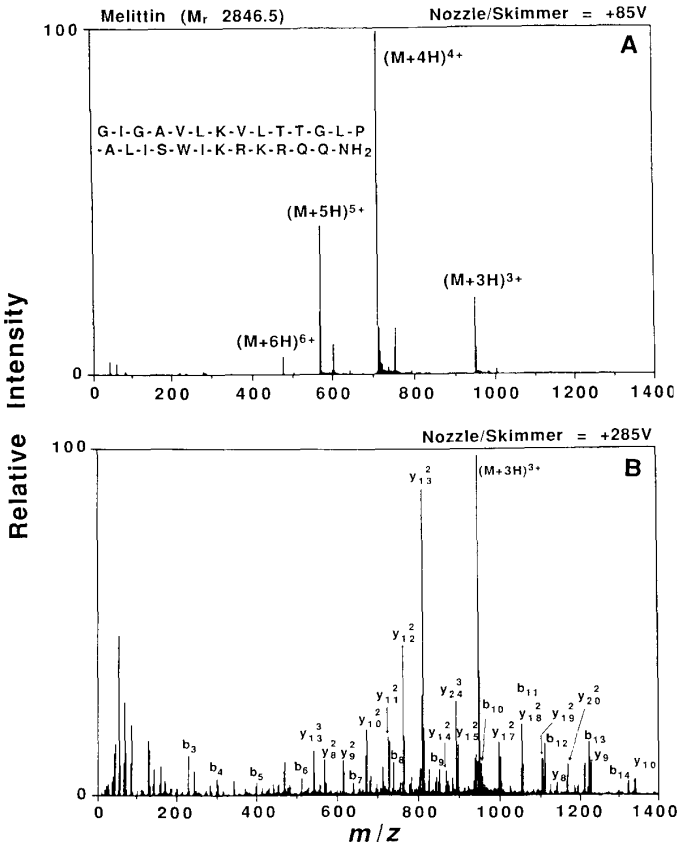

Figure 4. (**a**) ESI/MS spectrum for melittin at low nozzle-skimmer bias (+85 V) and (**b**) high nozzle-skimmer bias (+285 V). The superscripts on the sequence assignments refers to the charge state of the ions formed by CAD in the interface at Δ(N-S) = 285 V.

heating takes place in the interface to remove more weakly associated solvent molecules, yielding relatively clean mass spectrum with little "background" or noise. These operating conditions and spectrum quality are typical of ESI/MS. In addition to the peaks for the melittin molecular ions, only a few small peaks are observed at low *m/z* ($m/z < 200$). We also note other contributions that yield ions at the high *m/z* side of each multiply charged molecular ion. Such contributions are not observed in the absence of melittin. One prominent species corresponds to an ion with a molecular weight 164 higher than that of melittin. Collision-activated dissociation of this higher molecular weight species yields product ion spectra essentially identical to those obtained for melittin [11], indicating a weakly bound adduct. As would be expected, small increases of Δ(N-S) to 150–200 V somewhat decrease the abundance of these adducts but do not significantly affect protonated molecular ion intensities.

Increasing Δ(N-S) further to 285 V causes sufficient collisional heating to induce dissociation of most molecular ions, yielding the spectrum given in Figure 4b. The mass spectrum has increased greatly in complexity; the polypeptide is largely dissociated, although the less energetic CA at higher *m/z* of the lower charge states leaves the $(M + 3H)^{3+}$ molecular ion as the base peak [4]. The various dissociation products, distributed across the entire *m/z* range, have now been assigned using the conventional notation augmented by a superscript that indicates the charge state (absence of a superscript signifies a singly charged ion) [11]. Figure 4b also shows significant contributions at low *m/z* that are attributed to individual amino acid residues and other singly charged CAD products, perhaps arising from the prevailing multiple-collision conditions. All assignments are entirely consistent with our previous MS/MS study of this polypeptide [11].

Tandem mass spectrometry of the fragment ion peaks at *m/z* 812 and *m/z* 542, attributed to the y^2_{13}, y^3_{13} doubly and triply charged sequence ions (due to cleavage of the leucine–proline bond), afford the CAD spectra (sometimes referred to as MS/MS/MS [15]) shown in Figure 5. The relatively large peak widths for the precursor ions are a result of the modest resolution of Q1 and their relative intensity ($\sim 10 \times$ full scale). These spectra provide direct confirmation of the assignments for these species [11]. In addition, these yield sequence-specific ions from a portion of the

