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High-Accuracy Molecular Mass Determination of Proteins Using Matrix-Assisted Laser Desorption Mass Spectrometry

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A method for obtaining protein molecular masses with an accuracy of approximately ±0.01% by matrix-assisted laser desorption using an internal calibrant is described. The technique allows accurate mass determinations of protein sample sizes as small as 1 pmol. High concentrations of organic and inorganic contaminants (e.g. 1 M urea) do not strongly affect either the signal intensity or the mass assignment. The ability to assign an accurate molecular mass to a protein is contingent on the observation of clearly resolved protonated molecule ions in the mass spectrum.

The matrix-assisted laser desorption technique pioneered by Hillenkamp and Karas (*1*, *2*) allows the use of a time-of-flight mass spectrometer to mass analyze gas-phase protein ions. In the original technique, the protein sample of interest is mixed with a large molar excess of nicotinic acid in solution and then a small amount of this solution is dried onto a sample probe. The probe is then placed into a time-of-flight mass spectrometer, where the surface of the protein/nicotinic acid deposit is irradiated by a short pulse of ultraviolet light from a neodymium-yttrium aluminum garnet laser (Nd-YAG) (frequency quadrupled output, 266 nm). The absorption of the laser light causes the desorption of ions related to both nicotinic acid and the intact protein molecule. The protein-related ions produced by this effect are predominantly of charge states $z = +1$ and $+2$. It has subsequently been shown that analogous negatively charged ions are produced, with the predominant charge states being $z = -1$ and -2 (*3*, *4*).

The signals produced by the original method using nicotinic acid were characteristically much broader than the instrumental mass resolution of the mass spectrometer used, resulting in a mass resolution ($m/\Delta m$, measured at the full width, half maximum, fwhm) of approximately 50 and a mass accuracy of 0.1% (*5*). It has been recently shown that one large component of the peak width is caused by a photochemically generated adduction of the nicotinic acid "matrix" molecule to the protein analyte (*3*). Several new matrix types have been discovered that can be used to desorb protein molecules, which produce much less intense and more easily resolved photochemically generated adducts (*6*). The best of these matrices are the cinnamic acid derivatives *trans*-3-methoxy-4-hydroxycinnamic acid (ferulic acid) and *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). The extended conjugation of the chromaphore in these materials increases the range of laser wavelengths useful for desorption (compared to nicotinic acid) allowing the use of the frequency tripled output of a Nd-YAG laser (355 nm) (*7*). This wavelength is not absorbed by the analyte molecules of interest, minimizing the possibility of direct absorption of the laser energy by the protein.

The use of cinnamic acid derivative matrices, particularly sinapinic acid, for protein analysis has allowed our group to resolve the protonated molecule ion signal from the photochemically generated adduct signal by using a simple, linear time-of-flight mass spectrometer. With the peaks resolved ($m/\Delta m = 300$–500, fwhm), the time-of-flight of the centroid of these peaks can be accurately determined. However, to assign the mass that corresponds to this time-of-flight, it is necessary to calibrate the instrument. The relationship between time-of-flight (t) and the mass-to-charge ratio (m/z) of an ion is given by

$$(m/z)^{1/2} = At + B \quad (1)$$

where A and B are calibration constants that depend on the accelerations given the ion and the ion drift length in the instrument. By knowing the mass-to-charge ratio of any two ions in a spectrum and by measuring their flight times, it is possible to determine A and B uniquely. This technique of calibrating time-of-flight spectra has been used extensively in plasma desorption mass spectrometry (*8*), secondary ion mass spectrometry (*9*), and laser desorption mass spectrometry (*10*), where the time-of-flight of two low-mass ions (e.g. $[H]^+$ and $[Na]^+$) are determined accurately and used to extrapolate to higher mass.

The use of intense low mass signals to calibrate high mass protein signals was originally attempted in our laboratory but was found to be too inaccurate for most applications. The very long flight times of protein ions compared to the low mass calibrants meant that the centroids of the calibrant ions had to be determined very exactly so that the extrapolation to long flight times would be sufficiently accurate. Given the time resolution of the best commercial transient recorders (5 ns),

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such an accurate determination could not be achieved, resulting in a mass assignment error of typically ±0.1%.

