Exploring Infrared Wavelength Matrix-Assisted Laser Desorption/Ionization of Proteins with Delayed-Extraction Time-of-Flight Mass Spectrometry

Wenzhu Zhang, Shufang Niu, and Brian T. Chait
The Rockefeller University, New York, New York, USA

We report a study of the application of delayed extraction (DE) to infrared-wavelength matrix-assisted time-of-flight mass spectrometry (IR-MALDI-TOF-MS) of proteins. The shapes of the spectral peaks obtained with DE-IR-MALDI-MS are compared with those obtained from the same samples and matrix using continuous extraction (CE) IR-MALDI-MS. Application of DE results in significant improvements in the peak resolution, revealing spectral features (in proteins with molecular masses <12 kDa) that were not resolved in the corresponding CE-IR-MALDI mass spectra. Particularly significant is a series of peaks on the high mass side of the protonated protein peaks that arise through replacement of protons by adventitious sodium ions in the sample. We deduced that these sodium replacement species are a significant contributer to the broad tails (and resulting peak asymmetries) that are a feature of the DE-IR-MALDI mass spectra of proteins with molecular masses ≥17 kDa. The peak width reduction observed in IR-MALDI by DE suggests that, as in UV-MALDI, the initial velocity distribution for ions produced in the MALDI process contributes to the peak broadness in the CE mass spectra. In a systematic comparison between DE UV-MALDI and DE IR-MALDI, we determined that photochemical matrix adduction is present in UV-MALDI but absent in IR-MALDI. In addition, we find that protein ions produced by IR irradiation are less internally excited (i.e., cooler), exhibiting less fragmentation, more Na^+ replacement and/or unspecified noncovalent adduction, and more heme adduction with apomyoglobin. Thus, IR-MALDI appears to be a softer means for producing gas-phase protein ions than is UV-MALDI. It will be of considerable practical interest to determine whether large protein ions produced by IR-MALDI are sufficiently cool to survive transport through reflecting TOF mass spectrometers (without loss of small neutral species such as H_2O, NH_3, and CO_2) and the extended time periods required for detection by quadrupole ion trap and Fourier transform ion cyclotron resonance mass analyzers. (J Am Soc Mass Spectrom 1998, 9, 879–884) © 1998 American Society for Mass Spectrometry

Matrix-assisted laser desorption/ionization (MALDI) has become a method of choice for the production of gas phase ions from proteins and other biopolymers [1–7]. Although, the majority of current MALDI-mass spectrometry (MS) measurements of proteins make use of ultraviolet (UV) wavelength laser light, infrared (IR) wavelength laser light has also been shown to be effective for this purpose [8–18]. MALDI-MS at these longer wavelengths is of interest from both the practical and theoretical points of view. From the practical point of view, molecules constituting the natural environment of proteins (especially water) have strong vibrational absorption cross sections at certain IR wavelengths (e.g., ~3 μm) and, therefore, could conceivably be used as MALDI matrices [13,19]. In addition, it has been reasoned that the deeper penetration of IR versus UV laser light allows more effective utilization of sample dispersed through a thick porous medium (as is the case for proteins dispersed in polyacrylamide gels or transferred from gels to blotting membranes) [10,16]. From the theoretical point of view, it is of interest to directly compare IR- with UV-MALDI in an attempt to understand the mechanisms underlying the MALDI process.

In a recent study carried out in our laboratory [20], we undertook such a direct comparison of UV- and IR-MALDI mass spectra of proteins using the same samples and matrices on the same sample surfaces. The spectra were obtained with a linear time-of-flight (TOF) mass spectrometer operating in the continuous extraction (CE) mode. The striking overall similarity of the

Address reprint requests to Brian T. Chait, 1230 York Ave., New York, NY 10021. E-mail: chait@rockvax.rockefeller.edu

1044-0305/98/$19.00
PII S1044-0305(98)00060-9

Received February 11, 1998
Revised April 2, 1998
Accepted April 6, 1998

UV- and IR-MALDI spectra led us to propose that MALDI at both wavelengths may have a common ionization mechanism, occurring as a natural consequence of the solid to gas phase transition induced by the UV or IR irradiation. At the same time we also noted fine differences. In particular, the IR-MALDI spectral peak shapes were less symmetric than those obtained with UV-MALDI—i.e., the IR-MALDI spectra were characterized by peaks with steep low mass edges and extended high mass tails. Although we provided possible explanations for these peak shape differences [20], we did not undertake a systematic experimental investigation of their origin. This is the subject of the present paper.

