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Laserspray Ionization, a New Atmospheric Pressure MALDI Method for Producing Highly Charged Gas-phase Ions of Peptides and Proteins Directly from Solid Solutions*

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The first example of a matrix-assisted laser desorption/ionization (MALDI) process producing multiply charged mass spectra nearly identical to those observed with electrospray ionization (ESI) is presented. MALDI is noted for its ability to produce singly charged ions, but in the experiments described here multiply charged ions are produced by laser ablation of analyte incorporated into a common MALDI matrix, 2,5-dihydroxybenzoic acid, using standard solvent-based sample preparation protocols. Laser ablation is known to produce matrix clusters in MALDI provided a threshold energy is achieved. We propose that these clusters (liquid droplets) are highly charged, and under conditions that produce sufficient matrix evaporation, ions are field-evaporated from the droplets similarly to ESI. Because of the multiple charging, advanced mass spectrometers with limited mass-to-charge range can be used for protein characterization. Thus, using an Orbitrap mass spectrometer, low femtomole quantities of proteins produce full-range mass spectra at 100,000 mass resolution with <5-ppm mass accuracy and with 1-s acquisition. Furthermore, the first example of protein fragmentation using electron transfer dissociation with MALDI is presented. ***Molecular & Cellular Proteomics 9:362–367, 2010.***

Two primary differences between ESI and MALDI methods are the sample environment (solution *versus* solid) and the observable charge state(s) (multiply *versus* singly charged). The multiply charged ions observed in ESI mass spectrometry (MS) enhance the yields of fragment ions, a key benefit in structure characterization, and allow analysis of high molecular weight compounds on mass spectrometers with a limited mass-to-charge (*m/z*) range. In contrast, MALDI MS is ideal for the analysis of heterogeneous samples because it often requires less sample, and spectra of singly charged ions are easier to interpret. We report here the astonishing observation of highly charged molecular ions by laser ablation of a solid matrix/analyte mixture typically used in MALDI MS analyses. The distribution and abundances of the observed ions are similar to those obtained by ESI. Importantly, the MALDI mechanism that produces singly charged ions can be "turned on" at the operator's will by changing only the matrix *or* matrix preparation conditions; this capability is not available with any other ionization method. These findings show for the first time that singly charged ions as well as multiply charged ions are available in MALDI. Besides having important mechanistic implications relating to MALDI and ESI, our findings have enormous practical analytical utility.

ESI and MALDI combined with MS revolutionized the study of biological materials and earned the Nobel Prize in Chemistry for their ability to ionize proteins for analysis using MS. However, after two decades of extensive studies, the mechanism for ion formation in MALDI remains controversial (1–8). At the heart of these debates lies the predominance of singly charged ions in MALDI mass spectra; the exception being very high mass compounds. A mechanism for the formation of multiply charged ions in MALDI has previously been proposed (1) based on molecular modeling studies (9, 10) and glimpses of multiply charged ions have been observed in lower molecular weight compounds (11–14). The formation of these multiply charged ions has been attributed to sample preparation, high laser fluence, a metal-free sample stage, use of an IR laser, and atmospheric pressure (AP)[1] conditions. Multiply charged ions were also recently observed by laser ablation of a liquid surface in the presence of a high electric field (15). The inability in that experiment to observe ions from a solid MALDI matrix/analyte sample or in the absence of an electric field suggests an ionization process involving liquid droplets in a

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[1] The abbreviations used are: ACN, acetonitrile; AP, atmospheric pressure; Cyt., cytochrome; DHB, dihydroxybenzoic; ESI, electrospray ionization; ETD, electron transfer dissociation; FF, field-free; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; *m/z*, mass-to-charge; TG, transmission geometry.

high field similar to ESI (16) or other liquid based, field-induced ionization methods (17, 18).

