Direct Comparison of Infrared and Ultraviolet Wavelength Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry of Proteins

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In an effort to gain an understanding of the processes governing matrix-assisted laser desorption/ionization (MALDI), we made a direct comparison of ultraviolet (UV)- and infrared (IR)-MALDI linear time-of-flight mass spectra of proteins obtained from the same samples and matrices (on the same sample surface), using two different lasers, each having short duration (<10-ns) pulses, i.e., a tunable wavelength Nd:yttrium aluminum garnet (YAG) pumped optical parametric oscillator laser operating at 2.94 μm and a Nd:YAG laser operating at 355 nm. We observed that (1) the IR-MALDI and UV-MALDI spectra of a given protein from the same matrix were strikingly similar; (2) protein ions produced by IR-MALDI experienced less fragmentation than those produced by UV-MALDI; and (3) photochemical adducts produced during UV-MALDI were absent in IR-MALDI. These results lead us to speculate on the mechanisms for the ionization process in UV- and IR-MALDI. Because photons with a wavelength of ~3 μm are unlikely to effect electronic excitation of the matrix at the irradiance used for MALDI, we propose that ionization in IR-MALDI occurs as a natural consequence of the solid-to-gas phase transition induced by the IR irradiation, and involves proton transfer reactions in the intermediate phase between solid and gas. The strikingly similar UV- and IR-MALDI mass spectra leads us to the additional proposal that ionization in UV-MALDI may also be a natural consequence of the phase transition and that electronic excitation may not play a primary role in the ionization process. (J Am Soc Mass Spectrom 1998, 9, 1–7) © 1998 American Society for Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [1–4] has become a versatile and important tool for the sensitive determination of the molecular masses of proteins [5–7]. Two parameters that appear critical for effective MALDI-MS are (i) the wavelength of the irradiating laser light and (ii) the chemical and physical properties of the matrix in which the sample of interest is imbedded [4, 7]. Although the laser wavelengths most frequently used for practical MALDI-MS of proteins are in the ultraviolet (UV) range (e.g., 266 nm [1, 3], 337 nm [2, 8], 355 nm [9]), useful mass spectra of proteins have also been demonstrated at infrared (IR) wavelengths [10–15]. MALDI at certain IR wavelengths (e.g., ~3 μm) is of potential practical interest because molecules constituting the natural environment of proteins (especially water) have strong vibrational absorption coefficients at these wavelengths, leading to their possible use as MALDI matrices [16, 17].

Hillenkamp and coworkers [10] first obtained mass spectra of proteins at IR wavelengths with an erbium-yttrium aluminum garnet (Er:YAG) laser emitting at 2.94 μm. These workers used matrices (e.g., succinic acid, urea, glycerol) that absorb photons efficiently at ~3 μm (i.e., close to the O–H and N–H stretch absorptions, but not at wavelengths normally employed for UV-MALDI) as well as matrices that are also suitable for UV-MALDI (e.g., caffeic acid [18]). The same group subsequently demonstrated IR-MALDI-MS of proteins with a TEA-CO_2 laser emitting at 10.6 μm [11], obtaining mass spectra that they reported to be almost identical to those obtained at 2.94 μm and very similar to those obtained at UV wavelengths. These investigators reported a series of other interesting observations, including: (1) a general tendency towards the formation of higher charged states in IR-MALDI as compared to UV-MALDI; (2) a substantially greater removal of sample-matrix material per laser shot by IR-MALDI than by UV-MALDI; and (3) a higher mass resolution (in a reflectron time-of-flight mass analyzer) for bovine trypsin in IR-MALDI compared to UV-MALDI, which they presumed was due to a lower degree of metastable decay and/or matrix-adduct formation in the IR. Although the many similarities between UV- and IR-MALDI led these investigators to infer that the desorp-

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tion mechanism may be similar at both wavelengths, no mechanism was suggested for ionization in IR-MALDI. Nevertheless, they pointed out that direct multiphoton ionization (or photochemical processes) induced with the CO_2 laser was improbable because of the low energy (~120 meV) of the IR photons. Hillenkamp's group and others subsequently successfully applied IR-MALDI to studies of oligonucleotides [19–21] as well as to components of protein mixtures transferred to membranes after separation on electrophoretic gels [13–15].

