

Collision-induced Dissociation of 30 m/z Unit Wide Windows of Electrospray-generated Ions Sampled Under Lens Conditions of Nominally Zero Potential Gradient

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Windows 30 m/z units wide of ions generated in electrospray and sampled under lens conditions of nominally zero potential gradient conditions were mass selected with Q1 in a triple-quadrupole mass spectrometer, fragmented in Q2, and their product ions mass analysed with Q3. A variety of analytes (equine myoglobin, bovine insulin, leucine enkephalin and PPG 1000) were examined. The windows selected sometimes contained desolvated (bare) analyte ions; more often they did not and contained only 'background' ions. For protein samples, multiply charged product ions having m/z values both lower and higher than those of the precursor ions were observed even when the precursor ion window could not have contained naked protein dimer ions. To observe significant product ions, the precursor ion window typically contained ions residing within background 'humps' that trailed protein peaks. These ions were speculated to be diverse, solvated multimeric clusters of the analyte which fragmented in Q2 to yield the characteristic product ion spectra. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Electrospray is arguably one of the most versatile sample introduction methods in mass spectrometry, being amenable to a variety of analyte ions ranging from simple inorganic ions¹ to complex biological macro ions.² For most analytes, pre-formed ions in solution are electrosprayed (sometimes with the aid of a nebulizer gas³) into air at 1 atm pressure. The mechanism of ion production in electrospray is a subject of much interest and discussion.^{4,5} However, there is little doubt that a variety of charged species, including ions, clusters and droplets, are produced and can potentially be sampled for analysis through an orifice. In order to minimize sampling of solvent and other polyatomic molecules, it is common practice to employ a 'curtain gas' or 'counter-current bath gas,' which sweeps the region immediately upstream from the orifice and presumably keeps this region relatively free of these molecules. On some instruments, e.g. the SCIEX TAGA 6000E, turning off the curtain gas results in a significant reduction of the electrospray signal, presumably due to a significant increase in clustering in the lens region of

the mass spectrometer. Furthermore, it is also common practice to transmit the sampled ions under an appropriate potential gradient in that region to ensure efficient transmission as well as fragmentation of any clusters that may be present.

The importance of this 'front-end' collision-induced dissociation (CID) has been highlighted in studies carried out by Smith and co-workers^{2,6} and Thomson.⁷ In studies of fragmentation of multiply charged protein ions, Smith *et al.*² observed that the product ion spectra of $[M + nH]^{n+}$ ions of cytochrome *c* ($n = 9–12$) contained low abundances of other multiply charged cytochrome *c* ions, e.g. for $n = 10$, low abundances of $[M + 9H]^{9+}$, $[M + 10H]^{10+}$ and $[H + 11H]^{11+}$ were also present. They attributed these low-abundance ions to the presence of a small percentage of dimer ions that were sampled; these dimers fragmented to monomer units of different charges during CID. However, in a subsequent study where mild interface conditions were emphasized and were highly disparate monomer abundances were observed, Smith *et al.*,⁶ reassigned those monomer ions with m/z values lower than that of the precursor ion to products of CID of the solvated clusters that they mass selected. They proved, by means of a precursor ion scan, that some of the $[M + 16H]^{16+}$ product ion of cytochrome *c* could be formed from declustering of adducts of the $[M + 16H]^{16+}$ ion with solvent molecules. In Thomson's work,⁷ model protein ions, e.g. myoglobin, were also electrosprayed and

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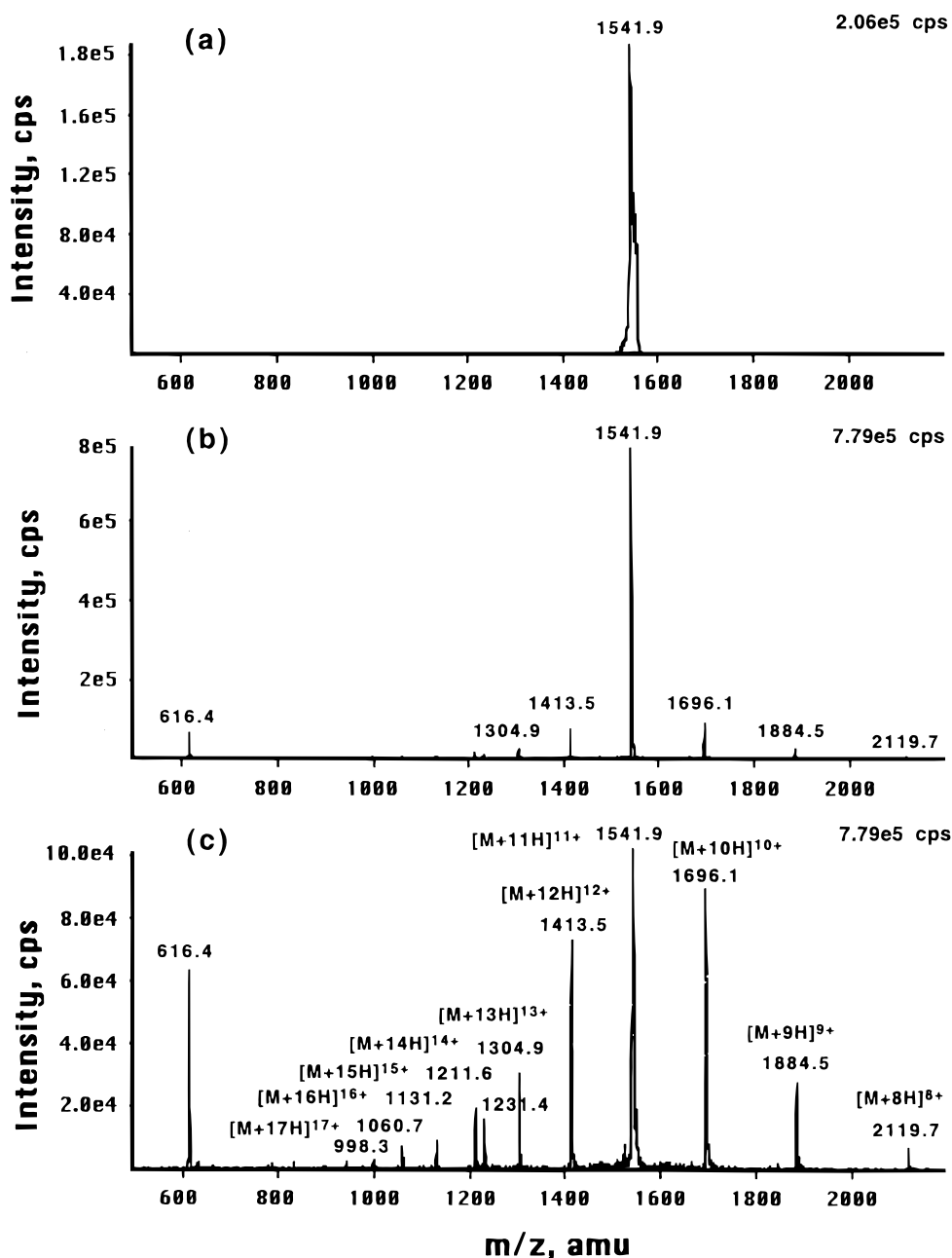