Here, we show analytically useful ESI-like MALDI mass spectra obtained using standard MALDI conditions but using a nontraditional source (19) mounted in place of the standard atmospheric pressure ionization source on a mass spectrometer most commonly used with ESI. The utility of this MALDI MS method for extending the mass range of mass spectrometers as well as the capability of peptide/protein sequencing using electron transfer dissociation (ETD) (20) is demonstrated. Because highly charged ions have not previously been observed with any MALDI ion source configuration, we briefly discuss the fundamental concepts that lead to their production. Key aspects of laserspray ionization (LSI) are laser ablation using a UV laser aligned in transmission geometry (TG) (21–23), field-free (FF) at AP (24), using a heated AP to vacuum ion transfer capillary. In order to emphasize the MALDI sample preparation but distinguish laserspray from conventional AP-MALDI, the new ionization method will hereafter be referred to as FF-TG AP-MALDI.

EXPERIMENTAL PROCEDURES

Lysozyme, cytochrome *c* (Cyt. *c*), angiotensin I, angiotensin II, and 2,5-dihydroxybenzoic acid (2,5-DHB) were obtained from Sigma-Aldrich. HPLC grade water and acetonitrile (ACN) were obtained from Fisher Scientific. For FF-TG AP-MALDI, proteins and peptides were dissolved in a 30:70 ACN/water solution and premixed with 2,5-DHB, a common MALDI matrix, prepared as a concentrated solution in a room temperature 1:1 ACN/water solution. The premixed matrix/analyte solution was deposited on a glass microscope slide (Gold Seal, about 80% transmission) using a standard solvent-based MALDI preparation method (25) followed by evaporation of the solvent. Mass spectra were acquired on an Orbitrap Exactive (Thermo Fisher Scientific) mass spectrometer after removal of the Ion Max source and overriding the interlocks. Ions were generated when a glass slide containing matrix/analyte, positioned closely (1–3 mm) in front of the mass spectrometer ion entrance orifice, passed through the focused (~100-μm) laser beam (337 nm, Spectra Physics VSL-337ND-S). The laser was aligned with the ion transfer tube entrance aperture so that the laser beam passed through the focusing optics, the glass slide, and the capillary that transports ions from AP to vacuum. The transfer capillary was heated to 350 °C, and the laser fluence per pulse was typically 1–2 J cm^{-2}. The laser fluence is higher than is commonly used in vacuum reflective geometry MALDI because in TG the rapidly expanding matrix/analyte jet must penetrate the entire sample layer for ions to be observed. The temperature of the ion transfer capillary was used to control the desolvation conditions for the temperature study with angiotensin I.

Electrospray ionization used the heated electrospray (HESI) probe of the Orbitrap Ion Max source with tune conditions that are typical for protein analyses. For the ESI mass spectrum, 1 μl of a 1 pmol μl^{-1} solution of lysozyme was injected into a 1:1 ACN/water solution infused at a flow rate of 2 μl min^{-1}. AP-MALDI ETD experiments were obtained on a Thermo Fisher Scientific LTQ with ETD capabilities using the same FF-TG AP-MALDI ion source configuration discussed above and ETD conditions described elsewhere (20).

RESULTS AND DISCUSSION

Lysozyme was chosen as a well studied protein for initial experiments. A typical FF-TG AP-MALDI mass spectrum for lysozyme is shown in Fig. 1*A* and can be compared with the ESI spectrum shown in Fig. 1*B*, also acquired on the Orbitrap Exactive from the same 1 μM solution at 100,000 resolution and using 1-s acquisition. Observation of efficient production of such highly charged ions (up to 13+) in MALDI is unprecedented. As with ESI, the higher charge states allow AP-MALDI mass spectra to be obtained for compounds with molecular weights beyond the *m/z* limit of the instrument (4000). The capability to produce highly charged ions in high abundance in AP-MALDI and ESI strongly suggests that these ions are formed via related mechanisms.