A convenient alternate to using low-mass calibration ions was the use of well-characterized high-mass calibrant signals. There are a reasonably large number of well-characterized proteins in the 10000–20000 mass range that are commercially available in a pure form. If one of these proteins is added to a sample to be analyzed, the two ions corresponding to that protein (i.e. $[M + H]^+$ and $[M + 2H]^{2+}$, where M represents the intact protein or protein subunit) could be used to calibrate the entire spectrum. This method of adding a single calibrant has proved to be both useful and accurate, allowing the determination of protein molecular masses to an accuracy of 0.01% when the protonated molecule ion signals are resolved. The utility of this method was greatly enhanced by the good properties of sinapinic acid as a matrix when examining mixtures of proteins and peptides. This material will normally produce protonated molecule ion signals from most (if not all) of the protein or peptide species in a mixture and is largely insensitive to the presence of relatively high concentrations of ionic contaminants (e.g. 0.1 M buffers, such as sodium acetate or Tris HCl and 1.0 M urea). Therefore, a calibrant protein can be added to a sample with very little sample cleanup required. The sample concentrations that are generally required by the technique are in the range of 0.1–10 μM and the sample amount introduced into the mass spectrometer is routinely on the order of 1 pmol.

EXPERIMENTAL SECTION

Instruments. The mass spectrometer used was built at Rockefeller University, and the details of the instrument have been described (*3, 6*). The Nd-YAG laser used (HY 400, Lumonics, Inc., Kanata, Ontario, Canada) has an output pulse length of approximately 15 ns and is equipped with second-, third-, and fourth-harmonic generation and separation assemblies. All of the work described in this paper was carried out using the 355-nm, frequency-tripled output. The laser has been modified by the addition of a mode selection aperture in the oscillator cavity and the removal of its output amplification rod to improve the beam's spatial homogeneity. The laser irradiance used for producing a mass spectrum was approximately 1 MW/cm^2. This irradiance level was not protein dependent; i.e. the same irradiance was used for all of the samples run. The laser was operated with a continuous flashlamp triggering frequency of 5 Hz to maximize the shot-to-shot stability of the laser and Q-switched with a Pockels cell on every second lamp flash, producing an output frequency of 2.5 Hz.

Spectra were obtained by adding together the transient signals recorded from 50 to 200 laser shots using a CAMAC crate based transient recorder (TR8828D, Lecroy, Chestnut Ridge, NY) and crate controller (6010 MAGIC controller, Lecroy). The summed spectrum was transferred to a VAX Workstation 3200 for data analysis. Peak centroids and mass calibrations were calculated by using custom software developed in our laboratory. This software finds peaks above a given threshold value. The program then calculated a centroid consisting of a 100-ns region around the peak, selecting the 100-ns region that had the largest integrated intensity in the vicinity of the peak. The use of a selected region about the top of a peak for centroid calculation, rather than a region determined by an intensity threshold, is necessary to achieve accurate mass assignments in the case of multiple peaks that are not fully resolved in the sense of the 5% valley definition commonly used in magnetic sector mass spectrometry. The choice of a region corresponding to the part of the signal containing the real isotopic distribution of the analyte molecule reduces effects caused by peak asymmetry. This peak searching routine has been used for ten years for assigning molecular masses in plasma desorption mass spectra and was used with minor modifications for laser desorption mass spectra.

Sample Preparation. The desorption matrix that was used for all of the spectra taken for this paper was 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Aldrich Chemical Co., Milwaukee, WI). A 50 mM stock solution of the matrix was made by dissolving sinapinic acid in aqueous 30% acetonitrile (v/v). The water (distilled-in-glass grade, Burdick & Jackson Laboratories, Muskegan, MI) used to make the solution contained 0.1% trifluoroacetic acid (Aldrich) to assist in dissolving proteins. This stock solution must be stored in the dark and cannot be kept for more than a few days without losing its effectiveness.