Figure 1. Product ion spectra of 10 μM equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; ~ 30 m/z unit precursor ion window centred on m/z 1542; axial acceleration voltage (Q0 – Q2), 52 V, i.e. for the $[\text{M} + 9\text{H}]^{9+}$ ion, $E_{\text{lab}} \approx 468$ eV: (a) no collision gas in Q2; (b) 4 mTorr of nitrogen; (c) results from (b) shown with an expanded abundance scale.

sampled into the mass spectrometer under mild lens conditions. In some experiments, the first quadrupole (Q1) of a triple-quadrupole mass spectrometer was operated as a high-pass filter to transmit multiply charged protein ions larger than a given m/z value; these transmitted ions were mass resolved by means of the third quadrupole (Q3). In the absence of collision gas in the second quadrupole (Q2), only the 'expected' multiply charged protein ions were observed; that is, if Q1 was set to transmit ions beyond an m/z value of 700, only ions with $m/z > 700$ were observed in mass scanning Q3. In the presence of collision gas, however, 'unexpected' ions were encountered; in the same example, more highly charged protein ions having m/z values smaller than 700 would be recorded. In other

experiments, Q1 was set to pass a multiply charged protein precursor ion under unusually low resolution, and Q3 was scanned under unit mass resolution to resolve the product ions after CID in Q2. What was observed was that, aside from the unreacted precursor ion, other multiply charged protein ions having charges both larger and smaller than that of the precursor ion were also present. Agreeing with the earlier interpretation of Smith *et al.*,² Thomson⁷ attributed his observations to the presence of a small fraction of protein dimers that were mass selected along with the monomers.

Stimulated by these reports, we decided to investigate these phenomena and this paper describes results of some of these investigations.