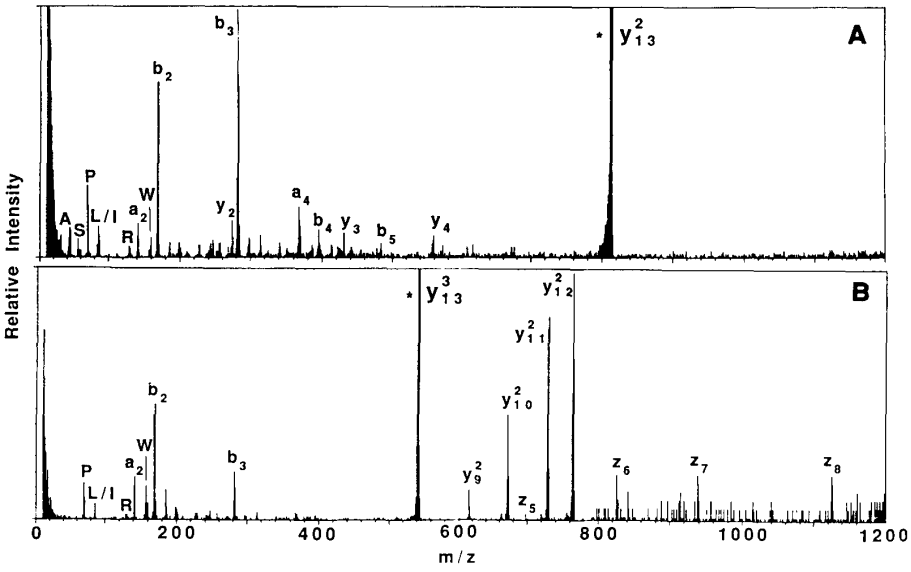

Figure 5. Tandem mass spectra of the (**a**) y^2_{13} (doubly charged) and (**b**) y^3_{13} (triply charged) product ions selected after CAD of melittin in the atmosphere-vacuum interface at Δ(N-S) = 285 V.

melittin molecule unprobed by direct study of the intact molecular ions using the low-energy collisions allowed with our tandem quadrupole instrument. These experiments suggest a generally useful analytical strategy: (1) Initially, MS/MS of the various molecular ion charge states is conducted to determine the *m/z* values of product ions. (2) Subsequently, CAD in the interface is followed by CAD of ions of interest in Q2 (for sequence determination, for example) that are already attributed to specific charge states. In this manner, it should be possible to significantly extend the useful range of application of MS/MS studies.

Collisional Activation of Proteins in the ESI/MS Interface

At higher voltages in the atmosphere-vacuum interface, the degree of CA (or "heating" due to the quasi-thermal nature of the excitation) becomes sufficient to result in cleavage of covalent bonds and dissociation of species as large as proteins. As shown in Figure 3, for horse heart cytochrome *c*, increasing Δ(Or-Q0) to 60 V results in dissociation of the highest charge states (for example, compare intensities for the 18+ state). This arises from the greater translational excitation for higher charge states (i.e., proportional to *z*), which is partially transferred to internal energy through collisions. In fact, this method was used for our initial MS/MS studies of cytochrome *c* molecular ions [8, 13]. The nozzle-skimmer arrangement, however, provides more efficient dissociation in the interface because collisions occur in a region of better-defined pressure. (Available results suggest CA in this region becomes inefficient for pressures above ~10 torr.) Such conditions serve to limit the kinetic energy distribution of ions entering Q1. Figure 6 shows mass spectra from a similar experiment for horse heart myoglobin (M_r 16,951), where Δ(N-S) is varied from +85 V to +285 V. We find that at higher voltages the higher charge states are largely dissociated, leading to the fragmentation evident across the entire *m/z* range. Because neither mass spectrometric operation conditions nor ion kinetic energy change significantly as Δ(N-S) is increased, the contributions clearly arise from the large number of dissociation products. The quasi-thermal internal energy distribution, that is, the effective temperature obtained in the nozzle-skimmer region (different for each charge state), is not readily determined because of difficulties in defining actual pressures, collisional cross sections, and energy transfer efficiencies. However, as described previously [13], collisional heating in this region can be used to increase the efficiency of subsequent CAD processes in the tandem mass spectrometer. In such an application, molecular ion internal energy is elevated to a level that minimizes the CAD collision energy subsequently required after mass selection.