Earlier studies showed that ions produced by UV-MALDI have broad initial velocity distributions [21–24], a phenomenon that contributes to the broad peaks observed in MALDI mass spectra obtained with CE in linear TOF mass analyzers. Delayed extraction (DE) techniques have been used to overcome the effect of the broad initial velocity distributions, leading to significant improvements in the peak resolution obtained with UV-MALDI-TOF-MS [25–29] and more recently with IR-MALDI-TOF-MS [30]. Here, we report a study of the application of DE to IR-MALDI-TOF-MS. Peak shapes obtained with DE IR-MALDI-MS are examined and directly compared with those obtained with DE UV-MALDI-MS. The results allow us to provide a detailed explanation for the peak shape differences observed in our earlier studies and to illustrate the subtle differences between IR- and UV-MALDI.

Experimental

The UV- and IR-MALDI linear time-of-flight mass spectrometer has been described in [20]. Briefly, IR photons were produced by an optical parametric oscillator laser tuned to a wavelength of 2.94 μm (pulse duration ~5 ns), whereas UV photons (355 nm) were produced by frequency tripling the fundamental from a Nd:YAG laser (pulse duration ~10 ns). The desorbed ions were extracted by either CE or DE into an ~1.8 m flight tube, and detected by a hybrid detector (a microchannel plate followed by a gridded electron multiplier) [31]. To ensure high sensitivity, it is necessary to efficiently transport the majority of the ions of interest from the ion source to the detector. We, therefore, aligned the TOF analyzer [using an imaging detector (Model 3025-FM, Galileo Electro-Optics, MA)] so that the center of ion beam struck the center of the detector and used an electrostatic particle guide [32] to prevent the beam from spreading beyond the periphery of the detector. In the CE experiments, ions with charge state q were extracted into the flight tube with energies of $q \times 30$ keV, whereas in the DE experiments the same ions were extracted with energies between $q \times 27$ and $q \times 28$ keV, depending on the fine adjustment of the ion extraction pulse amplitude (see Figure 1 and discussion below).

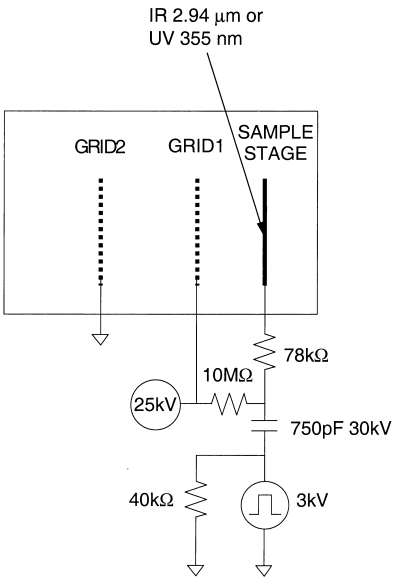

Figure 1. Schematic diagram of the pulsing system in the DE MALDI-TOF mass spectrometer.

The DE pulsing system is shown schematically in Figure 1. A pulse having a rise time of ~2 μs is delivered to the sample probe using a high voltage (HV) switch. The HV switch was constructed in-house, and is a modified version of a previously published design [33]. The delay time between ion production and application of the extraction field was adjusted between 1.75 and 3.5 μs using a high precision digital delay generator (model DG535, Stanford Research System, Palo Alto, CA). The pulse voltage (supplied by a 0–3000 V power source, Model 215, Bertan Associates, Syosset, NY) was manually scanned to obtain the optimum TOF peak resolution in the mass range of interest. The optimum condition for each protein sample was first obtained using UV irradiation, whereafter these same ion extraction conditions were used for the corresponding IR-MALDI measurement, a procedure that was observed to yield optimum extraction conditions for both wavelengths (data not shown). Comparisons between UV- and IR-MALDI mass spectra were made on the same proteins using the same matrix (sinapic acid). DE spectra were calibrated using a previously described procedure [24] that yielded mass determination errors $\leq \pm 1$ Da for proteins with molecular masses <17 kDa.

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).