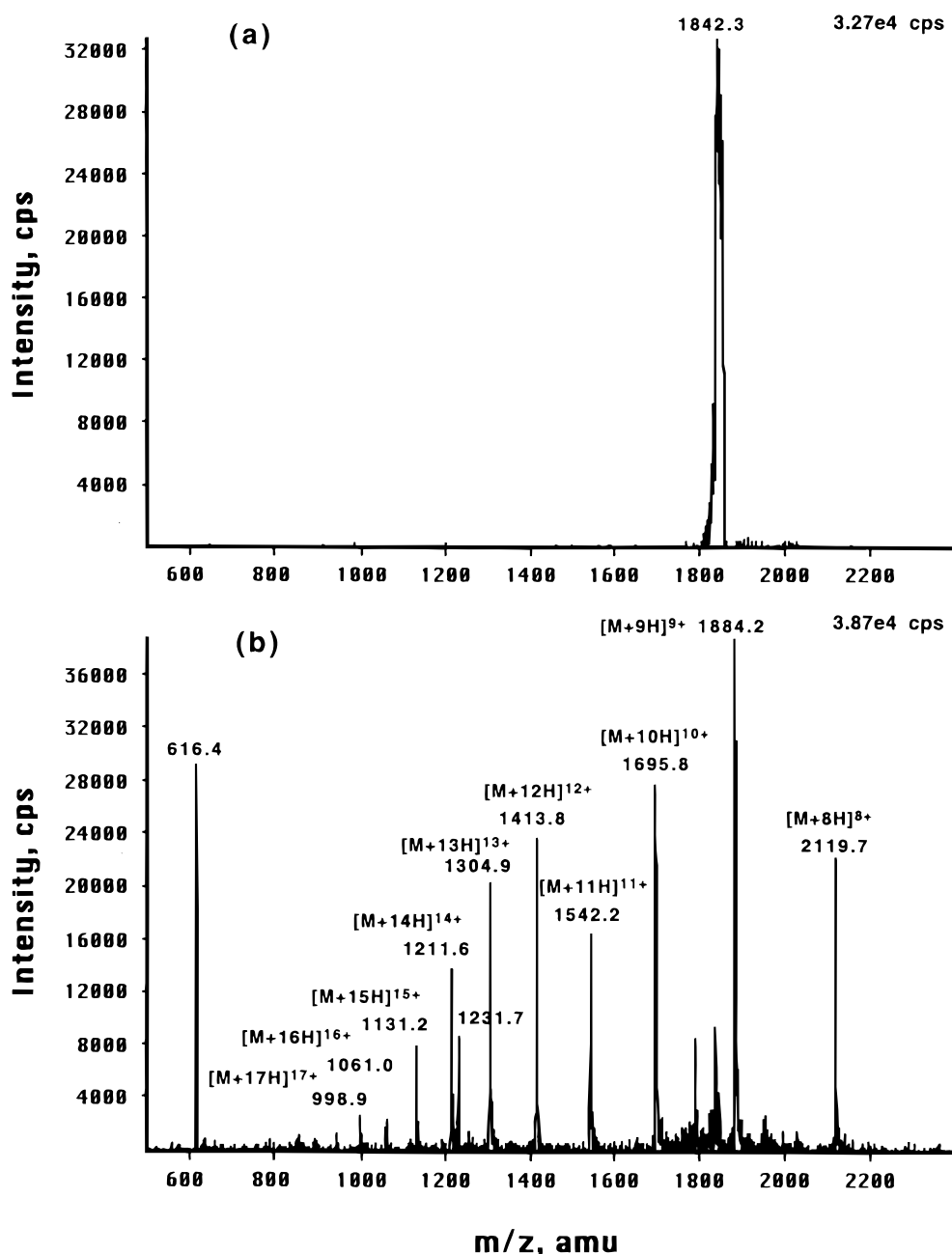


Figure 2. Product ion spectra of 10 μM equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; ~ 30 m/z unit precursor ion window centred on m/z 1842; axial acceleration voltage ($Q0 - Q2$), 52 V: (a) no collision gas in $Q2$; (b) 4 mTorr of nitrogen.

EXPERIMENTAL

Experiments were carried out on a PE-SCIEX (Thornhill, ON, Canada) API 300 triple-quadrupole mass spectrometer. Samples (equine myoglobin, bovine insulin, leucine enkephalin and PPG 1000, all from Sigma, St Louis, MO, USA) were typically 10 μM in concentration dissolved in either water or mixtures of water and methanol; acetic acid, ammonia and ammonium acetate were used for pH adjustment. They were continuously infused using a Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) into the electrospray probe via a 150 μm o.d., 75 μm i.d. fused-silica tube (Polymicro Technologies, Phoenix, AZ,

USA). Nitrogen was used as the nebulizer gas. The pneumatically assisted electrospray probe was typically biased at 5 kV, 4 kV positive with respect to the interface plate. The optimum probe position was established from time to time, but was typically with the tip about 2 cm from the interface plate and with the spray off-axis from the orifice.

Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a typical scan speed of 10 ms per 0.3 m/z unit. Ten scans were summed to produce a mass spectrum. Most experiments were carried out with $Q3$ being the resolving quadrupole, and with the orifice (OR) and the quadrupole lens ($Q0$) biased to ground potential; that is, ions were sampled under a nominally zero potential gradient from OR to

