High-Resolution Tandem FT Mass Spectrometry above 10 kDa

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The Michelson interferometer revolutionized spectroscopy with its capabilities to measure a wide range of wavelengths simultaneously (Fellgett's advantage), with unusual precision (Conne's advantage), and without slits to reduce radiation intensity (Jaquinot's advantage).[1] The interferometer records overlapping frequencies that are converted to a wavelength spectrum by a Fourier transform (FT). For mass spectrometry (MS), Comisarow and Marshall introduced the same advantages into the ion-cyclotron-resonance technique to give us the powerful FTMS instrument; its trapped ion capabilities for sequential ion selection, reaction, and measurement also provide multiple "tandem" MS (MS^n).[2] Similarly revolutionary for MS have been matrix-assisted laser desorption ionization (MALDI)[3] and electrospray ionization (ESI),[4] which extend MS applicability from molecules of a few kilodaltons in size to those of hundreds of kilodaltons, or even larger.[5] However, these mass spectra also have orders-of-magnitude higher requirements with respect to the number of possible peak masses and the accuracy and resolution needed for their measurement; for this, FTMS has been developed to provide unique MS^n capabilities for molecular weight (MW) and structural characterization of biomolecules and polymers larger than 10 kDa.[6,7]

In FTMS, ions stored on the magnetic field axis (z-axis) between electrostatic trapping electrodes are excited with resonant radio frequencies (rf) into cyclotron orbits in the x,y-plane.[2] A coherent orbiting packet of ions of the same mass/charge (m/z) value induces into opposing parallel detector plates an rf signal. The frequency of this sine wave is determined by the ions' m/z and the magnetic field strength only, and the wave amplitude is proportional to the number of charges in the ion packet. Orbiting ions of many m/z values induce many such frequencies of differing amplitudes; FT of these overlapping signals produces the corresponding mass spectrum of m/z vs ion abundance.[2,6] A variety of research achievements from many laboratories were critical for the ESI/FTMS techniques employed here.[6,7] These include the resolution enhancements of Wilkins and Gross,[6a] Freiser's pulsed valve addition of collison and reagent gases,[6b] Russell's direct introduction of biomolecular ions by desorption ionization,[6c] the Hunt and McIver quadrupolar transmittance of ions through the fringing magnetic field[6e] with differential pumping,[6f] Marshall's ion excitation and detection techniques,[6g] and Laude's open cylindrical ion cell[6h] and optimized ion trapping.[6i,j] These are covered more thoroughly in an extensive review.[7]

FTMS has been applied successfully to ions from MALDI,[8] but ESI is especially valuable because its evaporative ionization minimizes molecular ion dissociation[4] and produces multiply-charged ions (with 16–36 protons in Figure 1) that can be readily dissociated to yield structural information by tandem MS (MS^n);[9] singly-charged ions larger than 3 kDa are difficult to dissociate.

Multiple charges (higher z values) also improve the FTMS resolving power (RP), as it is inversely proportional to m/z. For carbonic anhydrase (29 kDa), FTMS achieves RP = 5×10^5 (Figure 1),[10] vs $1-5 \times 10^2$ for conventional MS instruments. The resolved isotopic peaks are of special utility, as their unit mass spacing,

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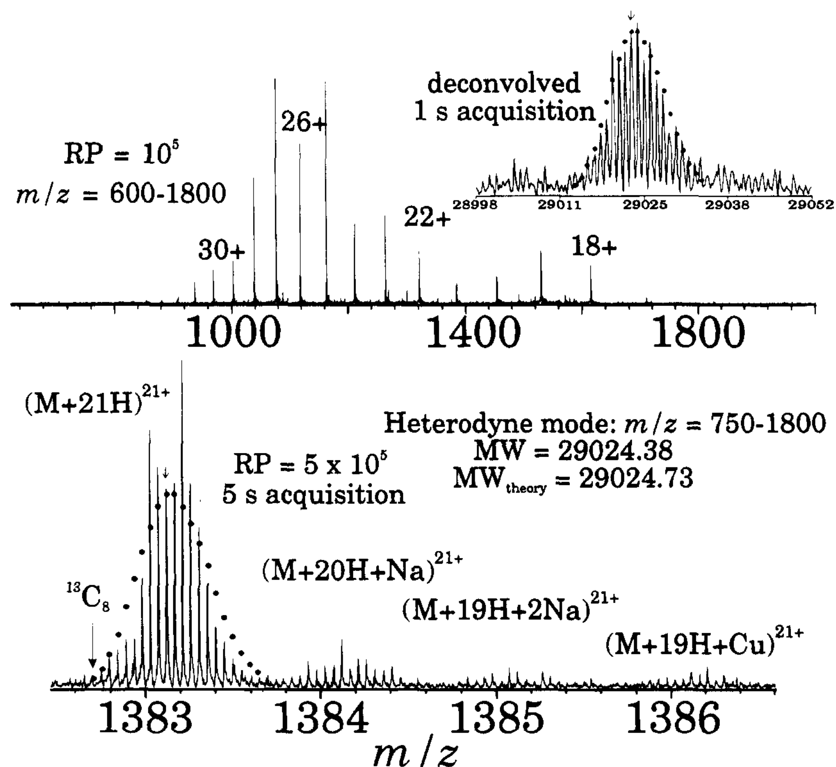

Figure 1. Broad-band ESI/FT mass spectra of bovine carbonic anhydrase. Top: Single 1-s transient showing $(M + nH)^{n+}$ for n = 16–36. Inset: Summed isotopic peak abundances. Bottom: Heterodyne spectrum of $(M + 21 H)^{21+}$ isotope peaks and Na and Cu adducts, RP = 5×10^5. Superimposed dots are the predicted isotopic abundances.