These data highlight the utility of collisional heating in the atmosphere-vacuum interface, where CAD processes can yield spectra with useful structural information for smaller ions and fingerprinting of larger species without the necessity of tandem instrumentation. The collisional heating in this region results in dissociation without any significant loss of total ion current [indeed, the higher Δ(N-S) bias typically im-

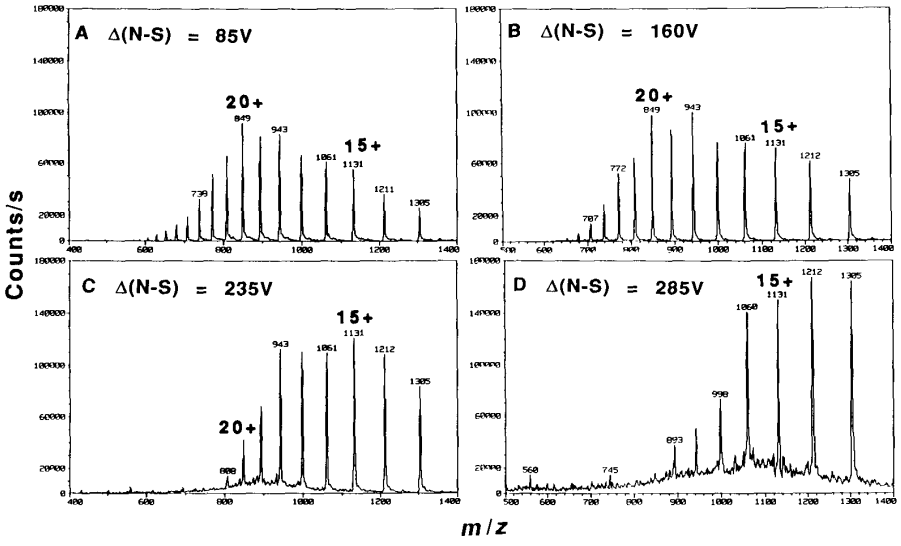

Figure 6. Electrospray ionization mass spectra for horse heart myoglobin obtained with the modified differentially pumped sampling region at various values of the nozzle-skimmer bias, Δ(N-S). The major peaks represent the series of protonated molecular ion charge states.

proves ion transmission in this region]. It also provides an effective route for generating fragment ions from large molecular ions. These can be studied subsequently by MS/MS to provide confirmation of sequence assignments and directly access previously intractable regions of the molecule by MS/MS of the intact precursor ion. The negligible "background" from ESI (evident by comparison of Figure 4a and b) also enhances the utility of this method compared to other matrix-assisted desorption ionization methods (e.g., fast-atom bombardment) due to the low intensity and limited number of solvent-related "matrix" ions.

Tandem Mass Spectrometry of Large Polypeptides

Collision-activated dissociation of multiply charged molecular ions of melittin in the triple-quadrupole instrument yields dissociation products that represent nearly the entire polypeptide sequence [11]. Tandem mass spectra of doubly charged precursor ions from smaller peptides have also been reported by other laboratories [16]. However, it must be noted that without knowledge of product ion charge state, a priori sequencing for larger molecules by CAD spectra is currently precluded due to the large number of possible products in various charge states.

Figure 7a gives the ESI mass spectrum for human parathyroid hormone (1-44), M_r 5064, dominated by the +5 to +8 protonated molecular ions. Smaller contributions are also noted from species of both higher and lower mass. Peaks on the low-*m*/*z* side (e.g., *m*/*z* 705.8, 823.5, 988.0) of each multiply charged parathyroid hormone molecular ion indicate a molecule of unknown origin with M_r 4935. Figure 7b gives the CAD mass spectra obtained with laboratory frame collision energies of 900 eV for the +5 to +8 precursor ion charge states. Tentative sequence assignments are given for several major dissociation products and are ascribed to, primarily, singly charged fragments originating from both ends of the molecule. In contrast to the previous success with the smaller peptide melittin [11], a large portion of the polypeptide sequence appears inaccessible under the present collision conditions with our current instrumentation. One would expect such limitations to obtaining "useful" CAD fragments to be increasingly manifest as molecular weight increases. Although excellent studies of CAD of singly charged peptides with M_r up to approximately 2500 have been reported with quadrupole [17] and tandem magnetic sector [18] instruments, relatively few reports [19] for larger molecules have been published. However, some sequence information appears to be readily obtainable from CAD of multiply charged molecular ions generated by ESI for large peptides with $M_r > 3000$ [20]. In fact, the MS/MS spectra of human parathyroid hormone represent the first case, to our knowledge, in which *any* useful sequence-related information for a biomolecule with $M_r > 5000$ has been obtained from the intact species mass spectrometrically. It appears that the multiple charging phenomenon generally enhances CAD efficiencies. Higher energy collisional processes in sector instruments may be anticipated to be somewhat more successful for these multiply charged ions, as for singly charged precursors. In addition, MS/MS of product