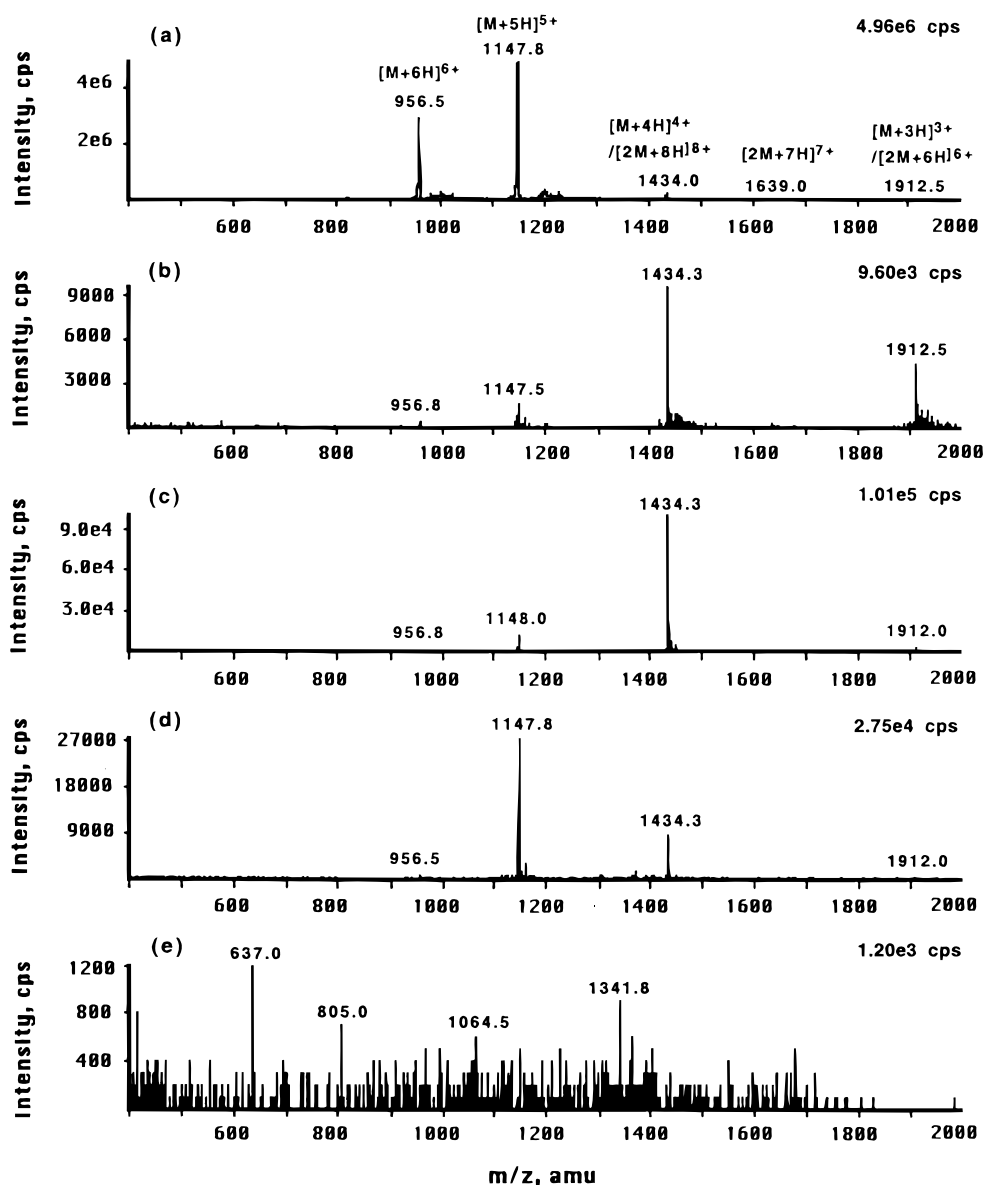


Figure 3. 10 μM bovine insulin in water-methanol (1:1) containing 0.2% acetic acid: (a) Q3 scan where $\text{OR} = \text{Q0} = 0$ V; and product ion spectra of 30 m/z unit wide precursor ion window centred on (b) m/z 1800, (c) m/z 1500, (d) m/z 1300 and (e) m/z 1100; axial acceleration voltage ($\text{Q0} - \text{Q2}$), 52 V.

Q0 (the skimmer, immediately downstream from the orifice, is permanently grounded). As a result, a Q3 scan would produce a mass spectrum collected under nominally zero collision energy conditions in the lens region. To generate a product ion spectrum, Q1 was set to pass a precursor ion window of typically ~ 30 m/z units at 10% peak maximum; nitrogen was used as the collision gas in Q2 at a pressure of 2–4 mTorr (1 Torr = 133.3 Pa). The collision energy at the laboratory frame of reference (E_{lab}) was typically 10–50 eV for singly charged ions.

RESULTS AND DISCUSSION

Our first attempts were to reproduce the findings of Smith and co-workers^{2,6} and Thomson.⁷ Figure 1 shows product ion spectra of an ~ 30 m/z unit wide

window centred on the $[\text{M} + 11\text{H}]^{11+}$ ion of equine myoglobin: (a) in the absence of collision gas in Q2 and (b) and (c) with 4 mTorr of nitrogen. It is evident in Fig. 1(c) that multiply charged equine myoglobin ions from +8H to +17H were present. In fact, it was not necessary to select a precursor ion window that included any of the $[\text{M} + n\text{H}]^{n+}$ ions to observe this effect. Figure 2 shows product ion spectra of a 30 m/z unit wide precursor window centred on m/z 1842, which is sandwiched between the $[\text{M} + 9\text{H}]^{9+}$ and the $[\text{M} + 10\text{H}]^{10+}$ ion at m/z 1884 and 1696, respectively. Again, it is evident that the product ions include multiply charged myoglobin ions from +8H to +18H. Ions such as these have been ascribed to the presence of a small percentage of protein dimers along with the monomer that was selected.^{2,7} The abundance of the multiply charged product ions seen in Fig. 2(b), their charges and the precursor ion window chosen appear to cast doubt on this interpretation. First, the m/z values of the relevant equine

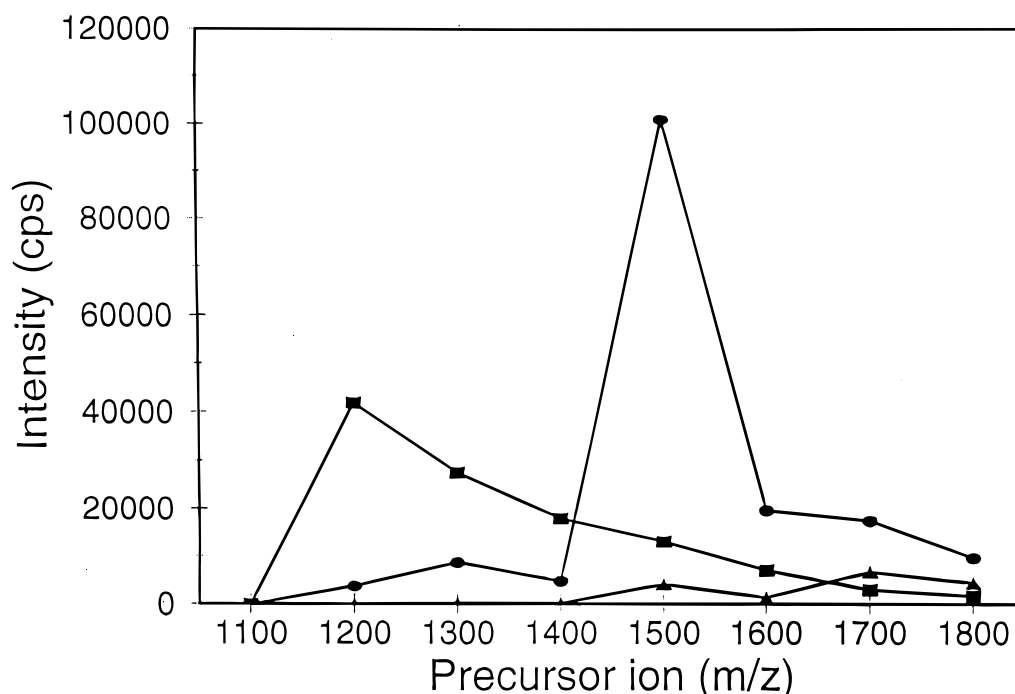


Figure 4. Abundances of $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ product ion of insulin versus the m/z values of the precursor ion window: data from Fig. 3. $\blacktriangle$, $[M + 3H]^{3+}$; $\bullet$, $[M + 4H]^{4+}$; $\blacksquare$, $[M + 5H]^{5+}$.