$m(^{13}C) - m(^{12}C)$ = 1.0034 Da, provides an accurate mass scale;[11] in Figure 1, the 21 isotopic peaks in one m/z unit show that this is the $(M + 21H)^{21+}$ ion. Although convenient "deconvolution" algorithms are available for identifying an m value from two or more peaks of the same m but different z values,[12] this becomes more ambiguous with increasing number of m values and increasing magnitude of z values in the spectrum.[12c]

This high RP capability also means higher mass-measuring accuracy. Distinguishing 21+ vs 22+ charges in the Figure 1 (lower) spectrum requires distinguishing $^1/_{21}$ vs $^1/_{22}$ of one of the 29 000 Da, or ~1 ppm mass-measuring accuracy. In a mixture, the 13+ isotopic peaks of cytochrome c (12.3 Da) overlapped those of the 18+ of apomyoglobin (17 kDa). An isotopic doublet was measured as $\Delta m/z = 0.20$, so that using the cytochrome c peak as a standard yields a value for apomyoglobin in error by 3 mDa.[13] Even with this, however, the accuracy of the daltons value can be in question; this requires the determination of the number of ^{13}C atoms in the measured isotopic peak. Because the all-^{12}C peak is insignificant above 10 kDa, the ^{13}C value must be determined by matching the relative isotopic abundances expected against those found; although the abundances at higher RP vary substantially from those expected (dots, Figure 1, bottom), averaging abundance values for all charge states from a 1-s data collection with broader peaks (Figure 1, top right) improves this match, which is improved further by averaging many such spectral measurements.

Noncovalent Adducts. ESI is so gentle that the resulting gaseous ions can be held together by hydrogen bonding or van der Waals forces that can be indicative of such competitive interactions in solution. For example, the degree of binding of the immunosuppressive drugs FK506 and rapamycin to the cytoplasmic receptor FKBP (11.8 kDa) in the gas phase qualitatively agrees with that in solution, and no gaseous complex is formed under denaturing conditions.[14] For the leucine zipper peptide GCN4-p1, the $(M + 4H)^{4+}$ monomer and its $(M + 8H)^{8+}$ noncovalent dimer have the same nominal m/z values, but the dimer has isotope peaks spaced at 0.125 instead of 0.25 m/z units. FTMS easily resolves these monomer and dimer peaks, enabling identification of an unexpected heterodimer.[15]

Molecular Weight. For this, the accuracy of standard gel electrophoresis is 5–10%. For identity confirmation of bovine carbonic anhydrase B, $C_{1310}H_{1994}N_{358}O_{383}S_3$, the Figure 1 data yield (frequency calibration only, without an internal standard) a value of 29 024.38 Da for the most abundant isotopic peak (MW) vs 29 024.73 Da calculated from the proposed sequence, providing a high confidence (12 ppm error) that the sequence is correct.[16] The MW value calculation uses the monoisotopic values ^{12}C = 12.0000, ^{13}C = 13.0034, ^1H = 1.0078, ^{14}N = 14.0031, ^{16}O = 15.9949, etc. The normal value, MW_{av}, using elements in their

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natural isotopic abundances (C = 12.0109, H = 1.0079, N = 14.0067, O = 15.9994), is 29 024.85 Da. However, the carbon value (as low as 12.0102 in petrochemicals) in biomolecules has a natural variation of ±0.0002 (±20 ppm),[17] or ±0.26 Da for the predicted MW_{av}; the C value 12.0111 actually predicts the highest abundance for the isotopic peak at 29 025.73 Da.

Other examples from these studies include thiaminephosphate pyrophosphorylase[18a] and equine alcohol dehydrogenase (E-chain),[18b] with a similar accuracy in determining their MW values of 23 015 and 39 821 Da, respectively. Separation of rabbit muscle creatine kinase by isoelectric focusing gave three bands with equivalent enzymatic activity; however, the ESI/FTMS spectrum of the combined bands showed $(M + nH)^{n+}$ ions with a single m value corresponding to MW = 42 982 (theory, 42 981). Its mutant with Cys replaced by Ser (S by O, Δm = 16) gave MW = 42 967.[19]

However, the ESI/FTMS mass spectrum of 45-kDa protein A from *Staphylococcus aureus* gave hundreds of peaks of unit mass separation at each molecular ion charge value.[18b] Coulombic ion repulsion affects ion trajectories[20a] to degrade resolution with more than ~5×10^5 ions in the cell, so all but the 50+ ions were ejected by stored waveform inverse Fourier transform (SWIFT),[20b] and those remaining were heated with infrared laser irradiation[21] to "boil off" noncovalent adducts. The resulting spectrum showed RP = 2.5×10^5 despite severe sodium contamination, with $(M + 50H)^{50+}$ yielding MW = 46 765, a value we believe to be accurate. However, the published sequence gives MW = 45 352, while FTMS of a recombinant protein A gave MW = 44 612.[18]