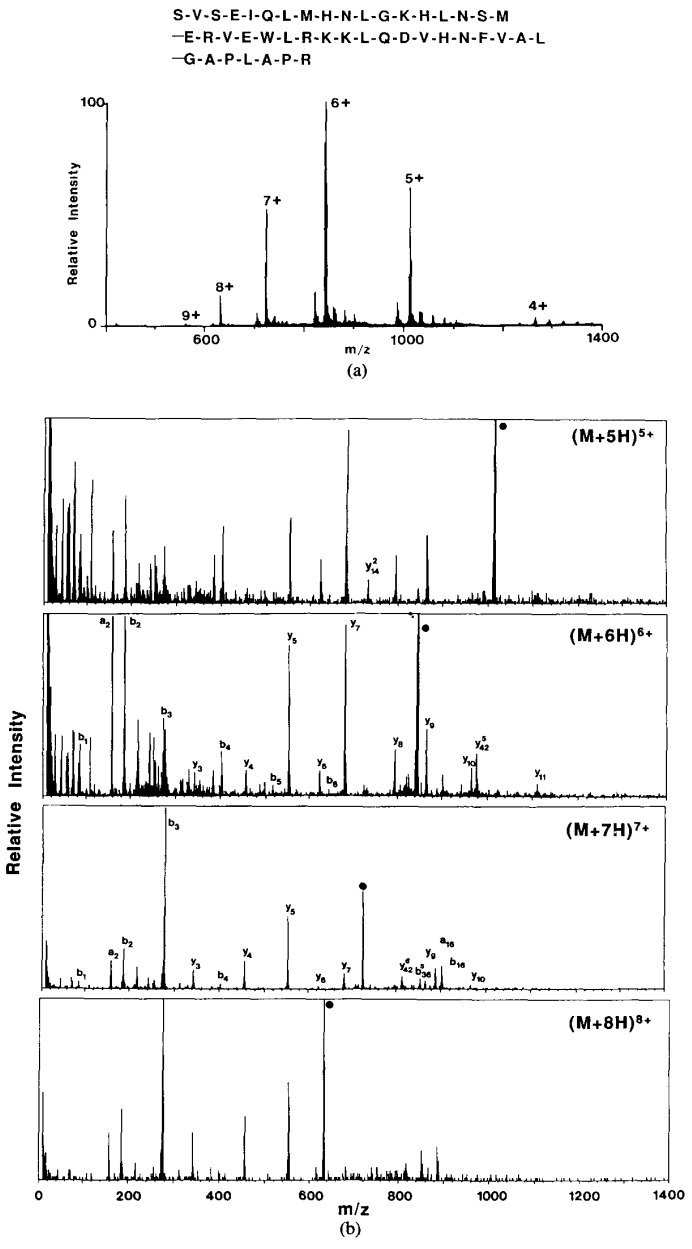

Figure 7. (**a**) ESI/MS spectrum for human parathyroid hormone (M_r 5064) showing dominant 4+ to 9+ multiply charged molecular ions; Δ(N-S) = 175 V. (**b**) 900-eV CAD mass spectra for the 5+ to 8+ multiply protonated molecular ions (indicated by •) showing several sequence assignments. Many similar product peaks are observed from different charge state precursors.

ions generated from collisional processes in the interface region should allow more sequence information to be obtained from larger peptides such as parathyroid hormone.

Collision-Activated Dissociation of Myoglobin

The mass spectrum obtained by CAD of the $(M + 20H)^{20+}$ molecular ion of horse heart myoglobin (153 amino acid residues, M_r 16,951) at a laboratory frame collision energy of 2000 eV with the original TAGA 6000E sampling orifice is given in Figure 8. As reported earlier for the somewhat smaller protein cytochrome *c*, fragmentation observed is distinctive of a particular charge state and is often concentrated in the region about the *m/z* of the molecular ion [8]. Also observed at these collision energies are relatively intense contributions at lower *m/z*, which we tentatively attribute to small (possibly singly charged) fragments from the ends of the polypeptide chains of *both* the molecular

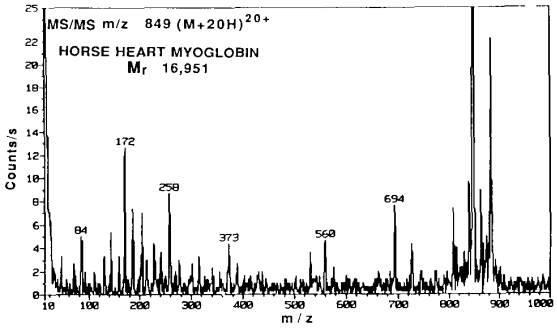

Figure 8. 2000-eV CAD mass spectrum for the $(M + 20H)^{20+}$ molecular ion of horse heart myoglobin obtained with the original TAGA 6000E sampling orifice and Δ(Or-Q0) = 5 V. The spectrum shows the more distinctive higher *m/z* region and the less distinctive lower *m/z* products (typical of most proteins studied to date). Noise is ≃ 1 count/s.

and larger fragment ions [8]. Such sequential dissociation processes can be minimized by lowering collision energies [8] but contribute to the present study due to the large collision cross sections of the molecular ions (estimated to be on the order of 10^{-12} cm^2) and the desire to maximize product ion intensities [13].