At UV wavelengths, efficient desorption/ionization requires that the matrix absorb the laser irradiation strongly via electronic transitions in the matrix [1, 3, 22]. Less is known about the absorption requirements for effective MALDI at IR wavelengths, where the laser photons are absorbed primarily via vibrational transitions in the matrix. Recently, using a tunable free-electron laser, Cramer et al. [23] observed mass spectra of insulin at wavelengths ~5.8 μm (i.e., close to the C=O absorption) that were comparable to ~2.94 μm. In a separate study, Sadeghi et al. [24] investigated the mass spectra of substance P, bovine insulin, and a dexyoligonucleotide $pd(T)_{10}$ desorbed from matrices of succinic acid, nicotinic acid, sinapic acid, and urea at wavelengths between 2.88 and 2.96 μm by using a tunable Cr:LiSAF laser-pumped optical parametric oscillator (OPO), and noted that tuning within this wavelength range did not significantly effect any of the IR-MALDI spectra.

In an effort to gain further understanding of the processes governing MALDI at UV and IR wavelengths, we undertook the first direct comparison of the UV- and IR-MALDI linear time-of-flight mass spectra of proteins obtained with the same samples and matrices (on the same sample surface), by using lasers having comparable, short duration (<10-ns) pulses. IR-MALDI spectra obtained by using a tunable wavelength Nd:YAG pumped OPO laser were compared to UV-MALDI spectra obtained with 355-nm frequency tripled light from a Nd:YAG laser with respect to features such as the distribution of charge states, peak shapes, and photochemical adduct peaks. We also compared the relative threshold energy density, sample consumption, resolution, and mass accuracy for IR- and UV-MALDI.

Experimental Details

The mass spectra were collected on a custom linear time-of-flight laser desorption mass spectrometer (30-kV ion acceleration) constructed at Rockefeller University and described elsewhere [3, 25, 26]. The original spectrometer layout was modified so that MALDI samples could be irradiated by light from two alternative laser sources (see Figure 1): (1) Nd:YAG laser (Model HY400, Lumonics Inc., Kanata, Ontario, Canada) producing frequency-tripled light at 355 nm with a pulse duration ~10 ns at a repetition rate of 2.5 Hz; and (2) an optical parametric oscillator (OPO) (Mirage 3000B, Continuum, Santa Clara, CA) pumped by a Nd:YAG laser

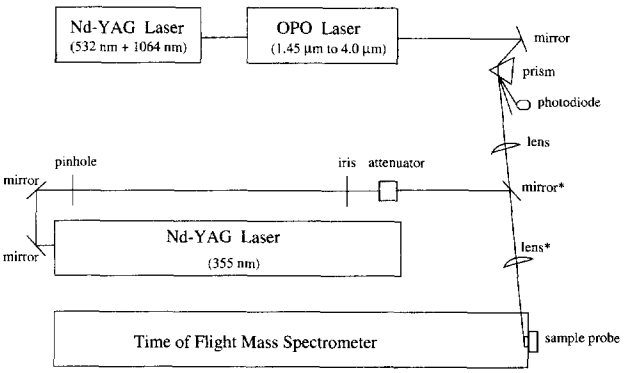

Figure 1. A schematic diagram of the IR- and UV-MALDI mass spectrometer. *The indicated mirror and lens were removed for taking IR-MALDI spectra. The pinhole and iris were inserted into the path of the Nd:YAG beam in order to obtain a smooth Gaussian beam spot.

(Surelite II, Continuum) at a repetition rate of 10 Hz. This latter system employs optical parametric oscillation and optical parametric amplification to convert Nd:YAG pump laser light (532 and 1064 nm) into midinfrared light (pulse duration ~5 ns) that is continuously tunable in wavelength from 1.45 to 4.0 μm by using a nonlinear three-wave mixing process in birefringent potassium titanyl phosphate (KTP).