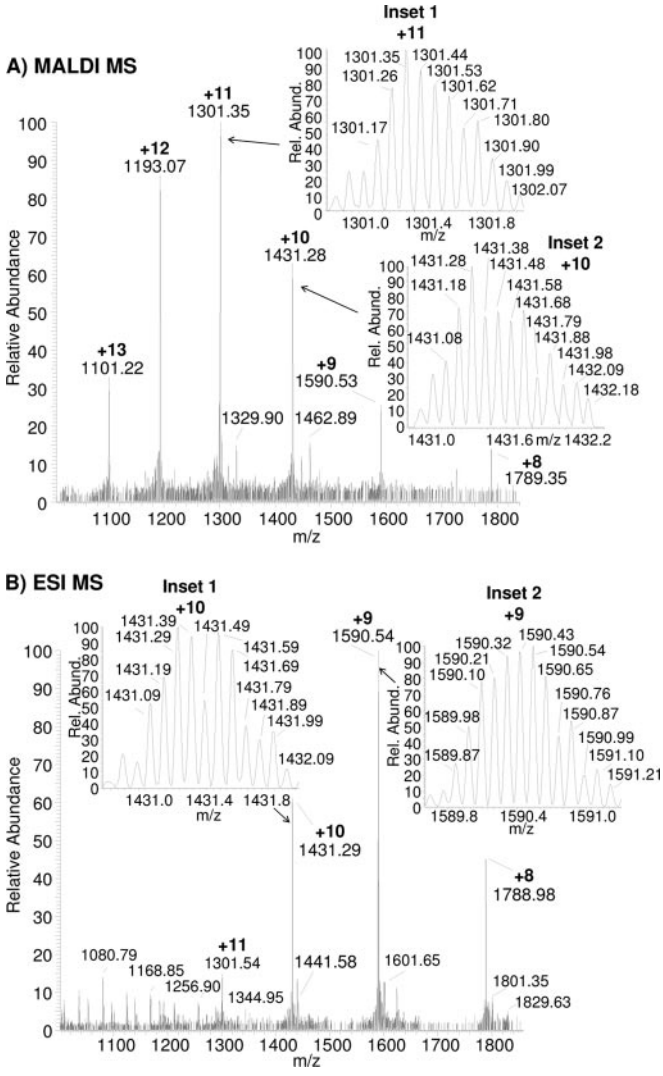
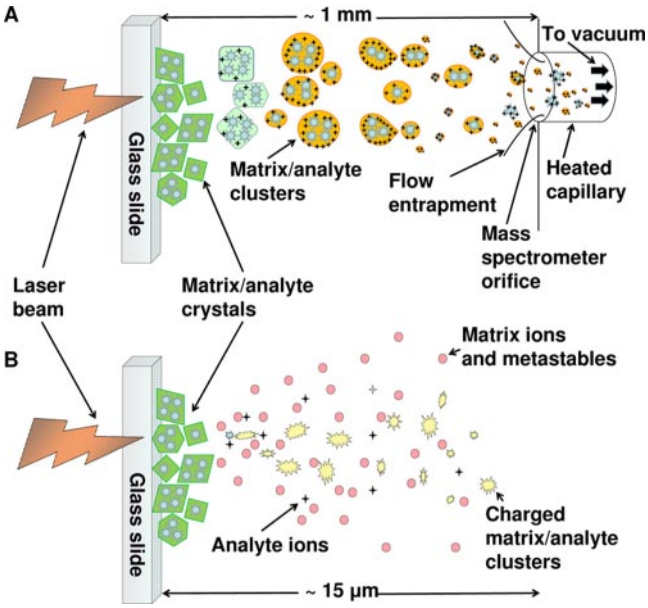


FIG. 1. **MALDI (*A*) and ESI (*B*) mass spectra of a 1 pmol/μl solution of lysozyme (1-s acquisitions, 100,000 resolution ($m/\Delta m$, full-width half-height at *m/z* 200)), charge states 8+ to 13+ for FF-TG AP-MALDI (1 pmol in 2,5-DHB loaded on a glass microscope slide) (*A*) and 8+ to 11+ for ESI (1 pmol injected into 1:1 ACN/water infused at 2 μl min^{-1}) (*B*).** *Insets* show the isotope distributions for the most abundant peaks and charge 10+ in each spectrum. The molecular weight can be calculated from each *m/z* value by knowledge of *z*, which is readily determined from the *m/z* spacing between ^{13}C isotope peaks.