Figure 9 gives CAD spectra for the 20+ to 22+ charge states of myoglobin, showing the higher *m/z* region. The spectra were obtained with the original TAGA 6000E design, under the same collision conditions as for Figure 8. In each case distinctive spectra are obtained, although they are limited by the low product intensities and the noise level of ~ 1 count/s. Several interesting features are evident when these spectra are compared. First, peaks at *m/z* 684 and 726 are evident for each charge state. Although not definitive, such behavior suggests that these ions have relatively low charge states. Second, a peak corresponding to a gain of one charge is evident in each spectrum. "Charge stripping" (i.e., ejection of an electron), or another process leading to loss of a small negatively charged particle, was observed previously in our tandem quadrupole studies of cytochrome *c* molecular ions [8]. Interestingly, this putative "charge stripping" peak was observed only for collisions of "cold" molecular ions [with Δ(Or-Q0) = 0 V], where normal CAD processes (e.g., fragmentation) were energetically precluded [13]. In the present studies at Δ(Or-Q0) = +5 V, very small internal excitation was afforded and, if the cytochrome *c* results can be generalized, the quasi-thermal distribution may be sufficiently broad to allow both "charge stripping" and CAD.

Improved tandem mass spectra were obtained by using the nozzle-skimmer arrangement (Figure 10). For this arrangement, CAD after collisional heating depends upon both the nozzle-skimmer bias Δ(N-S) and the skimmer-Q0 bias Δ(S-Q0). Figure 10 shows a better signal-to-noise ratio than Figure 9a due to the greater primary ion current. Of particular interest is

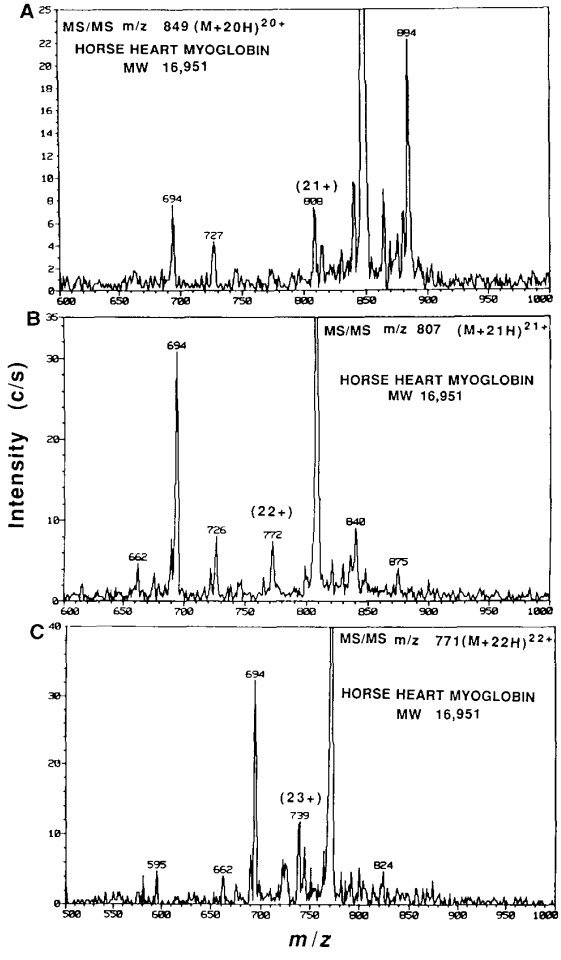

Figure 9. Collision-activated dissociation mass spectra for the 20+ to 22+ charge states of myoglobin obtained with the original TAGA 6000E sampling region (same conditions as for Figure 8). Note the peak in each spectrum attributed to "charge stripping" (gain of one charge).

the substantially reduced intensity of the "charge stripping" peak at *m/z* ~ 808. This observation is consistent with previous results for cytochrome *c* having greater internal excitation [8, 13]. Somewhat improved CAD efficiency is obtained by increasing Δ(S-Q0), reflected by the I_{885}/I_{849} ratio as shown in Figure 11. However, such operating conditions are of limited practical value here because of the much greater attenuation of the primary ion intensity. Apparently, increasing Δ(S-Q0) affects both the ion kinetic energy and ion trajectories sufficiently to greatly degrade transmission through the tandem quadrupole arrangement, decreasing the primary ion intensity (I_{849^+}) by a factor of over 40. Interestingly, the only product peak observable in Figure 11, which is also the major product under other conditions (*m/z* 885), is formed more than an order of magnitude more efficiently than under the condi-