Bovine insulin, bovine ubiquitin, bovine cytochrome c, equine myoglobin, and bovine carbonic anhydrase II were obtained from Sigma Chemical (St. Louis, MO). Bovine synapsin I [34] was a gift from Dr. Andrew Czernik at the Rockefeller University. The laser desorption matrix was 3,5-dimethoxy-4-hydroxycinnamic acid

(sinapic acid; Aldrich, Milwaukee, MI). Stock solutions of all the proteins were prepared to a concentration of 20 μM in 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL), and a saturated solution of sinapic acid (50 mM) was prepared in 2:1 (v/v) 0.1% TFA: acetonitrile. 1 μL of protein stock solution was mixed with 9 μL of matrix solution to yield a final protein concentration of 2 μM. A 0.5-μL aliquot of this protein/matrix solution (i.e., 1 pmol of protein) was deposited onto a stainless steel probe tip (2 mm in diameter) and allowed to dry at ambient temperature.

Results and Discussions

Comparison of Continuous and Delayed Extraction IR-MALDI

In our previous comparison between UV- and IR-MALDI using CE ion acceleration [20], we observed that the protein ion signals produced from single IR laser shots jittered about in time to a greater extent than the corresponding signals from single UV shots. This time jitter led to a general broadening of the IR-MALDI mass spectral peaks. The application of DE, in addition to producing the well-described decrease in spectral peak widths by compensating for the initial velocity distribution [25–30], greatly reduced the extent of the aforementioned IR-MALDI time jitter. To further improve the quality of the IR-MALDI mass spectra, we also summed only those individual spectra in which the intensities of the peaks of interest intensity were greater than 5% and less than 100% of the oscilloscope full scale range. This selection prevented spectra having either very small protein ion signals or saturated signals from being added into the summed spectrum. All the CE and DE UV- and IR-MALDI spectra reported here were collected using these same criteria and are sums of ~50 such single shot spectra.

Figure 2a compares the singly charged protein peak regions of the IR-MALDI mass spectra of bovine cytochrome *c* (MM = 12,231 Da) obtained using CE versus DE conditions from the same sample surface. Figure 2b provides an analogous comparison for bovine carbonic anhydrase II [molecular mass (MM) = 29,022 Da]. As we observed previously for IR-MALDI mass spectra obtained under CE conditions [20], the peak shapes are unsymmetrical, with steep low mass edges and extended high mass tails. Upon the application of DE, the resolution was improved by a factor of 3–4, although considerable high mass tailing is still evident. With the exception of a partially resolved peak (designated X in Figure 2a) located 21.0 ± 1.0 Da above the protonated cytochrome *c* peak, individual features in the high mass tails were not resolved. In an effort to resolve the components of the residual high mass tailing, we obtained DE IR-MALDI mass spectra of two proteins with lower molecular masses (Figure 3).

Figure 3 compares portions of the CE and DE IR-MALDI mass spectra of bovine insulin (MM = 5734 Da)

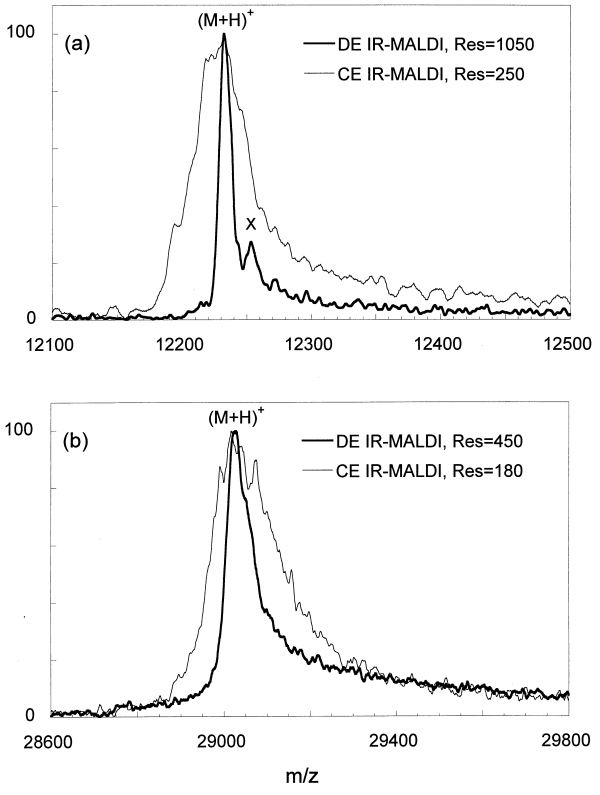

Figure 2. Comparison of CE and DE IR-MALDI mass spectra showing the singly protonated $(M+H)^+$ regions of (**a**) bovine cytochrome *c* and (**b**) bovine carbonic anhydrase II. Matrix: sinapic acid. The peak labeled X is located 21.0 ± 1.0 Da above the $(M+H)^+$ ion peak. "Res" designates the full width at half maximum resolution.