SCHEME 1. **Schematic representation of the FF-TG AP-MALDI source and active ionization mechanisms.** *A*, representation of the cluster model in which the absorption of photons by the matrix results in a free jet expansion, producing highly charged matrix/analyte clusters that become desolvated in the ion transfer tube, producing multiply charged ions. Ionization occurs over distances measured in mm. *B*, a representation of an expansion of *A* showing the chemical ionization process that occurs primarily near the sample surface and leads to primarily singly charged ions. This ionization process occurs in the first few μm from the surface.

Process for Producing Highly Charged Molecular Ions by MALDI—Here, we provide a brief description of mechanistic implications as well as the conditions that are necessary to observe highly charged ESI-like ions in MALDI MS. In ESI, the ionization mechanism is thought to involve highly charged solvent/analyte droplets (26–28). In MALDI, the most accepted model is a two-step ionization process that occurs in the expanding plume that is created by absorption of the laser energy by the MALDI matrix (4, 5). Primary photochemical ionization is followed by ion-molecule reactions similar to chemical ionization, leading primarily to singly charged analyte ions. Alternatively, a cluster model has been proposed in which charged clusters produce multiply charged ions in a MALDI process by a mechanism similar to ESI (1, 11, 12). To explain the dominant observation of singly charged ions, a "lucky survivor" mechanism was postulated in which highly charged positive ions, of *e.g.* proteins, were reduced to singly charged ions, presumably by ion-electron recombination (1). This mechanism was later modified to formation of singly charged ions directly from clusters (7). Others have also suggested that clusters are a source of ions in MALDI, including background ions (3, 6, 29). However, the validity of a cluster mechanism as a significant source of ions in MALDI MS has been questioned (5, 8). Given our observation of abundant, highly charged ions in FF-TG AP-MALDI, the potential mechanistic commonalities between ESI and MALDI must be reassessed.

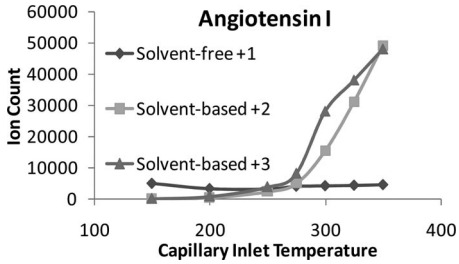


FIG. 2. **Ion count *versus* ion transfer tube temperature plot for the 1+ through 3+ charge states of angiotensin I.** The 2+ and 3+ ions are produced by the solvent-based and the 1+ ions are produced by the solvent-free MALDI sample preparation methods. The plot suggests two distinctly different ionization mechanisms, one of which (multiply charged ion formation) is highly dependent on the ion transfer tube temperature.

In analogy to ESI (16, 26–28) and the cluster (liquid droplet) model for MALDI (1, 11, 12), we propose a mechanism in which the laser-induced gas-phase clusters are highly charged. Similar to ESI, evaporation of solvent, but not charge, produces an unstable surface charge density, resulting in formation of smaller particles and eventually ion emission (Scheme 1). Unlike ESI in which an electric field induces excess positive or negative charges on solvent droplets, in the AP-MALDI experiments discussed here, cluster charging occurs in the absence of an electric field. Field-free production of charged clusters can occur at cluster birth by a statistical charge separation mechanism (28), photoionization, or electron loss from the cluster surface (7, 8).

Provided a threshold laser fluence is achieved (11, 12), cluster formation in MALDI is expected (1, 3–10, 29), and mechanisms exist for at least some of the clusters to become charged (1, 7, 8, 28). However, detection of higher charge state ions has remained elusive in MALDI until now. We propose that the highly charged clusters are initially produced during the explosive deposition of energy into the MALDI matrix in either AP- or vacuum MALDI at least with solvent-based matrix preparation using 2,5-DHB. However, for field emission of multiply charged ions, it is necessary to evaporate sufficient matrix, but not charge, from the clusters to achieve a surface charge density $>\sim 10^9$ V m^{-1} (30). One possibility for the absence of observable highly charged ions under vacuum conditions is that evaporative cooling quenches the initial rapid cluster evaporation process prior to achieving the necessary charge density.