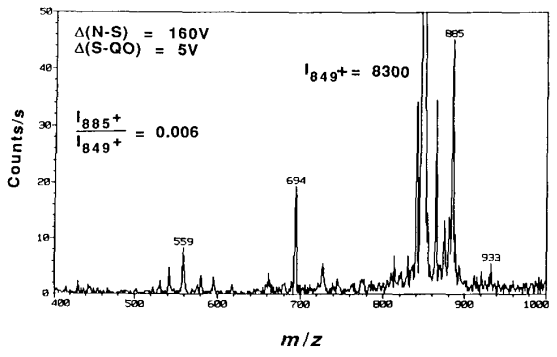

Figure 10. 2000-eV CAD mass spectrum for the $(M + 20H)^{20+}$ molecular ion of myoglobin using the modified ion-sampling region. Note the absence of the peak at *m/z* 808 ascribed to "charge stripping" in Figure 9a.

tions of Figure 10. This increase is much larger than can be ascribed to mass spectrometer "tuning," evaluated under a wide range of conditions. Discrimination of this magnitude is also unlikely because of the relatively small difference in *m/z*. This observation is qualitatively consistent with our previous observation of the effect of CA upon subsequent CAD efficiencies [13] and shows that such "heating" can also be controlled by manipulation of the Δ(S-Q0) electric field strength. Currently, optimum CAD product intensities are obtained with only moderate collisional heating in the interface. However, dissociation of very "hot" molecular ions is of fundamental interest and might be useful analytically if improved ion focusing can be obtained (preventing loss of precursor ion intensity).

The effect of Δ(S-Q0) voltage is more readily apparent from observation of metastable dissociation products obtained by removal of the collision gas (argon). While true zero collision conditions in Q2 are never obtained in the present instrumentation because of the

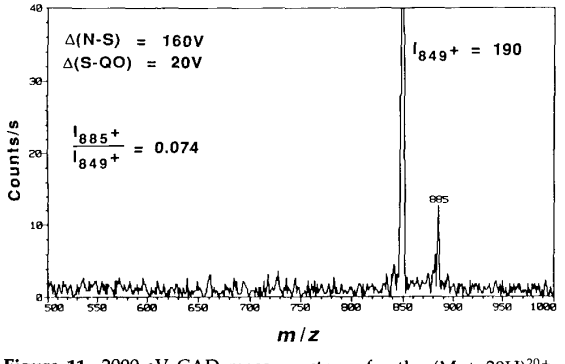

Figure 11. 2000-eV CAD mass spectrum for the $(M + 20H)^{20+}$ molecular ion of myoglobin obtained for ions collisionally activated in the interface. Although primary ion intensity is reduced greatly, the rate of formation of the *m/z* 885 ± 1 product has also greatly increased.

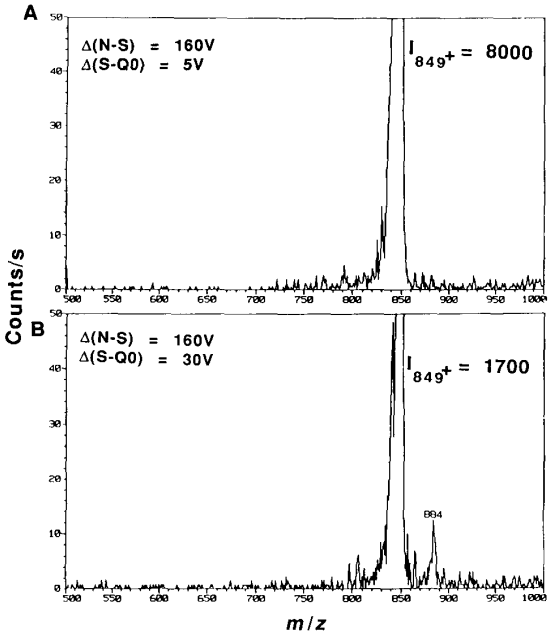

Figure 12. Metastable dissociation mass spectra (no collision gas introduced) for the $(M + 20H)^{20+}$ molecular ion of myoglobin at (**a**) higher and (**b**) lower degrees of CA. The "warmer" molecular ions (**b**) show the *m/z* 884 ± 1 product noted in the CAD spectra (Figures 9a and 11).