The output of the OPO laser is a mixture of light beams of three different wavelengths, i.e., the pump beam (corresponding to the photon with the highest energy in the three-wave process), the signal beam (the photon with the intermediate energy in the three-wave process), and the idler beam (the photon with the lowest energy in the three-wave process). The pump light was reflected into a beam dump by a sapphire beam separator installed adjacent to the output aperture of the OPO. The signal and idler beams along with a small amount of visible light (532 nm) were separated by an equilateral prism (Figure 1) made of infrared transmitting fused quartz (ESCO Products Inc., Oak Ridge, NJ). The three beams were separated (over a distance of 1.5 m in ambient laboratory air) by a few centimeters from each other just prior to entering the chamber window of the mass spectrometer. The visible beam was intercepted by a photodiode (Figure 1) and used to trigger the start of the time-of-flight measurement. The signal beam was blocked just prior to the mass spectrometer window. The idler beam (carrying the IR photons of interest) was focused through the window onto the sample-matrix film by using an infrared transmitting fused quartz lens of 8-in. focal length (ESCO Products Inc., Oak Ridge, NJ). The incident angle of this beam with respect to the sample surface normal was 78°. The spot illuminated on the probe was an ellipse with approximate dimensions of the minor and major axes of 130 and 650 μm, respectively [measured by burn marks on an infrared detector card (Sensor Physics, Oldsmar, FL)]. The incident angle of the UV beam was the same as the IR beam, and the UV

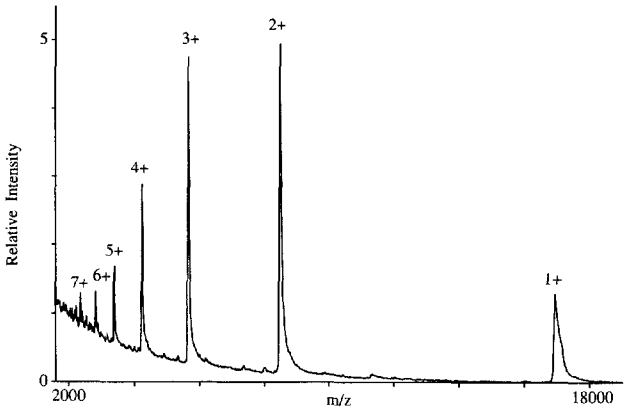

Figure 2. IR-MALDI spectrum of myoglobin in succinic acid. The charge states of the ions are indicated above the peaks. 100 shots were accumulated from one spot.

beam spot size on the sample (produced by a 6-in. focal length fused silica lens) was approximately 100 μm × 500 μm. The laser pulse energies of the separated signal and idler beams were measured just in front of the IR lens by using a calibrated disk thermopile detector (Model 818T-10 from Newport Laboratories, Irvine, CA). Although the laser was operated at a pulse repetition rate of 10 Hz, the IR-MALDI spectra were obtained by irradiating the samples with a series of single laser shots effected through a manually switched shutter having a fixed exposure time of 1/8 s (approximate pulse rate on the sample of 0.5–1 Hz). By contrast, data were obtained in the UV-MALDI mode with no manual shutter at a fixed rate of 2.5 Hz. The output energy of the OPO laser was controlled using two attenuators supplied within the Mirage 3000B OPO system that are used to separately control the energies of the 532-nm pump and 1064-nm seed light. The layout of the two lasers and their ancillary optics (Figure 1) was such that it was possible to switch between UV- and IR-MALDI on the same sample surface within ~1 min.

Time-of-flight (TOF) ion signals were recorded by a digital oscilloscope (Model 7200A with 500-MHz bandwidth, 1-Gsample/s Model 7242D plugin unit, LeCroy, Chestnut Ridge, NY) and transferred to a personal computer for data analysis using a commercial software package (TOFWARE, Ilys Software, Pittsburgh, PA).