(top panel) and bovine ubiquitin (MM = 8565 Da) (bottom panel). Again, application of DE has dramatically reduced the width of the intact protein ion peaks. The DE IR-MALDI spectra are characterized by an intense symmetrical protonated protein ion peak and a series of equally spaced peaks of lower intensity on the high mass side of the protonated protein ion peak. The average spacing between these equally spaced peaks (21.7 ± 0.4 Da) indicates that they originate through replacement of protons in the protein by adventitious sodium cations in the sample, giving rise to the $(M+H+n\mathrm{Na}-n\mathrm{H})^+$ ion species that have been observed extensively in the positive ion mass spectrometry of peptides and oligonucleotides. Formally such species are sodium salts of the biopolymer that may already be present in the solid sample prior to laser bombardment. Alternatively, the Na^+ replacement species may be produced during the MALDI process itself. Although we observed significant sample to sample variation in the intensity of these Na^+ replacement species (presumably due to different levels of Na^+ impurity in the different samples), there was a tendency for the IR-MALDI spectra to exhibit higher levels of the Na^+ replacement species than the UV-MALDI spectra. We deduce from these results that a significant component of the broad high mass tails observed from the higher

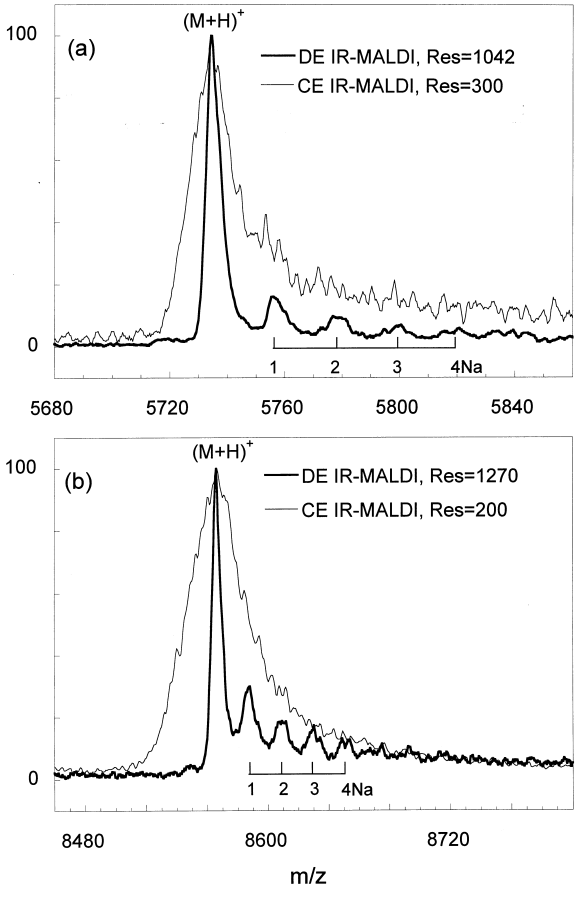

Figure 3. Comparison of CE and DE IR-MALDI mass spectra. (**a**) Bovine insulin. (**b**) Bovine ubiquitin. Matrix: sinapic acid.

molecular mass proteins shown in Figure 2 likely also arises from extensive Na^+ replacement.