Porcine serum albumin (PSA, $C_{2977}H_{4361}N_{789}O_{884}S_{35}$) was even more challenging; after all but the 43+ ions from ESI were ejected from the FTMS cell, ~1000 peaks of 1-Da spacing remained (Figure 2, top)[18b] Infrared heating[21] reduced this complexity dramatically; these data agree within a few daltons with values from the published sequence, MW = 66 740 (the MW_{av} value is 0.83 ± 0.60 Da higher). This sample was labeled "fatty acid free"; however, a "fatty acid and globulin free" sample from the same supplier gave an MW value 146 Da higher (Figure 2, inset), with Δm = 146 confirmed by a spectrum of the mixture (*vide infra*).[18b]

For nucleotides with their electronegative phosphate groups, negative ion ESI spectra are preferable.[22] For the 76-mer $tRNA^{phe}$ without cleanup, the spectrum (Figure 3, top right) is highly complex, with the 22-Da spacing of isotopic clusters indicative of Na (m = 23 Da) replacement of H.[23] Liquid chromatography (HPLC) desalting gave the Figure 3 (lower) spectrum, MW = 24 950.5 vs 24 950.3, calculated. A previously identified variant at MW + 15, $^6U{\cdot}^{67}A \rightarrow {}^6C{\cdot}^{67}G$, can be seen in Figure 3, as can another possible variant at approximately −15 Da not reported previously. A synthetic DNA 100-mer $A_{20}T_{43}C_{19}G_{18}$ gave a very complex spectrum; although MW = 30 702 agrees (±1 Da) with the expected value, other abundant ions indicate ESI dissociation as well as impurities.[23]

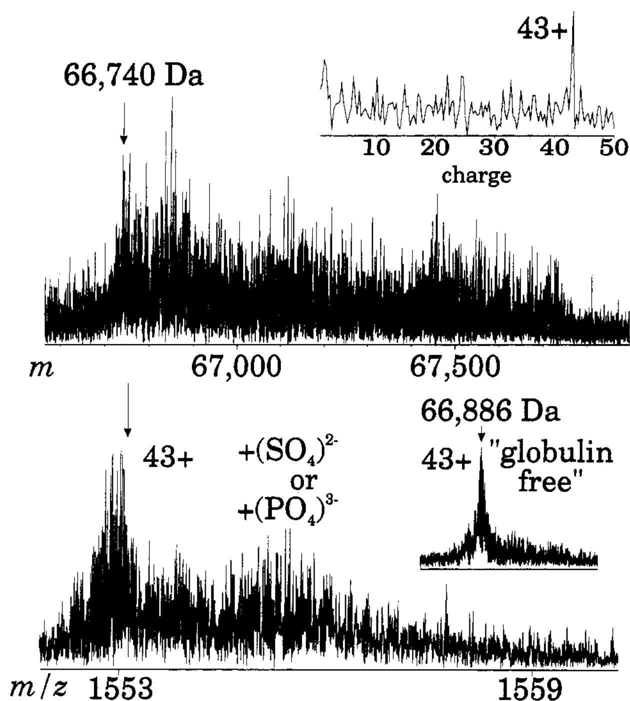

Figure 2. ESI/FT mass spectrum of porcine serum albumin (PSA) after (top) ejection of all ions except m/z 1500−1580; (inset) charge assigned as 43+ from FT of m/z values. Bottom: Expanded spectrum under same conditions after infrared irradiation of the 43+ ions; (inset) data from "globulin free" PSA.

ESI/FTMS also allows direct observation of individual oligomers of larger polymers, as illustrated by $(M + nNa)^{n+}$ ions of poly(ethylene glycol) of 21−23 kDa (Figure 4). Here FTMS has resolved over 5000 peaks from isotopic clusters representing 65 oligomers in 12 charge states.[12c]

ESI/FTMS Detection Limits. Gated trapping (500 μs) of ions in the cell while electrospraying 5×10^{-18} mol of ubiquitin (8.6 kDa) gave the Figure 5 (top) spectrum with RP = 2×10^5. However, this is only indicative of the basic sensitivity of FTMS; in recording this, orders-of-magnitude more sample were consumed in the continuous electrospray process.[24] Smith and co-workers have reported subfemtomole detection with direct introduction of mixture components separated by capillary electrophoresis.[25] As an alternative to introducing small samples, a valve with a 0.5-μL sample loop was introduced into the feed line before the electrospray needle.[24] Using a 10^{-7} M solution of ubiquitin, a 5×10^{-14} mol sample was introduced into a flowing (1 μL/min) sample-free solution in active electrospray. This "slug" of sample produced ubiquitin