In support of a requirement of desolvation for observation of multiply charged but not singly charged ions, the AP to vacuum ion transfer capillary (Scheme 1) temperature was varied for angiotensin I (molecular weight, 1295.7) using solvent-based and solvent-free 2,5-DHB sample preparation. Barely detectable 2+ and 3+ molecular ions were observed with the transfer capillary temperature set as low as 150 °C (no 1+ ions were observed). The 2+ and 3+ ion abundances

increase rapidly above 275 °C, and at 350 °C they dominate the mass spectrum (Fig. 2). Using the same matrix and analyte but prepared under solvent-free conditions (31, 32), 1+ molecular ions are exclusively produced, and little change in ion abundance is observed over the same temperature range, strongly implying that an entirely different ion formation mechanism is being sampled. This and other evidence suggest that the singly charged ions produced using the solvent-free sample preparation method are formed near the sample surface by the two-step chemical ionization mechanism normally observed in MALDI but that the multiply charged ions are produced from clusters far downstream from the initial laser/matrix interaction (Scheme 1) and are enhanced by improved desolvation conditions (higher ion transfer tube temperature). Interestingly, Fig. 2 suggests that higher sensitivity will be achieved by increasing the ion transfer tube temperature beyond 350 °C.

The unique configuration of the FF-TG AP-MALDI arrangement (Scheme 1) may also provide improved ion sampling by the combination of the forward momentum of the laser-generated plume and the flow-dominated entrapment by gas diffusing from AP to vacuum through the instrument orifice. The absence of an electric field is expected to minimize loss of ions at the entrance to the ion transfer tube (33) and favor sampling of higher mass and thus higher momentum species such as clusters relative to free analyte ions created in the expanding plume region near the sample surface. Thus, the high efficiency of producing multiply charged ions may partially be the result of preferential sampling at the capillary inlet orifice.

As noted, either singly or multiply charged ions can be selected in FF-TG AP-MALDI by choice of matrix or matrix/analyte preparation conditions. Thus, using 2,5-DHB and dried droplet solvent-based sample preparation, multiply charged mass spectra of peptides and proteins nearly identical to those in ESI are produced, whereas solvent-free sample preparation (31, 32) produces MALDI-like singly charged mass spectra for peptides. One difference in these distinct MALDI sample preparation methods is that analyte incorporation in the matrix (solid solution) is believed to occur for solvent-based preparations (34) but not in the solvent-free case (35, 36). Consistent with this concept, the addition of a drop of water/ACN solution to the solvent-free prepared sample on the glass slide and evaporation of the solvent resulted in the mass spectrum changing from all singly charged with solvent-free preparation to dominantly multiply charged peptide ions after addition of solvent.

Further support for the importance of analyte incorporation into the matrix is derived from solvent-based experiments using Cyt. *c* and various DHB positional isomers. Highly charged ions of Cyt. *c* were observed only for the 2,5-DHB isomer. Previous work (34) reported quantitative incorporation of Cyt. *c* only into the 2,5-DHB isomer. Importantly, changes only in the matrix/analyte conditions alter the