large collision cross sections for protein molecular ions and significant background pressures, previous studies have established that such conditions can yield pure metastable dissociation spectra [8, 13]. At low Δ(S-Q0) bias (5 V), no significant products are observed, as shown in Figure 12a. At Δ(S-Q0) = 30 V (Figure 12b), the greater internal excitation affords a product at $m/z \sim 884 \pm 1$, consistent with the major CAD fragmentation channel observed in Figure 11. Although assignment of sequence information for such protein fragment ions is currently beyond our experimental capabilities, we note, from CAD of the $(M + 20H)^{20+}$ molecular ions, that a peak at *m/z* 884 is consistent with a 19+ charge state product formed by loss of the *N*-terminal Gly-Leu residues (y^{19}_{151}). The complementary b_2 ion is present at *m/z* 172 (Figure 8). In fact, peaks at *m/z* 258 and 373 are also present, indicative of the b_3 and b_4 singly charged ions, while peaks at *m/z* 880 and 884 suggest the y^{19}_{150} and y^{19}_{149} complements. Consistent with this assignment, the peak at *m/z* 884 is absent from the CAD of the $(M + 21H)^{21+}$ molecular ion (Figure 9b), while we observed a prominent ion at *m/z* 840, consistent with the y^{20}_{151} fragment ion. It will be interesting to determine whether such similarities are common for other high-charge-state precursor ions. Highly similar fragmentation, differing in a systematic manner for precursor charge states, would allow comparison of spectra with the aim of examining

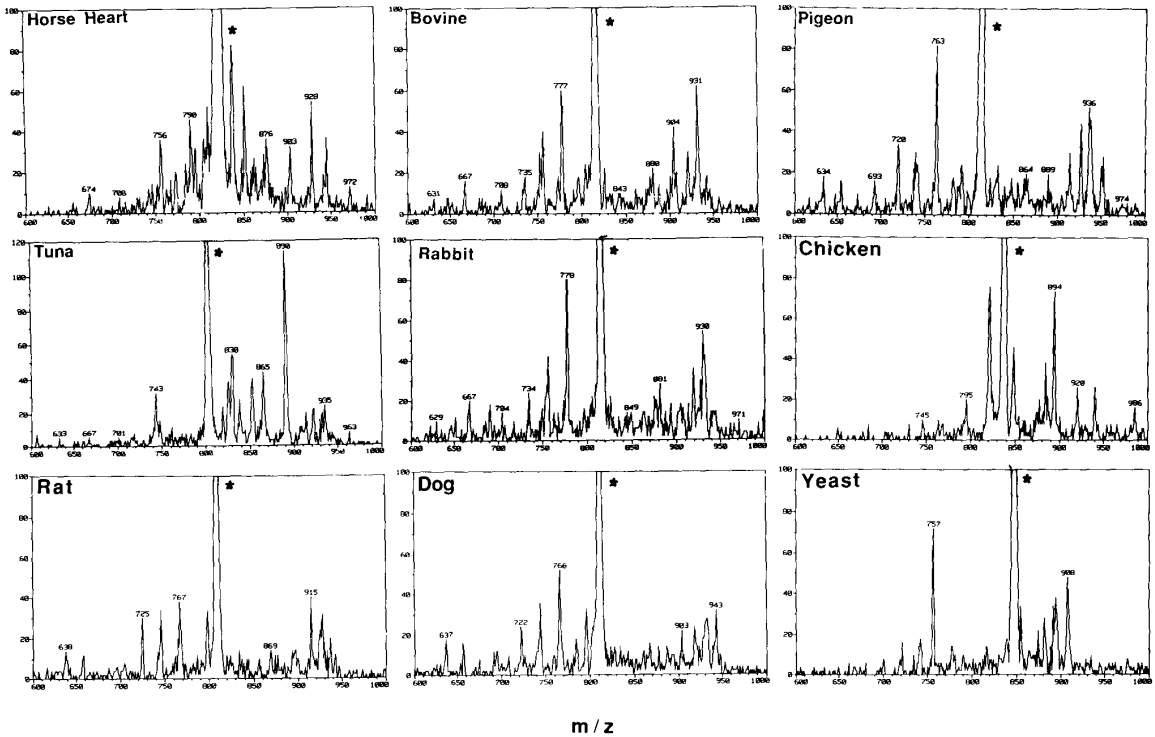

Figure 13. Collision-activated dissociation "fingerprints" obtained for the $(M + 15H)^{15+}$ ion (indicated by *) of cytochrome *c* from nine different species and yielding distinctive, sometimes subtle, differences in product spectra.

differences in charge states of the various products. In this manner, both mass and charge of the fragment might be obtained, as is currently accomplished for multiply charged parent ions [3–8].