Equine myoglobin, equine cytochrome *c* (acid modified), bovine ubiquitin, bovine transferrin, and human immunoglobulin G (IgG) were obtained from the Sigma Chemical Company (St. Louis, MO). Bovine synapsins 1a and 1b were obtained from Dr. Andrew Czernik (Rockefeller University). Stock solutions of all the proteins were prepared to a concentration of 20 μM in 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL), except IgG, which was prepared at 60-μM concentration. Laser desorption matrices were succinic acid (Sigma), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid; Aldrich, Milwaukee, MI), and 4-hydroxy-α-cyanocinnamic acid (4HCCA; Aldrich). Succinic acid was dissolved in 0.1% TFA to a final concentration of 30 g/L. Saturated solutions of sinapic acid (50 mM) and 4HCCA (26 mM) were prepared in 2:1 (v/v) 0.1% TFA:acetonitrile. In all the described experiments, 1 μL of protein stock solution was mixed with 9 μL of matrix solution to yield a final protein concentration of 2 μM (except for IgG at 6 μM). A 0.5-μL aliquot of this protein-matrix solution was deposited onto a stainless steel probe tip (2 mm in diameter) and allowed to dry at ambient temperature. Because succinic acid typically forms heterogeneous microcrystalline deposits, a slightly different method of sample preparation was devised to improve the homogeneity of the films. Thus, half of the desired sample-matrix solution was deposited on the probe and dried rapidly with a stream of forced room temperature air. The remaining half of the sample-matrix solution was applied to the dried crystals on the probe, causing them to redissolve, whereupon the solution was again rapidly dried with a stream of forced air. The resulting crystalline layer was found to be relatively homogeneous.

Results and Discussion

IR-MALDI of Bovine Myoglobin From Succinic Acid

Figure 2 shows an IR-MALDI mass spectrum of bovine myoglobin obtained using the present OPO laser system. The matrix (succinic acid) and wavelength (2.94 μm) were chosen to facilitate comparison with spectra obtained previously by Hillenkamp and coworkers [10], who used an Er:YAG laser. Although the OPO provides a light pulse of relatively short duration (5 ns) compared to that of the Er:YAG system (100–200 ns), the general features of the present spectrum were similar to those obtained previously at 2.94 μm [10] and also somewhat similar to UV-MALDI mass spectra of myoglobin. Our experience at obtaining IR-MALDI mass spectra of proteins was also quite similar to our previous experience with UV-MALDI. At low irradiation energies, no protein ion signal was detected. As we gradually increased the laser energy, the signals of interest began to appear occasionally. Further increases in laser energy led to an increased proportion of laser pulses that yielded readily discernible protein ion signals. We define an operational "threshold energy" as the laser pulse energy at which protein ion signals appear for >25% of the pulses (i.e., when the signal appearance ratio is >25%). Operation of the laser close to this threshold energy yielded tens of spectra from a single spot before the signal appearance ratio began to drop. The protein signals could be brought back by simply increasing the irradiation energy. In this way it was straightforward to obtain an accumulation of as many as 100 spectra from a single sample spot by gradually increasing the pulse energy up to 50% beyond the threshold energy (see Figure 2). It appears that the present IR-MALDI system removes somewhat less

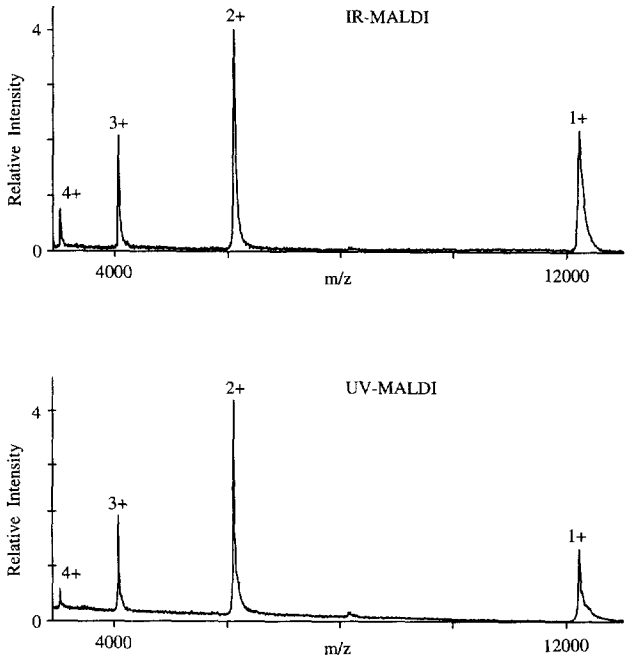

Figure 3. Direct comparison of IR- and UV-MALDI spectra for cytochrome *c* in 4HCCA. Top: IR-MALDI; bottom: UV-MALDI.