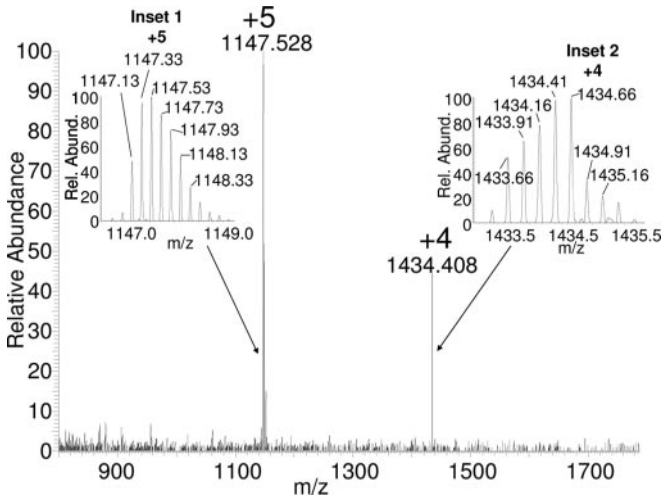


FIG. 3. **The FF-TG AP-MALDI mass spectrum obtained at 100,000 mass resolution from 40 fmol of bovine pancreas insulin loaded on the glass slide MALDI target plate in 2,5-DHB using the solvent-based dried droplet method.** The *insets* show the ^{13}C isotopic distribution of the 4+ and 5+ charge state ions. *Rel. Abund.*, relative abundance.

selected charge state population, thus assuring that the observed multiply charged ions are related only to the matrix and not instrument parameters. The ability to select different ionization pathways provides a unique opportunity to study fundamental processes related to formation of gas-phase ions from nonvolatile compounds as well as entire new applications.

Finally, it is unlikely that the multiply charged ions are a result of the ion source geometry because FF-TG MALDI has previously been used without such observations (23). This is demonstrated by changing the angle of the laser beam relative to the instrument orifice from 180° (Scheme 1) to 135°, making it possible to use FF reflective geometry so that the laser beam strikes the matrix/analyte mixture without passing through the glass plate. Multiply charged (2+) ions of angiotensin II were observed with FF reflective geometry AP-MALDI, thus demonstrating that TG alignment is not necessary for laser-generated multiply charged ion formation.

Analytical Applications of Highly Charged MALDI Ions—The ability to produce multiply charged ions by AP-MALDI has advantages relative to ease and speed of analysis, mass range enhancement, and fragmentation and potentially in tissue imaging studies (19). The speed of the method is shown by the ability to obtain a complete high resolution mass spectrum of a peptide or protein in 1 s. Because in AP-MALDI ionization occurs at atmospheric pressure, the time requirement for loading samples is also greatly reduced relative to vacuum MALDI, making high throughput analyses a reality.

Sensitivity is also an important issue as it is commonly believed that AP-MALDI is significantly less sensitive than vacuum MALDI. As with any new technology, the optimum parameters for the FF-TG AP MALDI method have likely not been achieved, and sensitivities much greater than reported