myoglobin dimer ions are: 1885 ($[2M + 18H]^{18+}$), 1785 ($[2M + 19H]^{19+}$) and 1696 ($[2M + 20H]^{20+}$). The precursor window centred on m/z 1842 would have contained very few of any dimer ions [see Fig. 2(a)]—too few to yield a product ion spectrum [Fig. 2(b)] whose intensity is comparable to that of the precursor ion. Second, product ions as highly charged as +18H are apparent in Fig. 2(b). Although some charge disproportion when the dimer fragments is evident in some of the spectra shown in Ref. 6, it is unlikely that one monomer would carry 80–90% of the charges and the other the remaining 20–10% [one reviewer suggested that to minimize Coulombic repulsion, a highly protonated monomer may preferentially interact with a less highly protonated monomer. In general, Coulombic repulsion would make any cation–cation reaction in the gas phase unfavourable. For any dimerization to occur in the gas phase, the monomers must, therefore, be very heavily solvated (or self-solvating) to reduce the effective charge. If that is the case, this preference will be diminished. Furthermore, there is evidence that protons attached to proteins and peptides in the gas phase are relatively mobile.^{8,9} To reduce Coulombic repulsion, mobile protons would tend to redistribute themselves somewhat evenly between the two monomer units, thus making highly uneven distributions unlikely]. However, if fragmentation of multimers had not occurred, it would have been difficult to reconcile the presence of abundant $[M + 8H]^{8+}$ and $[M + 9H]^{9+}$ product ions.

To confirm these results, we performed similar experiments on bovine insulin, which yields fewer and thus more widely spaced multiply charged peaks. Figure 3 shows the results of these experiments: (a) Q3 scan where OR = Q0 = 0 V; and product ion spectra of an approximately 30 m/z unit wide precursor ion window centred on (b) m/z 1800, (c) m/z 1500, (d) m/z 1300 and

(e) m/z 1100. Typical of full-scan mass spectra collected in this study, the multiply protonated protein peaks, such as $[M + 5H]^{5+}$ and $[M + 6H]^{6+}$, were often trailed by wide 'humps' (for lack of a better description), which were indicative of extensive and complex clustering. In the CID experiments, both precursor windows centred on m/z 1800 and 1500 would not be expected to contain significant numbers of insulin dimer ions (expected at m/z 1912, 1639 and 1434). However, their product ion spectra contained abundant multiply charged insulin ions with m/z values both lower and higher than those of the precursor ions. A comparison of the product ion spectra in Fig. 3 suggests that the abundances of the multiply charged insulin ions were related to the m/z values of the precursor window. This relationship is more convincingly displayed in Fig. 4, which shows the abundances of the $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ ions (m/z 1912, 1434 and 1149, respectively) with different precursor ion windows. The abundances of the $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ ions maximized at precursor ion windows centred on m/z 1500 and 1200, respectively, values just exceeding the m/z values of the ions and where clustering was apparently extensive [see Fig. 3(a), m/z 1200, at which a hump was evident]; the abundance of the $[M + 3H]^{3+}$ ion was relatively low.

Similar results were obtained in a study of leucine enkephalin, YGGFL. The electrospray mass spectrum of this pentapeptide is simple, showing the $[M + H]^+$ ion (m/z 556) and the $[2M + H]^+$ ion (m/z 1111) at an abundance a few per cent of that of the former. Figure 5 shows the abundances of the two leucine enkephalin ions with different precursor ion windows selected. As in Fig. 4, the abundances of both ions in the product ion spectrum maximized at precursor ion windows just exceeding the m/z values of the peptide ions. It is,