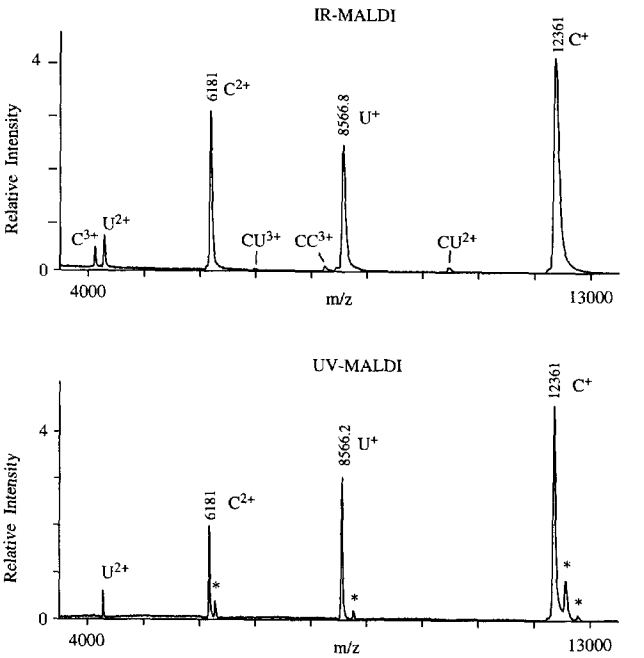

Figure 4. IR- and UV-MALDI spectra of a mixture of cytochrome *c* (designated C) and ubiquitin (designated U) in sinapic acid. Top: IR-MALDI; bottom: UV-MALDI. The singly and doubly charged ions from cytochrome *c* were used as calibrants to obtain the mass-to-charge ratio of ubiquitin. * designates peaks arising from attachment of photochemical adducts to the proteins.

material per laser shot than previously described systems in which only three to four shots could be obtained from a single sample spot [12]. Although we do not know the reason for this apparent difference, it could arise through our use of a highly sensitive linear TOF analyzer (as opposed to a reflectron [10, 11]) and/or our use of short duration IR laser pulses. At the same time, in agreement with previous studies [10, 11], we observe significantly greater sample/matrix removal per laser shot than for UV-MALDI of the same samples, for which several hundred shots can normally be obtained from a single sample spot at threshold energy. From a practical point of view, it is important to emphasize that, in general, the fraction of laser shots that lead to discernible protein signals (even significantly above the threshold energy) is lower for IR- than UV-MALDI, leading to longer times required for the accumulation of mass spectra.

The power density threshold for the observation of protein signals was measured to be $\sim 10^7$ W/cm^2, a value approximately tenfold larger than that previously determined for UV-MALDI at 266 nm ($\sim 10^6$ W/cm^2) [3]. However, it is difficult to make a direct comparison of the energy deposited in the matrix/sample film per unit volume in modes that lead to desorption and ionization because (1) absorption coefficients have not been accurately determined in solid microcrystalline matrix layers and (2) the partitioning of the primary electronic energy loss into vibrational/translational versus radiative modes has not been determined.

One apparent difference between the present IR-MALDI spectrum of myoglobin (Figure 2) and spectra of myoglobin that have been obtained previously using UV-MALDI is the relatively higher degree of charging observed at the IR wavelength [10, 12]. Unfortunately, a direct comparison from the same sample is not possible in the case of succinic acid because this IR matrix does not absorb sufficiently at UV wavelengths to give a MALDI spectrum. To make such direct comparisons, it is necessary to use the same MALDI matrix at both UV and IR wavelengths (see below).

Direct Comparison of IR-MALDI and UV-MALDI Mass Spectra

In the present work, direct comparison of UV- and IR-MALDI mass spectra was facilitated by our ability to rapidly (~1 min) switch between these two modes in the same TOF spectrometer (Figure 1), and by the capacity of certain matrices (e.g., 4HCCA, sinapic acid) to produce high quality spectra of proteins using irradiation in both wavelength ranges. Figure 3 compares mass spectra of equine cytochrome *c* obtained at 2.94 μm (top panel) and 355 nm (bottom panel) from a single sample film prepared from 1 pmol of the protein in 4HCCA. The overall appearance of the two spectra (including the distribution of charge states) are strikingly similar. Figure 4 shows a direct comparison of IR- and UV-MALDI mass spectra of a mixture of bovine ubiquitin (1 pmol) and equine cytochrome *c* (1 pmol) obtained from a different matrix (sinapic acid). Again, the overall appearance of the spectra obtained at these two wavelengths is strikingly similar. At both wave-

lengths, protein ions produced from sinapic acid (Figure 4) exhibit a considerably lower degree of charging compared to spectra produced from 4HCCA (Figure 3) [27].