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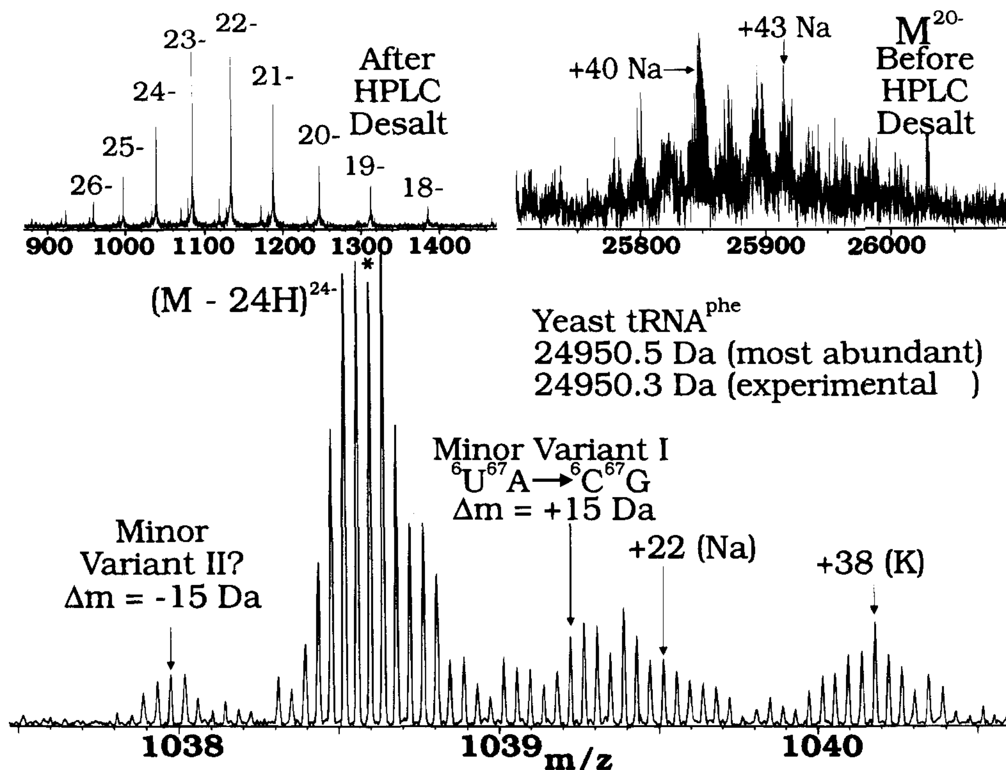

Figure 3. ESI/FT negative ion mass spectrum of phenylalanine transfer-RNA. Upper right: Broad-band spectrum of sample received. Upper left: Broad-band spectrum after HPLC purification. Bottom: Expanded 24− ion region.

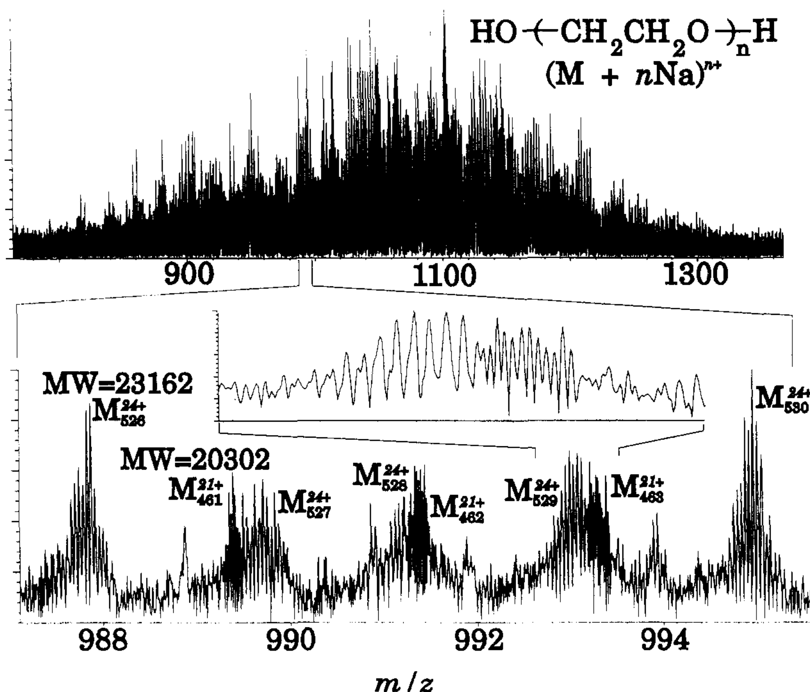

Figure 4. Broad-band ESI/FT mass spectrum (30 spectra coadded) of poly(ethylene glycol) "PEG 20,000", with regions expanded once and twice.

ions over an ~60-s period; the 1-s spectrum (RP = 4 × 10^4) of Figure 5 (bottom) gives MW = 8564.66 vs 8564.64 Da calculated and records peaks over an 8-kDa range. Although by most standards this is an unusually small amount of sample to yield such highly specific information, orders-of-magnitude improvement are obivously possible.

The ultimate of single molecule detection has been achieved by Smith and co-workers with suspended trapping of highly charged poly(ethylene glycol) ions.[5b] Although the isotopic peaks are not resolved in the m/z spectrum, sequential charge losses in 60 s of ion storage are used to determine z; in a specific case an ion of z = 2610 can be seen to lose charges stepwise to 2607, with the m/z spacings showing that m = 6.5 × 10^6 Da. More recent research of Smith's group has examined 100-MDa DNA molecules.