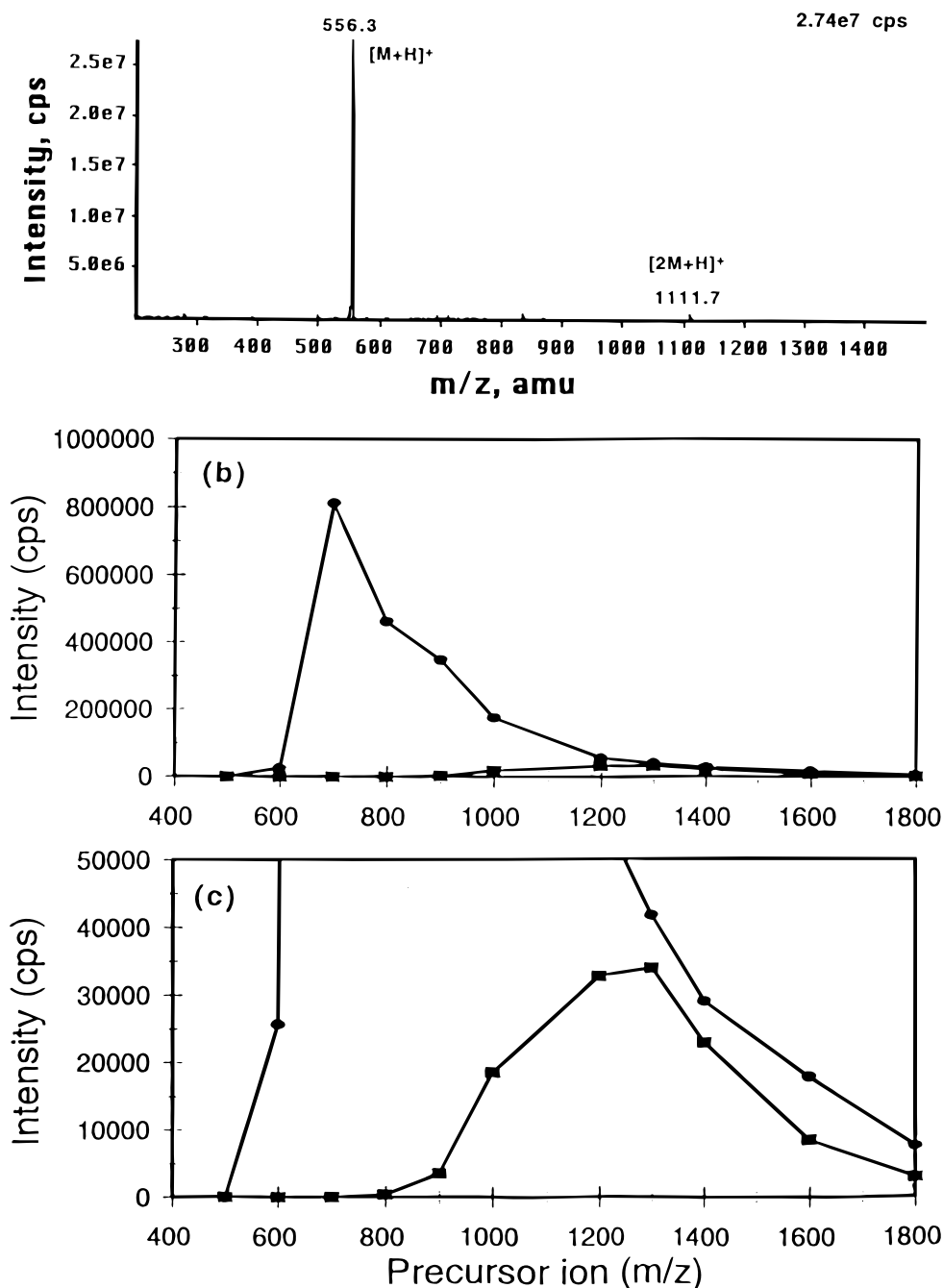


Figure 5. 10 μ M leucine enkephalin in water-methanol (1:1) containing 0.2% acetic acid; (a) Q3 scan; (b) abundances of $[M+H]^+$ (●) and $[2M+H]^+$ (■) versus m/z of precursor ion window; (c) expansion of (b); axial acceleration voltage ($Q0-Q2$), 20 V, i.e. for singly charged ion, $E_{lab} \approx 20$ eV.

perhaps, noteworthy that the leucine enkephalin dimer behaved similarly to its monomer ion.

Figure 6 shows the Q3 scan of a 1 mM polypropylene glycol (PPG) 1000 solution in methanol-water (1:1) containing ammonium acetate. As expected, we observe a distribution of primarily single ammonium adducts of PPG chains varying in the number of propylene units and maximizing at about 1000 Da; at about m/z 1600, the abundance of the PPG chains drop to a few per cent of the maximum although there exist PPG chains whose molecular masses exceed 2000 Da (see Fig. 7). Product ion spectra of precursor ion windows of ~ 150 m/z units wide are displayed in Fig. 7. The extra wide

windows were found necessary to yield product ions that were sufficiently abundant; the presence of NH_4^+ -containing PPG product ions in the product ion spectra is evident. It is, perhaps, noteworthy that the difference between the centre of the precursor ion window and the most abundant product ion of each scan appears to increase with increasing m/z values of the precursor window. It should also be pointed out that, when the precursor ion window was reduced to 1 m/z unit wide and included only a higher m/z PPG ion, no PPG product ions were produced, thus proving that these ions were not fragmentation products of longer PPG chains.

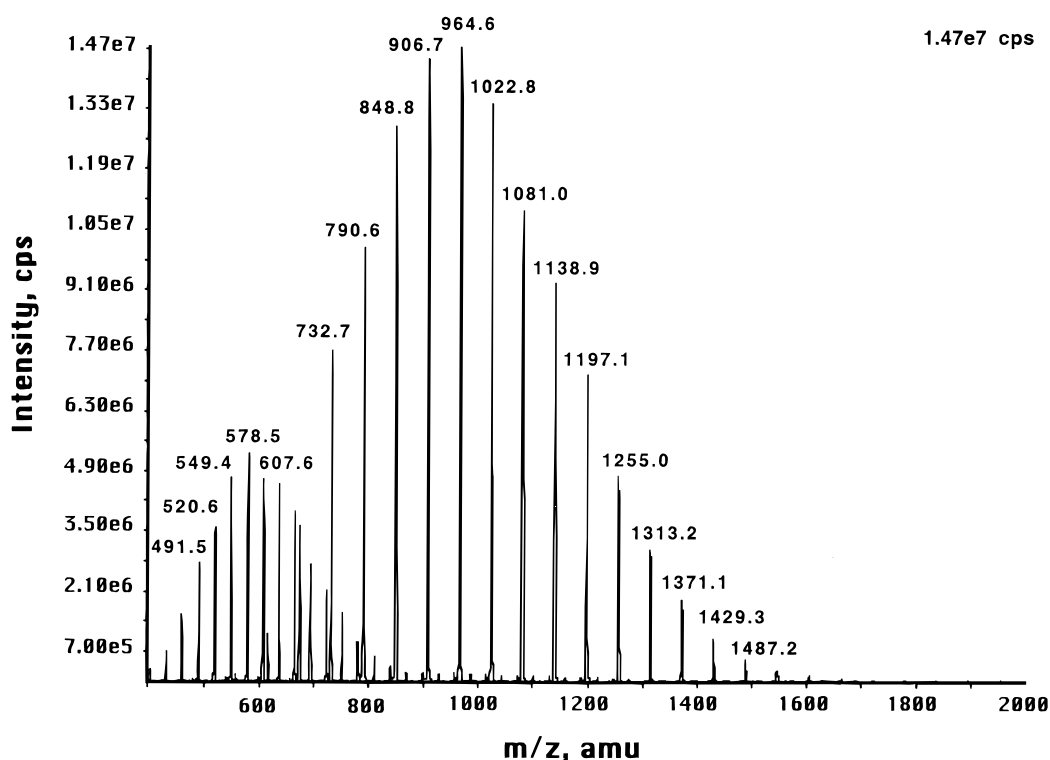


Figure 6. 1 mM polypropylene glycol 1000 in water-methanol (1 : 1) containing 1 mM ammonium acetate; Q3 scan.