Comparison of Delayed Extraction Spectra with UV- and IR-MALDI

We also made a detailed comparison of DE UV-MALDI and DE IR-MALDI spectra obtained under precisely the same ion extraction conditions. Figure 4 shows such a comparison for bovine ubiquitin (MM = 8565 Da) obtained from a single sample surface. The FWHM resolution of the singly protonated protein ion peak ($m/\Delta m$ = 1100) is identical for both UV- and IR-MALDI. Spectral features resulting from fragmentation and Na^+ replacement are readily discerned. With respect to fragmentation, the protein ions produced by UV-MALDI experience significant loss of H_2O/NH_3 ($\Delta \approx 18$ Da) and CO_2 ($\Delta \approx 44$ Da), whereas corresponding losses are less evident from the IR-MALDI mass spectrum. The lower degree of fragmentation observed in IR-MALDI indicates that IR irradiation produces cooler (i.e., less excited) ion species than does UV irradiation, confirming earlier conclusions by other workers [12,30] and us [20]. With respect to Na^+ replacement, we observe somewhat higher levels in IR- than in UV-MALDI, although the sample-to-sample variability of

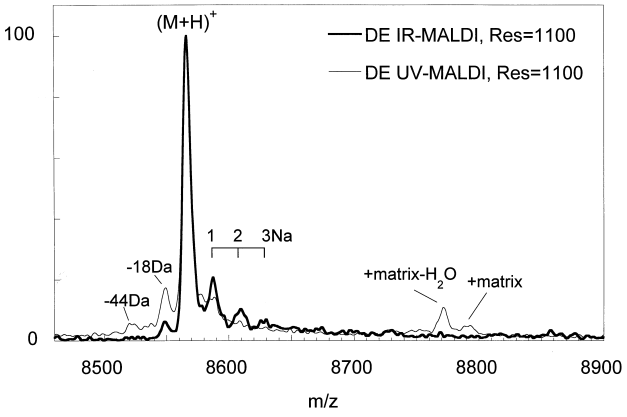

Figure 4. Comparison of DE UV-MALDI and DE IR-MALDI mass spectra for bovine ubiquitin. Matrix: sinapic acid.

Na^+ replacement (see above) demands that we be cautious about generalizing this observation. Finally, in accord with our earlier observations [20], photochemical matrix adducts produced during UV-MALDI are absent from the IR-MALDI mass spectra, presumably because the photon energy in IR-MALDI is too low to produce reactive species of the type observed in UV-MALDI [35].

In the top panel of Figure 5 we compare a portion of the DE UV-MALDI and DE IR-MALDI mass spectra of bovine cytochrome *c* (MM = 12,231 Da) obtained from the same sample/matrix preparation. The bottom panel shows a similar comparison of spectra obtained from equine myoglobin (MM = 16,952 Da). Identical FWHM resolutions were obtained with UV- and IR-MALDI. Thus, the FWHM resolution for cytochrome *c* was 1110 and that for apomyoglobin was 1000. Unlike ubiquitin, the spectral features arising from fragmentation, Na^+ replacement, and adduction are no longer all well resolved for these larger proteins. However, inspection of the spectral shapes allows us to infer that the same differences that we observed for ubiquitin are again manifested in the UV- and IR-MALDI mass spectra of these larger proteins. Thus, matrix adduction is absent from the IR-MALDI produced protein ions but present in those produced by UV-MALDI; protein ions produced by IR-MALDI exhibit a lower degree of fragmentation, resulting in steeper low mass peak edges; and the high-mass tailing in IR-MALDI is more pronounced than that observed in UV-MALDI. In addition, heme adduction is observed from the myoglobin sample in IR-MALDI (even though denaturing conditions were used during the preparation of the sample/matrix film), but not in UV-MALDI, providing further evidence that protein ions produced in IR-MALDI are cooler than those produced in UV-MALDI.

Moving to a still higher molecular mass protein, Figure 6 compares the doubly charged region of the DE MALDI mass spectra of bovine carbonic anhydrase II (MM = 29,022 Da) obtained with UV versus IR irradiation. We show the doubly charged ion peak rather