To prepare a sample for mass spectrometry, typically 5 μL of the matrix stock solution was placed in an Eppendorf tube and 0.5 μL each of protein solutions (1–10 μM) to be analyzed was added. The solution was briefly mixed by vortex stirring. A small aliquot (<0.5 μL) of the matrix/protein mixture was then applied to a 2 mm diameter, flat metal probe tip and dried at room temperature. The dried deposit was washed by immersing the probe tip in cold distilled water (4 °C) to remove any water-soluble contaminants, particularly inorganic salts (see below). The probe was then inserted through a vacuum lock into the mass spectrometer. The sample could be analyzed within 3 min of the probe's entry into the vacuum lock. Once the sample was inside of the mass spectrometer, it could be left in the vacuum for more than 10 h without any adverse effect on the laser desorption response. This long-term vacuum stability is caused by the low vapor pressure of sinapinic acid.

The probe tip used in our instrument was made of 2 mm diameter platinum rod. Platinum was chosen for this purpose because of its superior corrosion resistance and the ease with which it can be cleaned, not because of any special properties involving laser desorption. Probe tips made of stainless steel, silver, and tantalum have been used with similar results.

The washing procedure described above was important in obtaining high mass accuracies. If the sample contained significant amounts of sodium or potassium cations, the protein molecule ion signal would be distorted by the presence of unresolved cation adduct peaks. This change in the shape of a peak alters its time-of-flight centroid, resulting in an inaccurate determination of the parameters A and B in eq 1. Washes could be carried out using cold neutral or acidic water (0.1 N HCl). Basic solutions, e.g. water containing ammonia, completely quench protein ion production from the sample. Buffers containing the ammonium cation, such as ammonium bicarbonate, do not quench the protein signal.

The protein standards used were all purchased from Sigma Chemical Co. (St. Louis, MO). The specific preparations used (including Sigma reference numbers) were skeletal muscle myoglobin (equine, M-0630), heart cycochrome *c* (equine, C-3256), pancreatic trypsinogen (bovine, T-1143), subtilisin Carlberg (*Bacillus subtilis*, P-5380), and ribonuclease A (bovine, R-5500). The sample of monoclonal immunoglobulin G_1 (IgG_1, *Mus musculus*) was a gift from Professor T. P. King (Rockefeller University).

RESULTS AND DISCUSSION

A general feature of the mass spectrometry of intact proteins that is different from that of small molecules is what is meant by the phrase "the mass of a protein". Proteins contain a relatively large number of atoms, leading to a statistical distribution of molecular masses that depends on the isotopic distribution of all of the atomic species present in the molecule. As a practical method of characterizing the molecular mass of a protein, it is therefore of greater relevance to calculate and measure the center of the isotopically generated distribution rather than the monoisotopic molecular mass. All of the masses quoted in this paper are the average molecular masses, calculated by using the IUPAC standard average atomic masses (*11*).

It is our general experience that the ions that are observed in matrix-assisted laser desorption mass spectra correspond to intact, covalently bonded peptide chains. While there can be low intensity (<10%) peaks corresponding to dimers of individual chains when the protein is present at high concentration (>10 μM) in the matrix solution, proteins that are association multimers of individual subunit chains produce signals due to the subunits rather than the intact protein. As an example, mammalian hemoglobin is a tetramer of two α and two β subunit chains and four noncovalently bound

iron-containing heme groups. The laser desorption mass spectrum of this molecule produces three signals, corresponding to the α and β chains and the heme group. No significant signal from the intact tetramer/heme complex is observed. There are indications that some proteins may retain their quaternary structure during the desorption process (*12*), but this does not appear to be a phenomenon that can be generalized to all proteins.