The results that we have presented are consistent with the hypothesis that a significant fraction of the ions exiting Q0 and entering Q1 were very heavily clustered and complex. We speculate that these ions included not only solvated monomers but also large multimeric species of the analyte ions (e.g. multimeric species of myoglobin, leucine enkephalin and PPG). These multimeric species could be dry particles and/or solvated clusters of the analyte. A reoccurring trend in our experiments was that the m/z values of the precursor ion window selected were non-critical, and there were always some characteristic product ions observed, as long as the precursor window was a few tens of m/z units wide and that it resided within the multiply charged envelope of the protein ions. To produce these results, the clusters themselves must, therefore, have been highly variable; that is, they had to comprise a highly variable number (but not the nature) of monomer units and solvent units as well. The humps displayed in Figs 3(a) and 8 (Q3 scan of myoglobin) contained ions that could be deconvoluted as adducts between the protein monomer and a small molecule (or a number of small molecules). For example, the series of peaks marked with asterisks in Fig. 8(b) were calculated to have a molecular mass of 17114 ± 3 Da, 162 Da above the molecular mass of apomyoglobin (16952 ± 2 Da). Obviously, the humps contained many species other than the +162 Da adduct; however, most of them were buried in the 'chemical noise.' It is our interpretation that many multimeric species of varying degree of solvation, thus populating virtually all m/z values within a given range, constituted the ions carried by the hump. The existence of an elevated background between analyte peaks was most evidently displayed in Figs 3 and 8; it should be noted that such a background also

existed in the other Q3 scans although they were less prominent.

Desolvation and fragmentation of mass-selected multimeric species in Q2 accounted for the observation of monomer units of varying degrees of protonation in the product ion spectra. The relatively high pressure and, consequently, the large number of collision events attributable in Q2 of the API 300 mass spectrometer probably resulted in a relatively low threshold for this process. The fact that few dimeric ions (the only exception being those of leucine enkephalin, Fig. 5) were observed in the product ion spectra means that the multimeric species were held by weak forces and few naked oligomers could be produced from or survived the CID process. The monomers within the multimeric protein clusters could bear different numbers of attached protons. For this reason, product ions of proteins having m/z values both higher and lower than those of the precursor ions were possible [Figs 1(b) and (c), 2(b) and 3(b)–(e)]. (Product ions with higher m/z values could not have been formed from cleavage of proton adducts from more highly charged, and hence lower m/z values, ions; no significant small ions were observed in the low- m/z segments of any of the above product ion spectra.)

It should, perhaps, be emphasized that the diverse multimeric clusters referred to in this paper are likely to contain more than a few monomer units. For bovine insulin, the multiply charged ions are separated by a few hundred m/z units (Fig. 3). Even with heavy solvation, it is difficult to envisage how relatively intense product ion spectra, such as those shown in Fig. 3(b)–(d), could be formed from dimers of insulin. Furthermore, it should also be repeated that these large multimeric clusters were observed to exit from Q0; put differently, their