Primary Structure Information. For linear macromolecules, dissociation of their molecular ions can provide fragment ions whose masses are indicative of

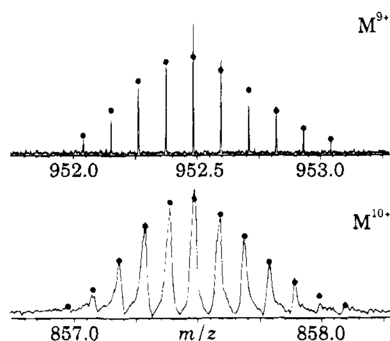

Figure 5. Partial ESI/FT mass spectrum of ubiquitin ions (top) trapped during a 10^{-4}-s spraying 20 μM at 150 mL/min (5×10^{-18} mol) and (bottom) formed by the separate introduction of 5×10^{-14} mol of sample. Superimposed dots are the predicted isotopic abundances.

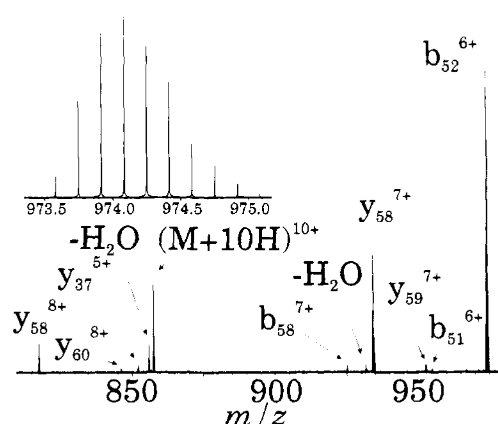

Figure 6. Heterodyne MS/MS ESI/FT spectrum, 2M data points, of bovine ubiquitin $(M + 10H)^{10+}$ ions dissociated by SORI, RP > 9×10^5. Inset: b_{52}^{6+} product.

the sequence of the backbone structure units. For such MS/MS and MS^n measurements, FTMS has several unique advantages. Collisional de-excitation of large ions, during and after FTMS measurement, causes them to spiral back down to the magnetic axis (or they can be forced back by quadrupolar axialization),[26] where they can be remeasured to improve signal/noise.[27] The mass spectrum of an individual product ion can be obtained by removing all other ions from the cell with SWIFT,[20b] dissociating the ion, and measuring its products; many such MS/MS spectra can be measured simultaneously with the Hadamard transform technique.[28] For MS/MS, the unit mass scale provided by the resolved isotopic peaks has a special advantage; dissociation of a selected ion single *z* value often yields products of only one *z* value,[9] so that *m* cannot be assigned by deconvolution.[12a] The sophisticated tandem double-focusing instrument has proven invaluable for oligopeptide MS/MS sequencing,[29] but the energy release in primary ion dissociation limits RP to 10^3-10^4 for secondary mass spectra. In contrast, FTMS *m/z* measurement is independent of ion kinetic energy, with the MS/MS spectrum of ubiquitin (8.6 kDa) showing RP = 9×10^5 (Figure 6).[30]

For dissociative characterization of the molecular ions, the most simple approach is through collisional activation (CA) of the entering ESI ion beam. This "nozzle-skimmer" (NS) ion dissociation in the high-pressure region provides a variable degree of CA.[9,31] The NS spectrum of carbonic anhydrase (Figure 7) shows ~100 molecular and fragment isotopic multiplets (tabulated in Figure 9) with *z* values identifiable from their isotopic spacings, a complexity only distinguishable with high resolution.[16,32]

In developing MS^n techniques for dissociating ions trapped in the cell, a primary requirement is efficiency. The larger the molecule, the more steps *n* of MS^n will be necessary for structural characterization, requiring a more complete recovery of the dissociation products. Optimization of a variety of methods for collisionally activated dissociation gave the highest (92%) efficiency[30] using sustained off-resonance irradiation (SORI).[33] Although infrared multiphoton dissociation (IRMPD) yields generally the same products as SORI and other collisional techniques, it is convenient for changing the degree of dissociation (Figure 8).[21b] The b- and y-type ions contain the N- and C-termini, respectively, so that in this 76-mer ubiquitin the b_{52} and y_{24} ions are complementary. Dissociation by accelerating ions into a surface appears to have the advantage of a one-step excitation; the complementary ion pairs from splitting carbonic anhydrase ions are much more equivalent in abundance.[34]

For carbonic anhydrase, MS/MS and MS^3 with SORI and IRMPD have extended the NS sequence information of Figure 7 to that of Figure 9.[16,32] Note the high tendency for cleavage on the N-terminal side of Pro residues.[9] Although this provides far from complete sequence information, these spectra would be critically valuable for verifying the sequence of carbonic anhydrase and detecting isomeric variance. Further, if the molecular weight of a recombinant product did not agree with that expected, the NS and MS/MS data would help locate the position of the structural variation. For example, NS dissociation of the creatine kinase and its mutant indicated the ^{282}Cys → ^{282}Ser substitution position in this 380 amino acid protein.[19] The abundant y_{98} ions, corresponding to ^{282}Cys–^{283}Pro bond cleavage with H rearrangement, are absent in the ^{282}Ser mutant; H transfer should be favored from the SH of Pro vs the OH of Ser.