An important consideration in accurately determining the molecular mass of a protein from the mass spectrum is the identification of the ion species present in a particular signal. Proteins produce two major signals in laser desorption time-of-flight mass spectra, corresponding to a singly charged species and a doubly charged species, which has an m/z value approximately one-half of the singly charged species. By analogy with plasma desorption mass spectrometry, the singly charged species can be assumed to the either (1) the intact protein plus one proton ($[M + H]^+$) or (2) alkali-metal cation ion adducts ($[M + Na]^+$, $[M + 2Na + H]^+$, etc.). Similarly, the doubly charged species can be the intact protein plus two protons or alkali-metal cations. Our observations of the signals produced by a large number of different protein and peptide samples (>100 different compounds) led us to the conclusion that the most abundant molecule ion species is formed by protonation. This conclusion is based on the accuracy of mass determinations that have been performed on these molecules. The addition of alkali-metal cations also seems to occur but is rarely the most intense signal (see below). An additional feature of the intact ion signal, whether singly or doubly charged, is an adduct signal caused by the photochemically generated addition of a matrix molecule to the protein. In the case of sinapinic acid, this matrix adduct ion is 206 Da higher in mass than the protonated molecule ion and appears to be the addition of a matrix molecule with an accompanying loss of water (*6*). The ion species with m/z 206 is always observed in the low mass portion of the mass spectrum, suggesting that it is readily formed from sinapinic acid by UV photon excitation.

Figure 1 shows a detail of the mass spectrum of equine cytochrome *c*, obtained before and after the washing procedure outlined in the Experimental Section. The ions shown correspond to singly charged species. A number of unresolved signals caused by single or multiple cation adduction are removed by the washing procedure, leading to a decrease in the peak width. The presence of these unresolved ion peaks in the molecular ion signal is undesirable because they skew the centroid of the signal toward higher mass, resulting in poor mass assignments. The removal of the sodium adduct peaks by washing suggests that the major ion species produced by matrix-assisted laser desorption are singly and doubly protonated protein molecules. This assumption has been used throughout the rest of this report and may be justified by the high degree of accuracy obtained in protein molecular mass determinations. A laser desorption mass spectrum of cytochrome *c* has been reported (*12*) that uses the frequency quadrupled Nd-YAG output (266 nm) which contained an additional peak caused by the loss of the covalently bound heme moiety from cytochrome *c*. This loss has not been observed with a wavelength of 355 nm, even with increased laser irradiance.

When the technique of washing sample deposits was initially attempted, there was some concern about the amount of protein that might be lost by the washing procedure. We find that the sensitivity of the technique remains about the same, with or without washing. Indeed, the signal intensity observed is increased by washing when a sample is particularly contaminated with inorganic salts (e.g. 0.5 M NaCl). Typical sensitivities are on the order of 1 pmol loaded onto the probe

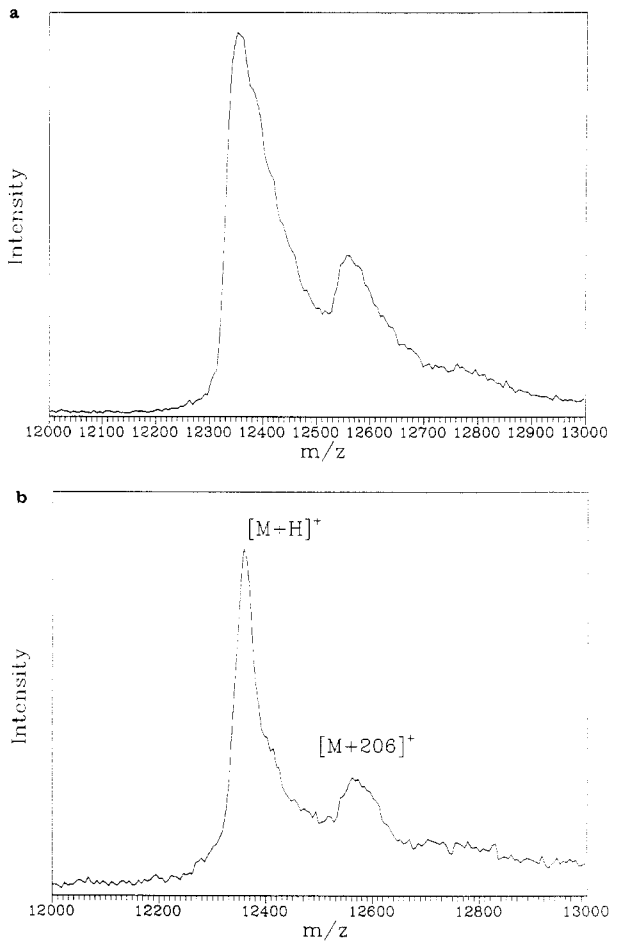

Figure 1. Singly charged molecular ion region of mass spectra obtained from a sample loading of 0.5 pmol of horse heart cytochrome *c*: (a) unwashed; and (b) washed in 4 °C water for 15 s. Both spectra are displayed using 20-ns time bins and horse skeletal muscle myoglobin was used as a calibrant.