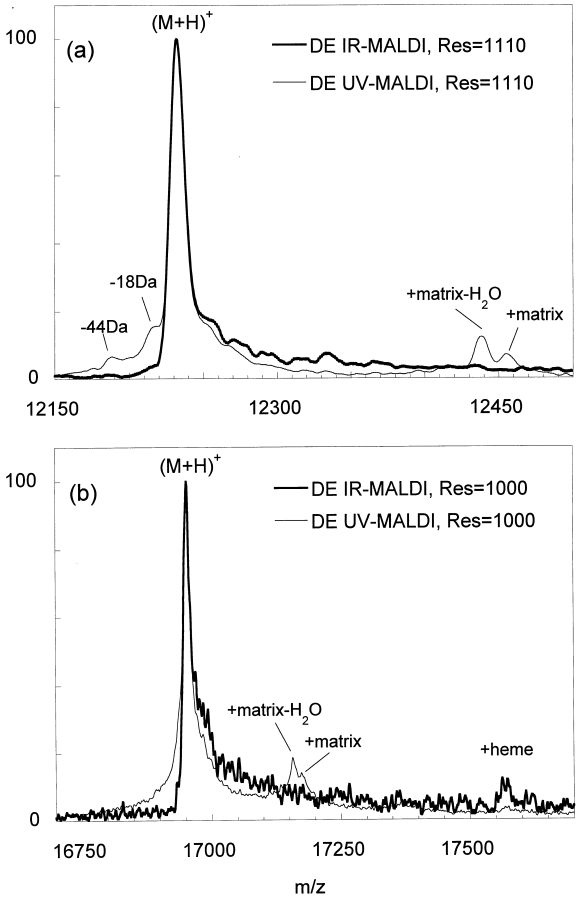

Figure 5. Comparison of DE UV-MALDI and DE IR-MALDI mass spectra. (**a**) Bovine cytochrome *c*. (**b**) Equine myoglobin. Matrix: sinapic acid.

than the singly charged peak because it has significantly higher intensity and therefore lower statistical fluctuations. Although the spectral features corresponding to fragmentation and Na^+ replacement (observed for smaller proteins) are no longer resolved, the observed peak shape differences are in concert with those obtained when comparing the lower mass proteins (Figures 4 and 5). Thus,

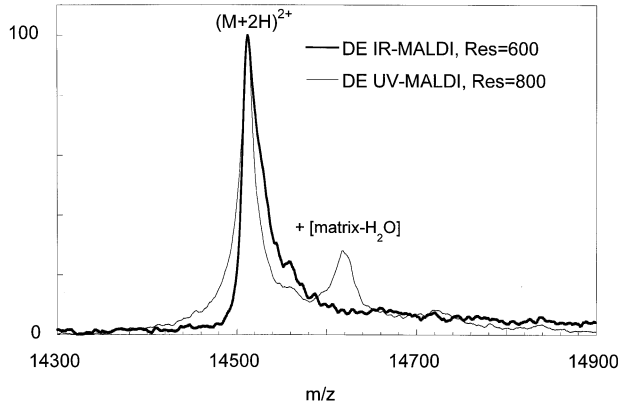

Figure 6. Comparison of DE UV-MALDI and DE IR-MALDI mass spectra for the doubly charged ion region of bovine carbonic anhydrase II. Matrix: sinapic acid.

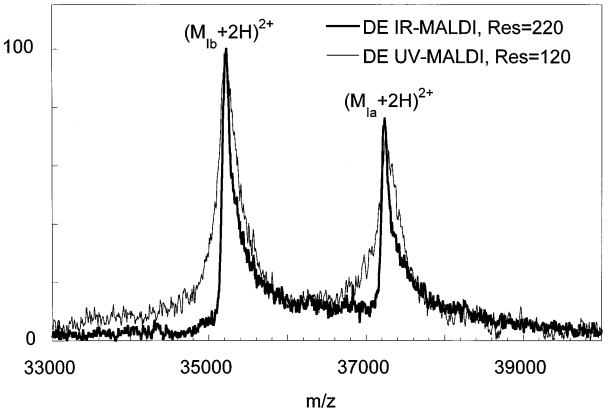

Figure 7. Comparison of DE UV-MALDI and DE IR-MALDI mass spectra for the doubly charged ion region of bovine synapsin Ia and Ib. Matrix: sinapic acid.

the lower degree of fragmentation in IR-MALDI results in a peak (Figure 6) with a more sharply rising low mass edge, whereas the higher level of Na^+ replacement or noncovalent adduction in IR-MALDI give rise to a more prominent high mass tail. Also, the matrix adduct peak is absent in IR-MALDI, but present in UV-MALDI. Overall, the carbonic anhydrase ion peak obtained by UV-MALDI is narrower than that obtained by IR-MALDI. Comparison of the less intense singly charged carbonic anhydrase II ions obtained with UV and IR irradiation yields results that closely mirror those obtained for the doubly charged species (data not shown).