The natural and recombinant protein A samples gave MW values 1413 Da higher and 740 Da lower, respectively, than that of the published sequence. IRMPD of the molecular ions from both samples gave 12 sequence specific peaks whose masses correspond to those expected for the first 21 C-terminal residues and the first 35 N-terminal residues, except that the latter were higher in mass by 14 Da. MS^3 of b_6 showed that this was consistent with the Val reported at

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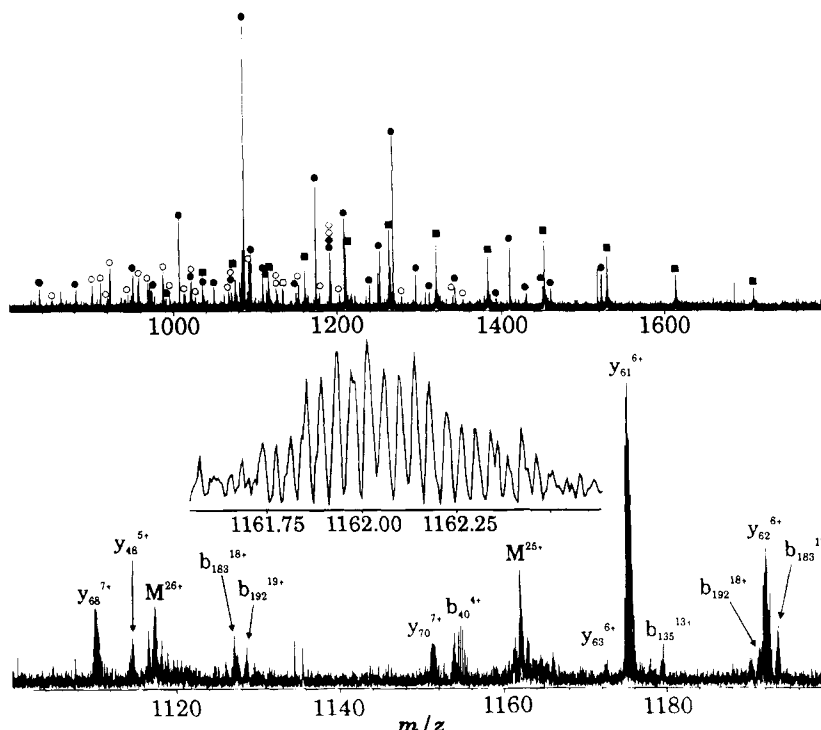

Figure 7. Nozzle-skimmer dissociation ESI/FT mass spectrum of bovine carbonic anhydrase, 25 scans summed, with one and two expansions. The $(M + nH)^{n+}$ (■), b-type (○), and y-type (●) fragments are tabulated in Figure 9.

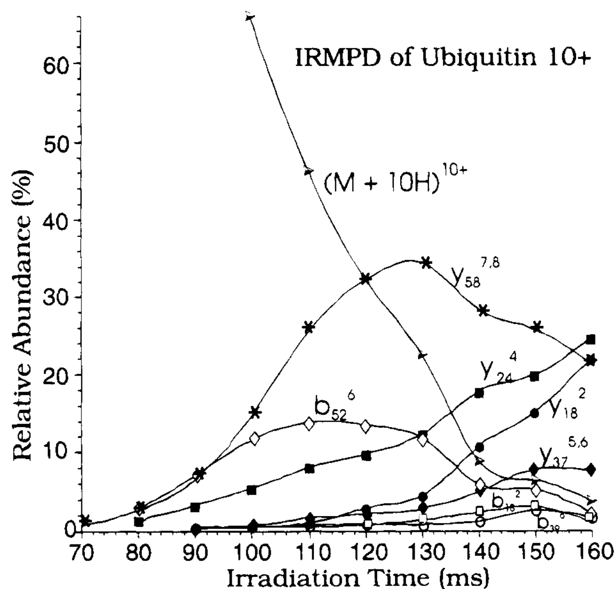

Figure 8. Infrared multiphoton dissociation of bovine 76-mer ubiquitin 10+ ions.

residue 4 actually being Leu or Ile, accounting for a small part of the MW discrepancies.[18b]

Extending the pioneering low-resolution MS/MS data already reported for albumin (67 kDa),[9] NS dissociation of the PSA samples differing in MW by 146 Da gave the same 19 fragment ion peaks corresponding to the first 34 N-terminal residues of the published sequence,[18b] verifying the seven such peaks found by Loo et al.[9] For other albumins the number of N-terminal sequence peaks found were as follows: goat, 17 (13); dog, 10 (10); and rabbit, 13 (5), with the last showing an A → T error at amino acid 26. The number identified by Loo et al.[9] (in parentheses) from the published sequence is notable, as they could only measure *m/z*, not *m*.

Nucleotides can also be sequenced by MS/MS. The major dissociation pathways elucidated by McLuckey[22a] for oligonucleotides provide the full sequence from the NS/FTMS spectrum[22b] of a 14-mer (Figure 10). The sequences of 21-mer $A_7T_5C_8G$ and 25-mer A_5T_{20} were verified with MW = 6291.15 and 7584.25, respectively (predicted 6291.10 and 7584.25) and fragment ions corresponding to 14 and 11 of the bases.[22b] Dissociation of DNA 50-mer anions of 15 307.6 Da (predicted 15 307.8 Da) produced 47 sequence specific peaks corresponding to the cleavage of 31 backbone bonds.