tip, although some samples may produce signals from less. The spectra shown in Figure 2 were obtained separately from sample/matrix deposits that were washed for 20 s in cold water. Figure 2a shows the spectrum obtained from a sample of bovine ribonuclease A (0.5 pmol) that was in a 5 μM solution containing 1.0 M urea. One microliter of this sample solution was mixed with 4 μL of the matrix solution and 0.5 μL of this mixture was deposited on the probe tip. The measured and calculated molecular masses (MM) were in good agreement (measured MM = 13 682, calculated MM = 13 682.3 (*13*)). Figure 2b is the mass spectrum obtained from 0.5 pmol of a monoclonal IgG_1 (*Mus musculus*) sample that was originally in a 5 μM solution containing 50 mM Tris HCl buffer (pH = 7.4). This sample was also diluted 4:1 with the matrix solution and 0.5 μL applied to the probe tip. A molecular mass was obtained (measured MM = 148 140), but because of the heterogeneity of the carbohydrate moieties attached to the polypeptide chains of IgG_1, this mass cannot be properly compared with a calculated value. The expected spread in molecular masses caused by heterogeneity in the IgG oligosaccharide chains is approximately 2100, assuming the carbohydrate structures proposed by Mizuochi et al. (*14*) and the presence of one asparagine linked biantenary complex type sugar chain in the two C_H2 (heavy chain) domains of the molecule. Any additional broadening of the signal in Figure 2b is probably caused by the presence of unresolved photochemically generated adduct ions of the type discussed above. These two examples demonstrate the selectivity of this technique for examining proteins, ie. a large molar excess of

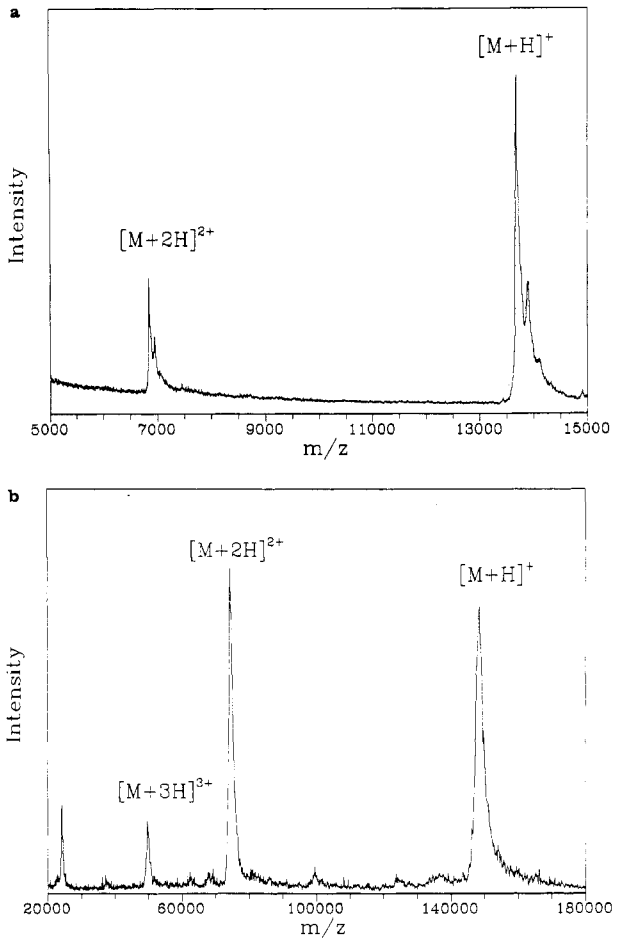

Figure 2. Mass spectra of two protein samples containing high contaminant concentrations: (a) bovine pancreatic ribonuclease A (5 μM protein solution in 1 M urea); and (b) monoclonal immunoglobulin G_1 (*Mus musculus*) (5 μM protein solution in 50 mM Tris HCl, pH = 7.4). Figure 1a is displayed in 20 ns time bins and Figure 2b is displayed in 50-ns time bins. In both cases, horse skeletal muscle myoglobin was used as a calibrant for molecular mass determination.