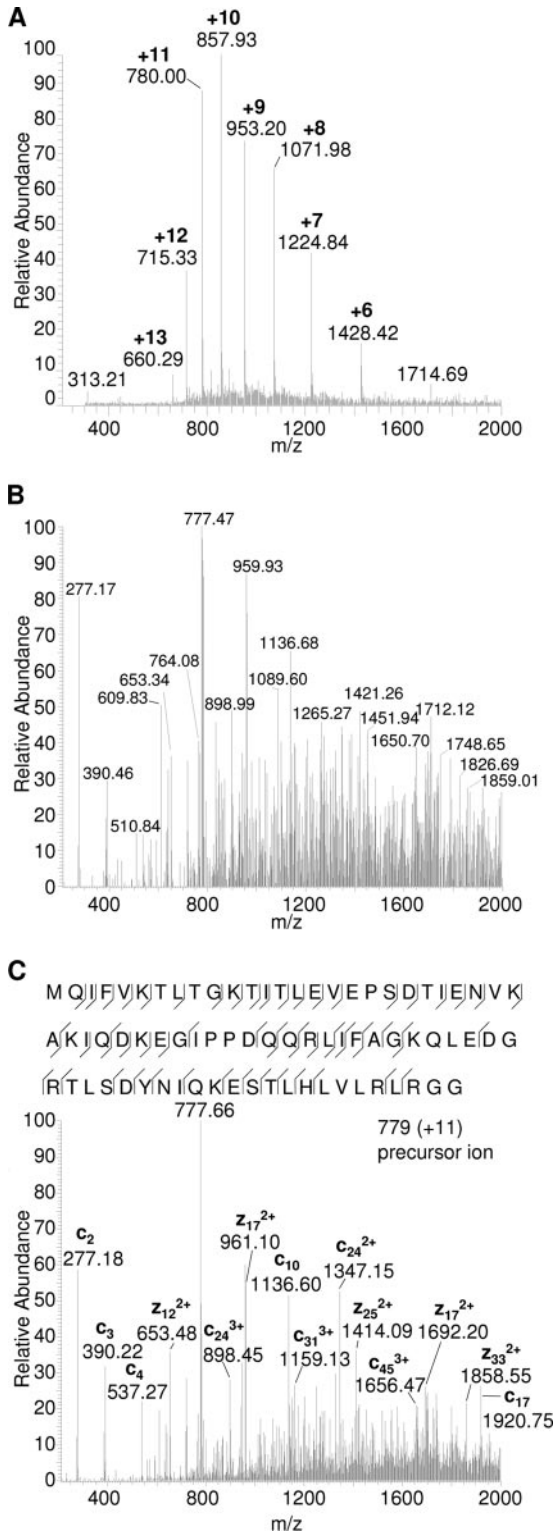


FIG. 4. *A*, the FF-TG AP-MALDI mass spectrum of a single scan acquisition from ~5 pmol of ubiquitin in 2,5-DHB loaded onto the glass slide. *B*, the single scan ETD acquisition from the mass-selected 11+ charge state (*m/z* 779) ions. *C*, the summed (40-s acquisition) proton transfer spectrum of the *m/z* 779 ion with the sequence coverage shown.

here can be expected. Nevertheless, the limit of detection, defined here as the signal necessary to observe the triply charged molecular ion and its three ^{13}C isotope peaks for angiotensin I (molecular weight, 1295.7) using 2,5-DHB as matrix and the solvent-based sample preparation method, was determined to be ~0.3 fmol applied to the glass slide (data not shown). The actual amount of sample consumed is estimated to be a less than 50 amol. A more meaningful gauge of sensitivity for most analyses is the amount of material required to produce a recognizable full-range mass spectrum. The mass spectrum obtained from 40 fmol of insulin in 2,5-DHB applied to the glass slide using the solvent-based dried droplet method is shown in Fig. 3.

The ability to obtain enhanced fragmentation in a MALDI process is another advantage of multiply charged ions. Important new fragmentation methods based on either electron capture (37) or ETD (20) have been applied to sequence determination of peptides and even proteins. Ubiquitin (molecular weight, 8561) was selected because it had been previously sequenced using ETD fragmentation (38). Fluoranthene was used as the electron transfer reagent as described previously (39). In initial experiments using FF-TG AP-MALDI, 2,5-DHB, and solvent-based conditions, ~5 pmol of ubiquitin was placed on the glass slide, and mass selecting the 11+ (*m/z* 779) charge state ion produced the single scan mass spectrum shown in Fig. 4*A*. The single acquisition ETD spectrum is shown in Fig. 4*B* and consists of numerous fragment ions of various charge states. Making use of the proton transfer reaction method (40) simplifies the fragment ion spectrum as previously demonstrated using ESI, thus allowing easier sequence interpretation (Fig. 4*C*). These results represent the first MALDI ETD mass spectra and provide sequence coverage of the protein as previously presented using ESI ETD (40).

The capability to observe either multiply or singly charged molecular ions by a MALDI process allows new experimental information that fills a gap in the long standing controversy regarding ionization mechanisms in MALDI and additionally provides a unique opportunity to study such processes as laser-induced cluster formation, charging, and desolvation as well as charge reduction mechanisms (1). The commonality with ESI also provides a means to probe the mechanisms by which analyte ions are released from highly charged droplets. Finally, these findings have significant analytical utility in areas such as high throughput analyses at high sensitivity, tissue imaging at AP using high resolution instrumentation, and identifying and characterizing proteins using fragmentation processes such as ETD.

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