Moving to larger species still, Figure 7 compares the high intensity doubly charged region of the DE MALDI spectra of bovine synapsin Ia and Ib (MMs = 74,519 and 70,441 Da) obtained with UV versus IR irradiation. Again, results for the less intense singly charged ion species mirror those shown in Figure 7 albeit with lower ion statistics. At this high mass, ion peaks resulting from the loss of small neutral entities, Na^+ replacement ions, and matrix adduct ions are not resolved from the doubly protonated protein ion. Nevertheless, comparison of the spectra shows that the IR-MALDI synapsin ion peaks exhibit a steeper rise on their low mass sides than do the corresponding UV-MALDI ion peaks. We surmise that this difference arises because of a lower degree of fragmentation in the IR-MALDI process versus a more extensive degree of fragmentation in the UV-MALDI process. Contrary to the data shown in Figures 4–6, the IR-MALDI peaks in Figure 7 have less prominent high mass tails, leading to peak widths that are overall narrower than those observed using UV-MALDI. In this case, it likely that the enhanced tailing on the peaks in UV-MALDI is mainly a result of the merger of the synapsin ion peaks with the peaks arising from matrix adduction.

Conclusion

We have successfully applied the DE technique to both UV- and IR-MALDI in a single TOF instrument and

have compared the spectra from a series of proteins using the same sample/matrix preparations. Application of DE improved the resolution significantly in both UV- and IR-MALDI, and identical resolutions were obtained in UV- and IR-MALDI under the same DE conditions for proteins with molecular masses up to 17 kDa. The peak width reduction in IR-MALDI by DE suggests that, as in UV-MALDI [21–24], the initial velocity distribution for ions produced in the process contributes to the peak broadness in the CE mass spectra. Furthermore, we deduce from the closely similar resolution enhancements that the initial velocity distributions in IR-MALDI are not markedly different from those occurring in UV-MALDI, in concert with a recent report of Berkenkamp et al. [36]. In our systematic comparison between DE UV-MALDI and DE IR-MALDI, we determined that photochemical matrix adduction is present in UV-MALDI but absent in IR-MALDI. In addition, we found that protein ions produced by IR irradiation are less internally excited (i.e., cooler), exhibiting less fragmentation, more Na^+ replacement and/or unspecified noncovalent adduction, and more heme adduction with apomyoglobin. Thus, IR-MALDI appears to be a softer means for producing gas-phase protein ions than is UV-MALDI. [We note here that Bahr et al. [29] have shown that certain matrices (notably DHB and "super DHB") also produce lower degrees of excitation in UV-MALDI]. Although first results of IR-MALDI reflectron TOF measurements on the small protein cytochrome *c* appear promising [30], it will be of considerable practical interest to determine whether large protein ions produced by IR-MALDI are sufficiently cool to survive transport through reflecting TOF mass spectrometers (without loss of small neutral species such as H_2O, NH_3, and CO_2) and the extended time periods required for detection by quadrupole ion trap and Fourier transform ion cyclotron resonance mass analyzers. At present, fragmentation (especially the extensive loss of small neutral molecules) appears to limit the utility of UV-MALDI mass spectrometry for the accurate molecular mass analysis of large proteins.

Acknowledgments

This work was supported by grants from the Department of Energy (DOE 45Q170) and the National Institutes of Health (RR00862). The authors thank Herbert Cohen for assistance in construction of the high voltage pulsing system.