Table I. Measured and Calculated Molecular Mass of Equine Heart Cytochrome *c*

$[M + H]^+$
12360.3
12358.0
12360.5
12360.7
12360.2
12360.6

average measured mass = 12360.1 ± 1.0 u

calculated mass = 12360.1 u

Δ = 0.0 u

buffers or denaturants do not interfere with the formation of protein ions. The only contaminant that has been definitely identified with a poisoning of the ion formation process is the ionic detergent sodium dodecyl sulfate (SDS), although particular proteins may not be strongly affected by its presence in sufficiently low concentration.

In order to demonstrate the accuracies obtainable by using laser desorption time-of-flight mass measurement, the masses of three proteins were repeatedly determined. In each of the three cases, equine myoglobin (molecular mass = 16950.5 Da (*15*)) was added to the sample to serve as a calibrant. Approximately 0.5 pmol each of the analyte protein and the calibrant mixed with 20 nmol of sinapinic acid were loaded

Table II. Measured and Calculated Molecular Mass of Bovine Pancreatic Trypsinogen

molecular mass, $[M + H]^+$	$[M + 2H]^{2+}$
23976.0	23978.8
23979.9	23983.4
23978.4	23983.4
23979.9	23983.6
23983.0	23977.8
23980.9	23978.4

average measured mass = 23980.3 ± 2.6 u

calculated mass = 23981.0 u

Δ = −0.7 u

Table III. Measured and Calculated Molecular Mass of the Protease Subtilisin Carlsberg (*Bacillus subtilis*)

$[M + H]^+$	$[M + 2H]^{2+}$
27286.6	27288.1
27286.8	27288.0
27287.0	27291.0
27287.8	27292.2
27287.2	27288.4
27287.3	27287.4

average measured mass = 27288.2 ± 1.7 u

calculated mass = 27288.4 u

Δ = −0.2 u

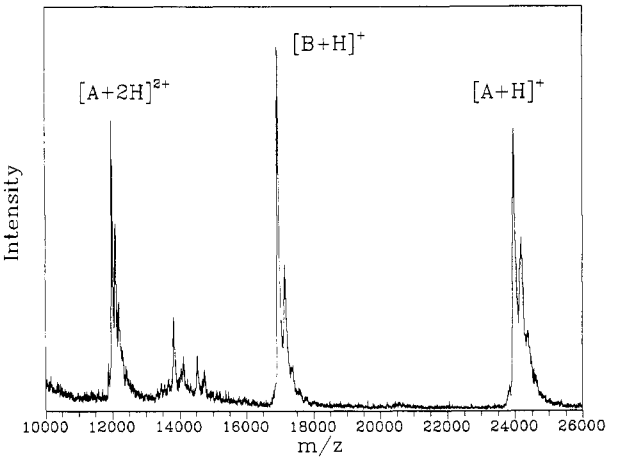

Figure 3. One of the mass spectra used in obtaining the data given in Table II. The region shown contains the singly and doubly charged ions of trypsinogen (A, 0.5 pmol) and the singly charged species of myoglobin (B, 0.5 pmol). Note the resolved adduct ions (see text) for each of these species. The additional signals in the mass spectrum were caused by contaminants in the trypsinogen sample.

onto the probe tip from 0.5 μL of solution. The results for the three proteins examined, i.e. equine cytochrome *c* (calculated MM = 12360.1 (*16*)), bovine trypsinogen (calculated MM = 23981.0 (*17*)), and subtilisin Carlsberg (*Bacillus subtilis*) (calculated MM = 27288.4), are given in Tables I, II, and III, respectively. The mass determinations were made by using both the singly protonated and doubly protonated signals and the numbers shown have been calculated by removing the appropriate numbers of protons from the observed *m/z* value. The averages obtained show that the error in a single measurement is approximately 0.01% and the error of the statistical mean is somewhat better than 0.01%. A detail of one of the spectra used to obtain this data is shown in Figure 3. This spectrum shows the $[M + H]^+$ ion signals of the two proteins to be clearly resolved from the matrix adduct

peaks $[M + 206]^+$. This level of accuracy has been found to be quite typical in any case where a single resolved peak is present for a protein species.