Significant differences between the spectra obtained at IR and UV wavelengths become apparent only upon close inspection of the spectral peaks (Figure 4). Thus, for example, low intensity satellite peaks are seen in the UV spectrum (bottom panel), but are absent from the IR spectrum (top panel). The satellite peaks in the UV spectrum arise from adduction to the proteins of photodehydrated sinapic acid, as described previously [18]. The absence of such adduction in the IR-MALDI spectrum is not surprising because the photon energy is too low to produce significant yields of photoproducts. Comparison of the top and bottom panels of Figure 4 show that IR-MALDI produced broader peaks in this mass range (m/z = 12,361, Δt(IR) = 470 ns, Δt(UV) = 210 ns; m/z = 8567, Δt(IR) = 400 ns, Δt(UV) = 160 ns; m/z = 6181, Δt(IR) = 290 ns, Δt(UV) = 148 ns, where Δt is the full width at half maximum height). A large component of this additional peak breadth was traced to the fact that the protein ion signals produced from single IR laser shots were observed to jitter about in time to a greater extent than those from single UV laser shots. From our observation that the individual laser shots produced relatively narrow IR-MALDI peaks (data not shown), we conclude that the time jitter likely results from pulse-to-pulse fluctuations in the energy acquired by the ions (perhaps, for example, from surface charging effects). Because the spectra shown in Figure 4 are the sum of the spectra from 100 laser shots, this additional energy (time) jitter caused the integrated IR-MALDI spectral peaks to broaden. Although we do not have a detailed explanation of the origin of this additional energy (time) jitter, we observe the effect to be reduced in delayed-extraction [28–31] spectra of the same sample. The effect will be discussed in more detail in a separate paper describing the application of delayed extraction TOF mass spectrometry to an investigation of IR-MALDI [32]. Further differences can be discerned in Figure 4, where peaks arising from multiply charge clusters (UC^{3+}, CC^{3+}, UC^{2+}, where U designates ubiquitin and C designates cytochrome *c*) are seen in the IR-MALDI spectrum (top panel) but are absent from the UV-MALDI spectrum (bottom panel). We take this observation of cluster ions as evidence that IR-MALDI of proteins from sinapic acid is softer than UV-MALDI from the same matrix.

The mass determination accuracy was measured for the singly protonated ion of bovine ubiquitin, using equine cytochrome *c* as a bracketing internal calibrant (Figure 4). The measured molecular mass (MM) of ubiquitin was found to be 8565.2 ± 1.0 Da for the UV-MALDI determination and 8565.8 ± 2.0 Da for the IR-MALDI determination. These masses agree within experimental error to the value calculated from the sequence (i.e., 8564.9 Da). The larger error for IR-MALDI in this mass range reflects the broader peaks observed in IR- versus UV-MALDI.