Application of Collision-Activated Dissociation to Protein Fingerprinting

The above studies, together with previously published results [8], show that CAD can be effective for proteins as large as 17,000 u. Unfortunately, both the absence of product ion charge state information and limited mass spectrometer resolution make it extremely difficult to interpret the spectra in terms of amino acid sequence. However, the fact that significant CAD fragment intensities are observed (within the above limitations) has prompted us to examine the CAD of proteins with closely related primary structures.

Figure 13 gives CAD mass spectra obtained for the $(M + 15H)^{15+}$ charge state of cytochrome *c* from nine species. The spectra show the *m/z* 600–1000 region, where most product ions are observed. The mass spectra were obtained using the original TAGA 6000 E interface [sampling orifice Q0 arrangement; Δ(Or-Q0) = 5 V] at collision energies of ~1500 eV laboratory frame and an argon collision gas thickness of 5×10^{13} molecules/cm^2. These spectra are signal-averaged from five scans obtained over a 20-min period and were reproducible in terms of relative product ion intensities to better than ±20% on a day-to-day basis. The molecular weights for the various species span a moderate range ($M_r \sim 12,040$–$12,700$) and can be quite readily differentiated by careful measurement, as reported previously [3, 6, 7]. However, of greater significance is the observation of both the strong similarities and the important differences among the spectra. In particular, the bovine, rabbit, and dog proteins are highly similar (differing by less than four amino acid residues in the sequence) and show the expected similarities. However, significant differences are also observed. For example, although both the bovine and rabbit cytochrome *c* show products at *m/z* 778±1, such a peak is absent for the dog cytochrome *c*, which instead shows a distinctive fragment at *m/z* 766±1. The prominent *m/z* ~ 904 peak for bovine cytochrome *c* is absent for rabbit cytochrome *c*. Other peaks at lower intensities also show differences. (It should be noted that what appears to be noise in these spectra is almost certainly unresolved and minor CAD products above

the actual noise level of $\simeq 1$ count/s.) Cytochrome *c* proteins with greater differences in sequence show more substantial differences as expected.

These results clearly suggest the potential of MS/MS for "fingerprinting" of proteins, even in the absence of the ability to obtain and assign sequence information. Although nanomole quantities of protein were used in the present studies, greatly improved sensitivity should be obtainable. Recent studies have indicated that electrospray ionization efficiency is substantially suppressed at the flow rate and concentrations used for the present work [20]. Sample sizes at least two orders of magnitude smaller should be accessible if even minor gains are achieved in the performance of current instrumentation. Additional studies are in progress with the aim of obtaining such improvements.

Conclusions

It has been established that the CAD of proteins as large as 17 kilodaltons can be sufficiently efficient to yield measurable product ion intensities by using a tandem quadrupole mass spectrometer. The CAD spectra can be readily correlated with polypeptide sequences for molecules as large as 5000 u. As molecular weight increases, product ions are observed that represent more limited regions of the polypeptide chain. However, given the ambiguities that result from both limited resolution and the absence of product charge state information, it is currently difficult to obtain sequence information for molecules larger than $\sim 10,000$ u.

The CAD mass spectra of the proteins cytochrome *c* and myoglobin show a relatively limited number of CAD products. This is consistent with the large internal energy required for dissociation and the relatively small incremental steps provided by low-energy collisions. The present results show that CAD of protein molecular ions can yield distinctive product ion spectra of potential utility for qualitative "fingerprinting." Generation of such spectra apparently benefits from the progressively more limited suite of processes, which may be both energetically and kinetically feasible for very large molecules. The manipulation of molecular ion internal energy in the atmosphere–vacuum interface is feasible and can affect the relative abundance of CAD products, at least under the multiple-collision conditions required in this research.

Although obtaining direct sequence information from proteins is currently difficult, alternative approaches may alleviate this situation. For example, an instrument that provides sufficiently high performance to resolve isotopic contributions, allowing product charge states to be determined, would afford the potential of product determination to much higher molecular weights than is now practical. If the CAD processes for proteins with different charge states are similar, yielding CAD fragments systematically shifted due to changes in their charge state, then it may be possible to directly obtain limited sequence information. Alternatively, sequential dissociation steps (e.g., MS^n), perhaps using ion-trapping devices, may provide a useful approach (particularly in combination with high-resolution methods). As an example, CAD in the atmosphere–vacuum interface of melittin, followed by MS/MS of selected fragments, has been demonstrated and has allowed confirmation of previous assignments.

Finally, the use of higher energy collisional processes might be expected both to significantly increase CAD efficiency and to provide increased fragmentation via higher energy pathways. While the potential of such methods remains to be determined for larger proteins, it is clear that the high degree of multiple charging afforded by ESI has presented new opportunities and new challenges for application of mass spectrometry to large biomolecules.

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