The mass accuracy (0.01%) and the amount sample loaded into the instrument (~1 pmol) obtained by matrix-assisted laser desorption should be viewed in the context of the results obtained by other mass spectrometric methods. Comparing the sample usage between the desorption ionization methods and electrospray/ionspray ionization is difficult to do quantitatively, because sample usage is referred to in a different manner. The sample used in a desorption method is quoted as the amount of material that is actually inserted into the mass spectrometer, while electrospray sample consumptions are calculated on the basis of the amount of time that a continuously flowing stream of dissolved analyte is sampled by a scanning quadrupole mass filter. Therefore, to determine the actual sample consumption for the electrospray/ionspray examples listed below, the reader is referred to the works cited.

Several of the proteins used in our study have been analyzed by fast atom bombardment/liquid secondary ion mass spectrometry using a double focusing magnetic sector instrument (VG ZAB-SE) (*19*). The molecular mass determination accuracy for these two proteins was 0.006% for equine cytochrome *c* and 0.35% for trypsinogen (species unknown). The amount of protein used for these mass determinations was 1000–3000 pmol.

Another subset of the proteins used in the present report has been measured by electrospray or ion spray ionization coupled to a quadrupole mass filter by an atmospheric pressure inlet system. Masses for equine cytochrome *c* have been reported by several groups using electrospray, e.g. MM = 12358.7 ± 3.7 (*20*) and 12350 ± 4 (*21*). Equine myoglobin has also been measured by several groups, e.g. MM = 16949.0 ± 4 (*20*), 16904 ± 7 (*21*), and 16949.5 (*22*). Equine cytochrome *c*, bovine trypsinogen, and equine myoglobin have been measured by electrospray in our laboratory (*23*) and these measurements indicate a mass accuracy of approximately 0.01% in this mass range. No electrospray spectra of subtilisin Carlsberg or immunoglobulin G have been reported in the literature. Attempts to analyze these proteins by electrospray in our laboratory did not produce useful mass spectra. The amounts of sample used to obtain the mass measurements by electrospray or ionspray that are quoted above are 50 (*20*, *24*) to 500 (*21*) pmol for cytochrome *c* to 1 pmol for myoglobin (*22*). A general comment on the sensitivity of electrospray ionization sources has been made by Loo et al. (*20*) stating that the sample consumption for electrospray is in the range of 0.1–10 pmol of protein used during a quadrupole scan.

Plasma desorption mass spectrometry has been used to measure the molecular mass of equine cytochrome *c* (MM = 12365 ± 10 (*25*)) and equine myoglobin (MM = 16944 ± 40 (*8*)). Plasma desorption has also been used to analyze subtilisin BPN' (a protein somewhat homologous to subtilisin Carlsberg), with a mass accuracy quoted as 0.1% (*26*). The amount of sample used for analysis in these three cases was 500–1000 pmol.

Inspection of the results given above for matrix-assisted laser desorption and the other current mass spectrometric techniques for the analysis of proteins indicates that in terms of mass accuracy, the laser desorption measurements are at least as accurate as those obtained by the other methods. The same can be said for sample usage, although this is a less well defined parameter in the case of the flowing liquid techniques, i.e. electrospray and ionspray.

A goal of current research in our laboratory is to increase the mass resolution of the laser desorption technique (currently $m/\Delta m$ = 300–500, fwhm) to at least $m/\Delta m$ = 1000, the mass resolution currently achieved by electrospray/ionspray using a quadrupole mass filter analyzer. Increased mass resolution is frequently desirable when analyzing samples that may contain closely related components of closely related masses. The application of ion mirrors to time-of-flight mass spectrometers (e.g. refs 9 and 10) suggest that if a large component of the residual peak widths that we observe is caused by a spread in the kinetic energy of the protein ions resulting from the ion formation process, mass resolutions of $m/\Delta m$ > 1000 may be achieved.

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