Tertiary Structure Information. Trapped ion MS instruments have the advantage that the ions can be conveniently reacted with an extensive variety of gaseous reagents.[6b] Many specific bimolecular reactions have been reported that are characteristic of functional groups in low-mass ions.[35] As found in protein conformational studies in solution,[36] gaseous multiply-protonated ions exposed to D_2O in the gas phase should undergo favored H/D exchange at sites on the molecule's exterior. For gaseous cytochrome *c* cations with 10^{-7} Torr of D_2O, the H/D exchange exhibits pseudo-first-order kinetics with >98% completion in 30 min.[37] The 6+ to 17+ cations exchange at eight distinct levels (Figure 11) ranging from 64 to 173 out of the 198 exchangeable hydrogen atoms (those bound to heteroatoms or to the imidazole C-2 of histidine). The same levels are exhibited by several different charge values, despite the change in electrostatic repulsion, suggesting that these represent local

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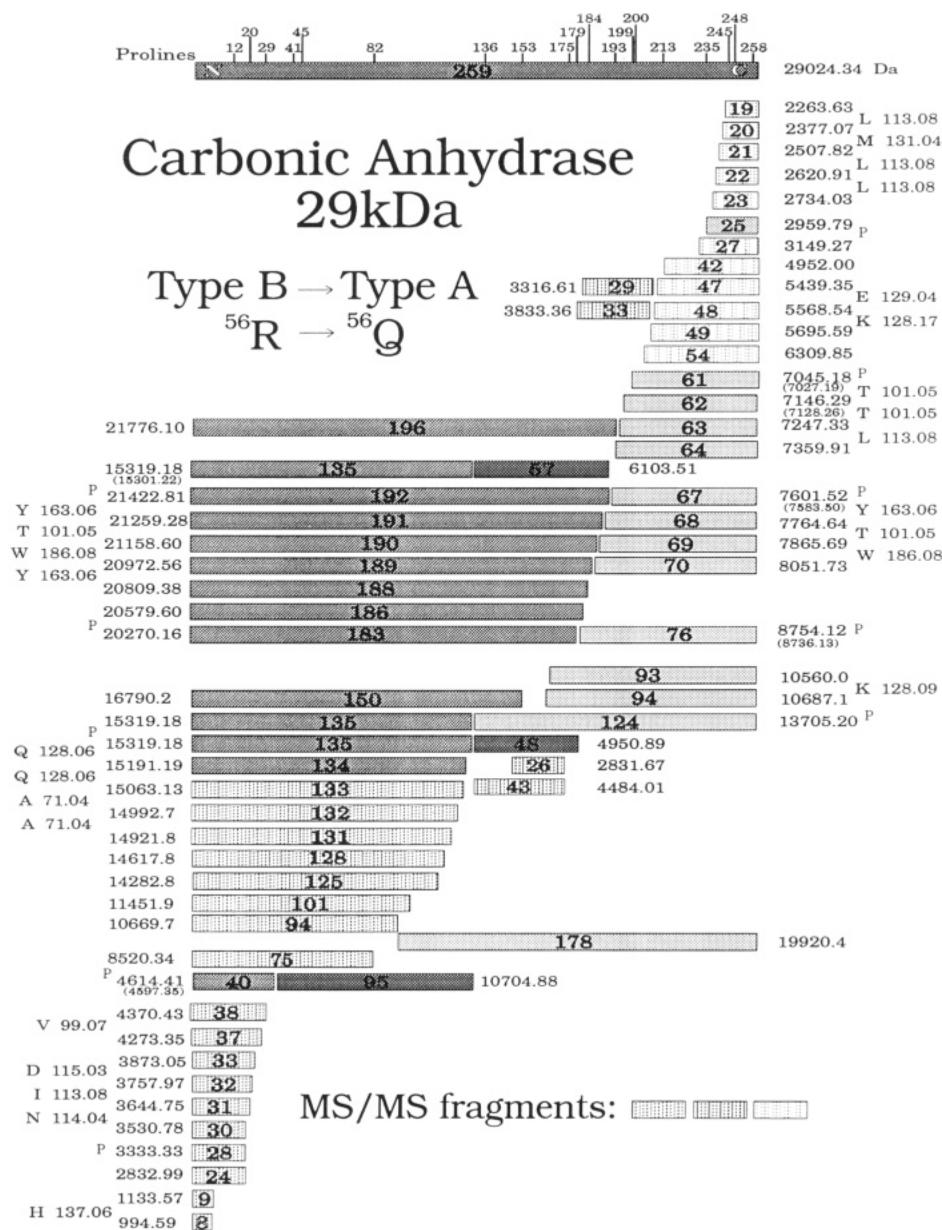

Figure 9. Masses of fragment ions identified in the NS and MS/MS spectra of bovine carbonic anhydrase, correlated with the published sequence and (top) Pro positions. Note the precision of the mass sums of complementary fragments vs the MW value and mass differences vs the amino acid mass.

minima on the potential energy surface representing possible gas-phase conformational structures. For ions exhibiting five of these lower levels with charges 8+ to 17+, infrared laser heating increases the ions' exchange to 133, presumably by an unfolding of the protein ion. Higher velocity collisions of quadrupolar ion axialization[26] yield 173 H exchanges, the highest level found for these gaseous ions. Further, charges can be removed from the ions with butylamine,[38] with the new charge value exhibiting either higher or lower exchange levels, consistent with both unfolding and folding in the gas phase.[37]