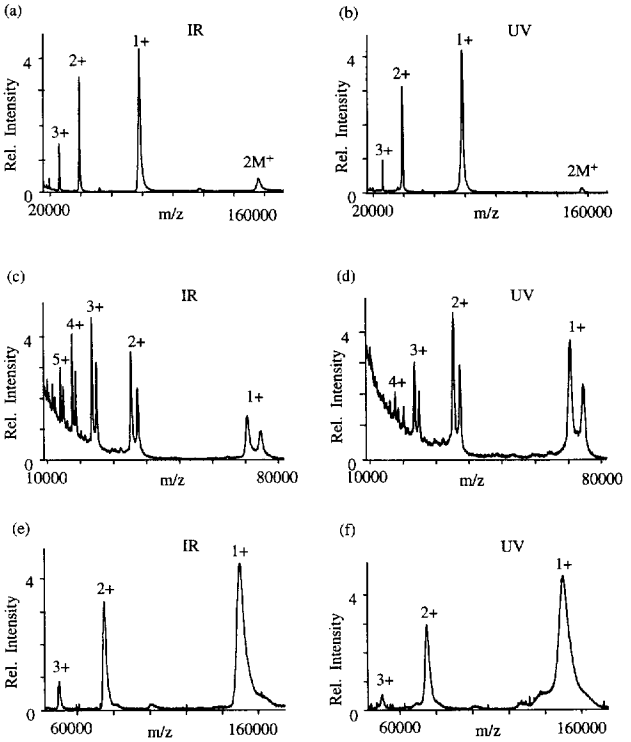

Figure 5. IR- and UV-MALDI spectra of three high mass proteins: transferrin, synapsin, and IgG in sinapic acid. **(a)** IR-MALDI of transferrin, **(b)** UV-MALDI of transferrin, **(c)** IR-MALDI of synapsin, **(d)** UV-MALDI of synapsin, **(e)** IR-MALDI of IgG, and **(f)** UV-MALDI of IgG.

IR-MALDI also yielded intense spectra of higher mass proteins [bovine transferrin (MM ~ 78 kDa), a mixture of bovine synapsin Ia and Ib (MMs = 74,519 and 70,441 Da) and human IgG (MM ~ 150 kDa)]. Direct comparison with UV-MALDI mass spectra from the same samples (Figure 5) showed a very close correspondence, in concert with the results obtained for lower mass proteins (Figures 3 and 4). Detailed inspection of the spectral peaks again showed differences. The IR-MALDI spectra exhibited peaks with a somewhat higher distribution of charge states and the peaks were narrower and notably steeper on their low mass sides. This sharper low mass tail may derive from a lower initial distribution of velocities for the IR-MALDI derived ions or possibly from a narrower initial distribution of velocities. Alternatively, or in addition, a lower degree of fragmentation (both prompt and metastable) in IR-MALDI compared with UV-MALDI may lead to a narrowing of the spectral peaks. Indeed, inspection of the regions of the spectrum on the low mass side of the singly and doubly protonated ion peaks of IgG provides evidence for a considerably higher degree of prompt fragmentation in the UV-MALDI mass spectrum. The relative contributions of initial velocity distribution and fragmentation to the peak shapes remain to be investigated in detail. In this regard, the application of delayed extraction [28–31] is proving useful and will be discussed elsewhere [32]. Nevertheless, what is

clear from our current observations is that IR-MALDI of larger proteins is considerably softer than is UV-MALDI. This observation is in concert with earlier findings of Hillenkamp and coworkers [12] from an indirect comparison of IR- and UV-MALDI of IgG using different matrices.

Conclusions

We have successfully used a tunable OPO laser to obtain high-quality IR-MALDI mass spectra of proteins and have made a direct comparison with UV-MALDI mass spectra of these proteins using the same sample/matrix films. The principal findings were that: (1) the resulting IR- and UV-MALDI spectra of a given protein were strikingly similar; (2) protein ions produced by IR-MALDI underwent less fragmentation than the corresponding protein ions produced by UV-MALDI; and (3) the photochemical adducts produced during UV-MALDI were absent in IR-MALDI.

We infer from the remarkable similarity of the IR- and UV-MALDI mass spectra that the mechanisms of desorption and ionization may be the same in both wavelength ranges. This conjecture appears reasonable for the desorption mechanism, which likely involves the production of an ejected plume or jet of material in both cases [33–40]. However, the mechanism for ionization in MALDI has remained more unsettled. In UV-MALDI, mechanisms for ionization involving electronic excitation/ionization have been proposed [41]. Because electronic excitation does not play a role in IR-MALDI, we propose here that ionization occurs as a natural consequence of the solid-to-gas phase transition induced by the IR irradiation and that ionization takes place through proton exchange reactions in the intermediate phase between solid and gas. We suggest that the protons derive from dissociation of the acidic matrix in this intermediate phase, and that the driving force for ionization is the relatively high proton affinity of the protein molecules. Furthermore, the strikingly similar UV- and IR-MALDI mass spectra lead us to propose that ionization in UV-MALDI may also be a natural consequence of the phase transition, and that electronic excitation may not play a primary role in the ionization process. This proposal is in concert with the recent results of Chen and Beavis [42], who observed that the distribution of charge states remains invariant for UV-MALDI over a wavelength range between 337 and 430 nm.

Acknowledgments

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