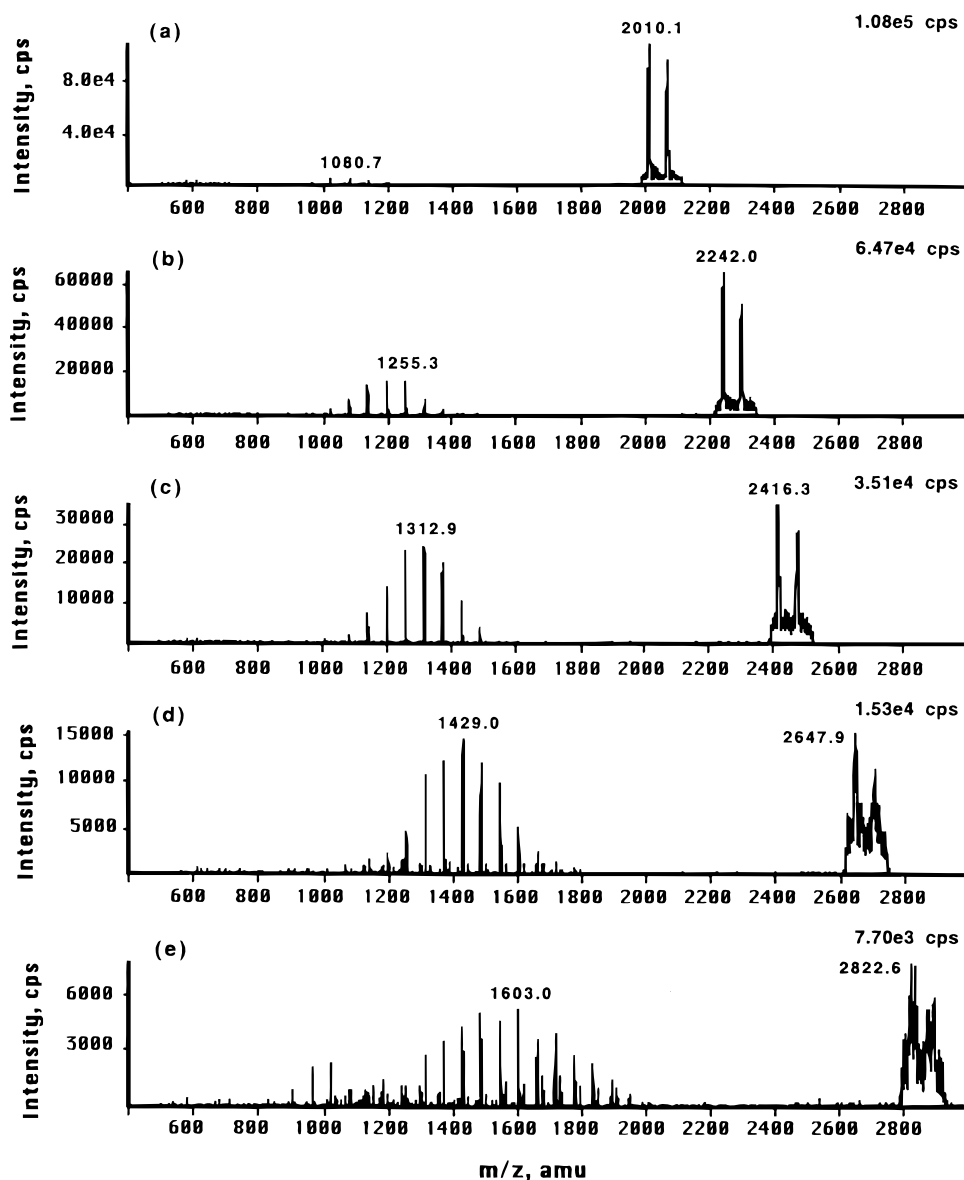


Figure 7. Product ion spectra of 150 m/z unit wide windows of polypropylene glycol 1000 centring on (a) m/z 2009, (b) m/z 2241, (c) m/z 2416, (d) m/z 2642 and (e) m/z 2819; axial acceleration voltage ($Q0 - Q2$), 50 V.

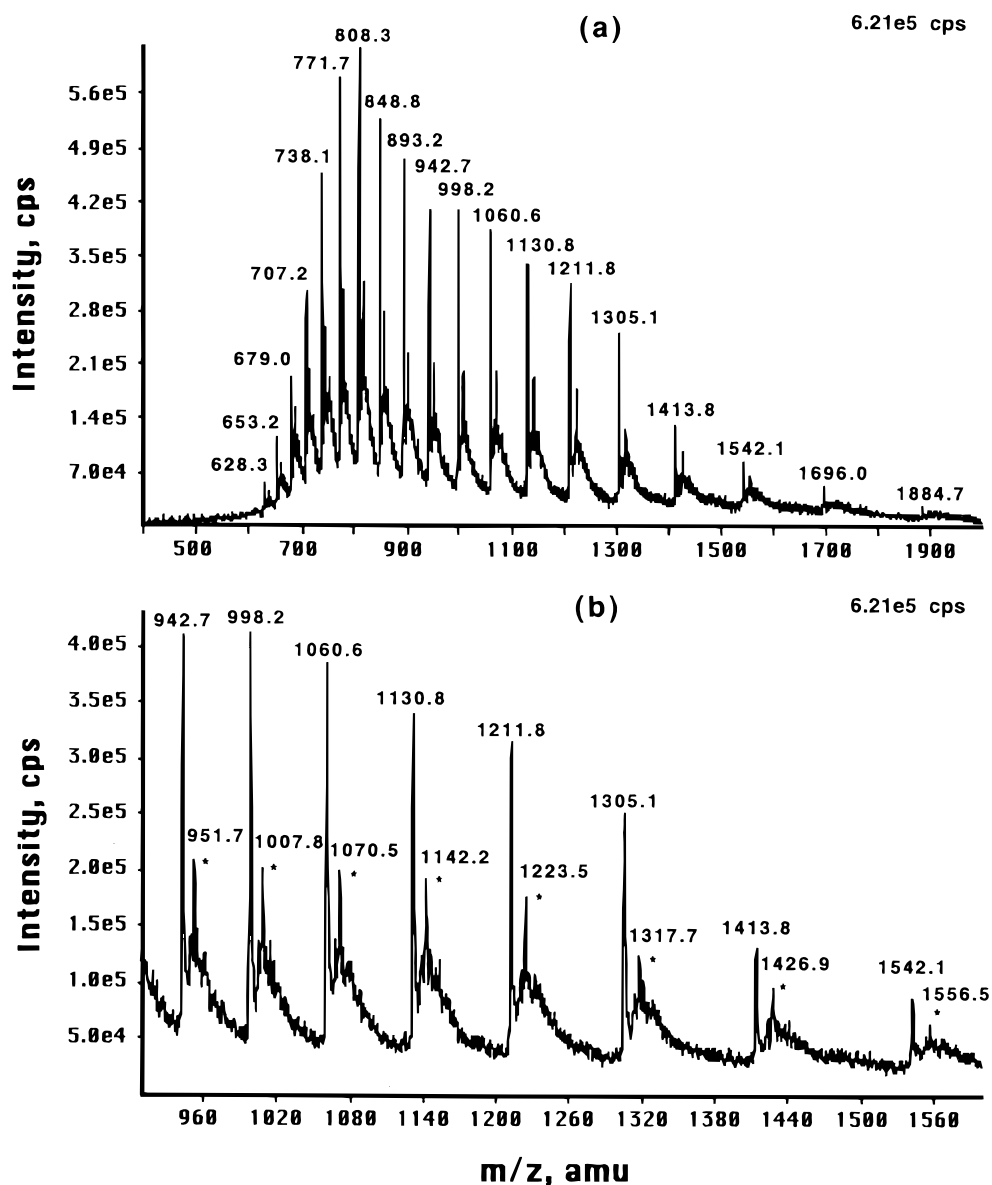


Figure 8. Q3 scan of 10 μ M equine myoglobin in water-methanol (1:1) containing 0.2% acetic acid; OR = Q0 = 0 V: (a) full spectrum; (b) expansion of (a); asterisks indicate a +162 Da adduct of myoglobin.

existence could only be traced back to that point. At present, it is unknown whether these species exist in the electrospray plume at 1 atm.

CONCLUSION

A significant quantity of electrospray-generated ions sampled under very mild lens conditions appeared to exit Q0 as large multimeric species. In fact, the number

of monomer units and exact composition of these species varied to such a large extent that individual ions were typically not resolved and they constituted the chemical noise in the electrospray mass spectrum; their existence, however, was inferred from the CID of a window of background ions, which yielded relatively high abundances of monomeric units. It is not known if these large multimeric species exist in the electrospray plume prior to sampling.

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