In solution, the native highly folded structure has been identified at the lowest H^+ concentrations (pH ~7), with the denatured structure dominant at pH ~3, but with some helical structure reappearing at the highest H^+ concentrations (pH 1). Figure 11 shows a qualitatively similar effect, with state III exchanging 64 H atoms for the 7+ ion, state II exchanging 132 H atoms (and 173 H for VIII) at somewhat higher charge values, but state I exchanging only 113 H atoms for 16+ and 17+ ions. However, the solvent water is thought to provide a dominant driving force for protein folding in solution,[39] which cannot be the case for the water-free gaseous ions, where presumably hydrogen bonding and van der Waals forces should dominate. Quoting Teeter, "it is the removal of water that induces a protein to form a three dimensional structure".[39]

The MS^n spectra of cytochrome *c* show dozens of fragment ions originating from most regions of the molecule.[9a,40] Planned studies will examine the H/D exchange in each of these subunits originating from molecular ions formed by deuteriation of conforma-

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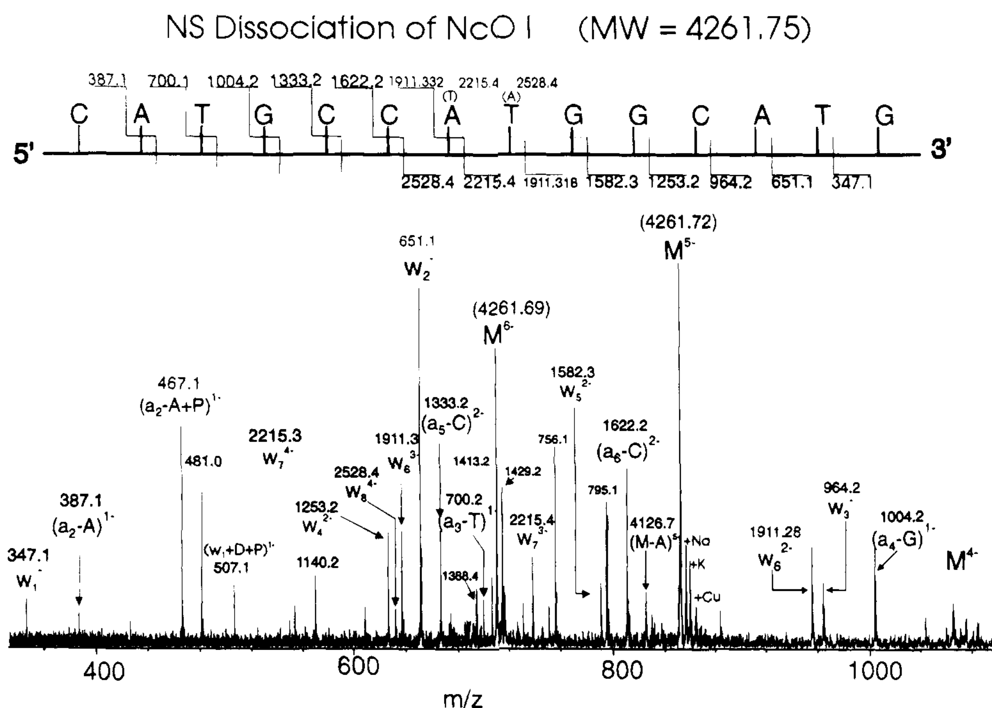

Figure 10. Negative ion ESI/FT mass spectra of a 14-mer oligonucleotide NcOI, predicted MW = 4261.75; a and w cleavages contain the 5′ and 3′-termini, respectively.

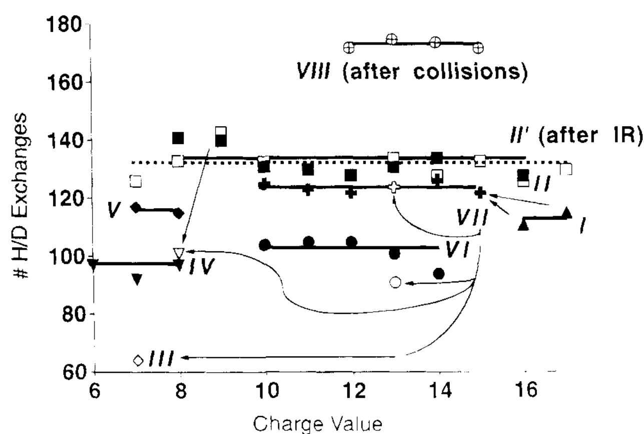

Figure 11. Gas-phase H/D exchange levels (10^{-7} Torr D_2O for 30 min) vs charge value of equine cytochrome *c* ions: solid symbols, states from electrosprayed ions; open symbols, states altered by irradiation, charge stripping, or collisions.

tional states in both solution and the gas phase to provide a much more critical comparison of solution and gas-phase folding, as well as mechanistic details for interconversion of this multiplicity of gaseous states.

Conclusions. Noncovalent and ionic adducts of ESI-formed macroions are so prevalent that low-resolution MW determinations (unresolved molecular ion adducts) must be prone to serious error; the spectrum of $tRNA^{phe}$ without desalting showed ~45 adducted Na atoms.[23] The 10^5-10^6 resolving power achievable even at 67 kDa indicates that molecules as large as 10^5-10^6 Da should also yield unit resolution mass spectra by extending the special methods for removing adducts and impurities and isolating single charge states. The high-resolution tandem mass spectrometry of even larger biomolecules and polymers presents a challenging future.

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