References

1. Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988,** *60,* 2299.
2. Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. *Rapid Commun. Mass Spectrom.* **1988,** *2,* 151.
3. Beavis, R.C.; Chait, B.T. *Rapid Commun. Mass Spectrom.* **1989,** *3,* 233.
4. Hillenkamp, F.; Karas, M.; Beavis, R.C.; Chait, B.T. *Anal. Chem.* **1991,** *63,* 1193A.
5. Chait, B.T.; Kent, S.B. *Science* **1992,** *257,* 1885.
6. Stults, J.T. *Curr. Opin. Struct. Biol.* **1995,** *5,* 691.
7. Beavis, R.C.; Chait, B.T. *Methods Enzymol.* **1996,** *270,* 519.
8. Overberg, A.; Karas, M.; Bahr, U.; Kaufmann, R.; Hillenkamp, F. *Rapid Commun. Mass Spectrom.* **1990,** *4,* 293.
9. Overberg, A.; Karas, M.; Hillenkamp, F. *Rapid Commun. Mass Spectrom.* **1990,** *5,* 128.
10. Eckerskorn, C.; Strupat, K.; Karas, M.; Hillenkamp, F.; Lottspeich, F. *Electrophoresis* **1992,** *13,* 664.
11. Strupat, K.; Karas, M.; Hillenkamp, F.; Eckerskorn, C.; Lottspeich, F. *Anal. Chem.* **1994,** *66,* 464.
12. Hillenkamp, F.; Karas, M.; Berkenkamp, S. *Proceedings of the 43th ASMS Conference on Mass Spectrometry and Allied Topics;* Atlanta, GA, 1995; p 357.
13. Berkenkamp, S.; Karas, M.; Hillenkamp, F. *Proc. Natl. Acad. Sci. USA,* **1996,** *93,* 7003.
14. Cramer, R.; Hillenkamp, F.; Haglund, R.F. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 1187.
15. Sutton, C.W.; Wheeler, C.H.; Sally, U.; Corbett, J.M.; Cottrell, J.S.; Dunn, M.J. *Electrophoresis* **1997,** *18,* 424.
16. Eckerskorn, C.; Strupat, K.; Schleuder, D.; Hochstrasser, D.; Sanchez, J.-C.; Lottspeich, F.; Hillenkamp, F. *Anal. Chem.* **1997,** *69,* 2888.
17. Caldwell, K.L.; McGarity, D.R.; Murray, K.K. *J. Mass Spectrom.* **1997,** *32,* 1374.
18. Sadeghi, M.; Olumee, Z.; Tang, X.; Vertes, A.; Jiang, Z.X.; Henderson, A.J.; Lee, H.S.; Prasad, C.R. *Rapid Commun. Mass Spectrom.* **1997,** *11,* 393.
19. Nelson, R.W.; Rainbow, M.J.; Lohr, D.E.; Williams, P. *Science* **1989,** *246,* 1585.
20. Niu, S.; Zhang, W.; Chait, B.T. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 1.
21. Beavis, R.C.; Chait, B.T. *Chem. Phys. Lett.* **1991,** *181,* 479.
22. Verentchikov, A.; Ens, W.; Martens, J.; Standing, K.G. *Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics;* Washington, D.C., 1992; p 360.
23. Boekelman, V.; Spengler, B.; Kaufmann, R. *Eur. Mass Spectrom.* **1995,** *1,* 81.
24. Juhasz, P.; Vestal, M.L.; Martin, S.A. *J. Am. Soc. Mass Spectrom.* **1997,** *8,* 209.
25. Colby, S.M.; King, T.B.; Reilly, J.P. *Rapid Commum. Mass Spectrom.* **1994,** *8,* 865.
26. Brown, R.S.; Lennon, J.J. *Anal. Chem.* **1995,** *67,* 1998.
27. Whittal, R.M.; Li, L. *Anal. Chem.* **1995,** *67,* 1950.
28. Vestal, M.L.; Juhasz, P.; Martin, S.A. *Rapid Commun. Mass Spectrom.* **1995,** *9,* 1044.
29. Bahr, U.; Stahl-Zeng, J.; Gleitsmann, E.; Karas, M. *J. Mass Spectrom.* **1997,** *32,* 1111.
30. Berkenkamp, S.; Menzel, C.; Karas, M.; Hillenkamp, F. *Rapid Commun. Mass Spectrom.* **1997,** *11,* 1399.
31. Beavis, R.C.; Chait, B.T. *Methods and Mechanisms for Producing Ions from Large Molecules;* Standing, K.G.; Ens, W., Eds.; Plenum: New York, 1991, p 227.
32. Macfarlane, R.D.; Torgerson, D.F. *Int. J. Mass Spectrom. Ion Phys.* **1976,** *21,* 81.
33. Continetti, R.E.; Cyr, D.R.; Neumark, D.M. *Rev. Sci. Instrum.* **1992,** *63,* 1840.
34. Zhang, W., Czernik, A.J., Yungwirth, T., Aebersold, R., and Chait, B.T. *Protein Sci.* **1994,** *3,* 677.
35. Beavis, R.C.; Chait, B.T. *Rapid Commun. Mass Spectrom.* **1989,** *3,* 432.
36. Berkenkamp, S.; Menzel, C.; Rohling, U.; Strupat, K.; Hillenkamp, F.; Presented at the *14th International Mass Spectrometry Conference,* Tampere, Finland, August 1997.