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Multipole-Storage-Assisted Dissociation for the Characterization of Large Proteins and Simple Protein Mixtures by ESI-FTICR-MS

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In Fourier transform ion cyclotron resonance mass spectrometry, collisionally activated dissociation (CAD) typically is accomplished within the analyzer ion cell. An alternative approach of multipole-storage-assisted dissociation (MSAD) has previously been demonstrated by inducing collisional fragmentation in the external multipole that is usually employed for ion accumulation. To explore the utility of MSAD for interrogating intact proteins and simple protein mixtures in a multiplexed manner, we have investigated the means of controlling the collisional energy and the fragmentation pattern for this experimental approach. With protein samples in the low micromolar concentration range, the two major experimental parameters affecting MSAD in the hexapole region were found to be the dc offset voltage and accumulation time. While low-energy MSAD of intact proteins yields fragment ions similar to sustained off resonance irradiation collision-activated dissociation (SORI-CAD), high-energy MSAD induces sequential fragmentation for intact proteins to yield a rich variety of singly charged ions in the *m/z* 600−1200 Da region. Each of the seven proteins (M_r range of 8.5−116 kDa) examined in this study exhibited their own characteristic MSAD fragmentation pattern, which could be used as a signature of the presence of a given protein, even in a mixture. In addition, any MSAD fragment can be isolated and dissociated further by SORI-CAD in an MS^3-type experiment inside the FTICR analyzer cell. This presents a novel way to interrogate the identities of these fragment ions as well as obtain amino acid sequence tag information that can be used to identify proteins from mixtures.

High-resolution mass spectrometry experiments provide exquisite information about the molecular masses of important biomolecules, such as intact proteins; however, for unambiguous identifications it is advantageous to supplement these measurements with ion fragmentation experiments to obtain in-depth information on protein sequence, post-translational modifications, and even higher order structure.[1] For example, a wide range of dissociation techniques for intact proteins have been implemented in the analyzer cell of Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, including sustained off resonance irradiation collision-activated dissociation (SORI-CAD),[2] electron capture dissociation (ECD),[3] infrared multiphoton dissociation (IRMPD),[4] and blackbody infrared radiative dissociation (BIRD).[5] The most common procedure for conducting these ion dissociation methods involves isolating an ensemble of parent ions at a given mass-to-charge ratio (*m/z*) inside the analyzer cell and then activating the trapped parent ions by these different methods to achieve fragmentation. While these dissociation methods have proven to be quite valuable, they have severe limitations for very large proteins and for the high-throughput investigation of protein mixtures.

Most current FTICR mass spectrometers also utilize a second ion-trapping/accumulation device: an external rf-only hexapole or octapole bounded by electrostatic elements.[6] Electrosprayed ions traverse the skimmer cone and are accumulated in the rf-only linear multipole storage trap by employing a dc-controllable gate electrode at the exit end of the multipole, as illustrated in Figure 1. The voltage and timing of this gate provides the ability to accumulate ions for a desired period of time, after which they can be transported out of the multipole and down to the FTICR analyzer cell for mass/charge measurement. Because electrospray ionization (ESI) is continuous, the multipole functions as a linear ion trap to admit and accumulate a sufficient ion population for eventual FTICR ion detection. New fragmentation techniques for FTICR-MS have been developed by exploiting ion dissociation in this linear ion trap, with either gas-phase collisional activation accomplished with multipole-storage-assisted dissociation (MSAD),[7−12] "ion threshing",[13] or photon-induced dissociation (termed external IRMPD)[14,15] While these approaches may seem

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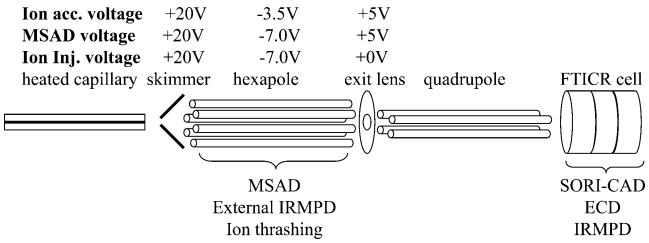

Figure 1. Ion optics of an FTICR mass spectrometer (not drawn to scale). During the ion accumulation stage, ions flow through the skimmer and are trapped in the hexapole, confined radially by the rf voltage of the rods and axially by the skimmer voltage, exit lens voltage, and hexapole dc offset voltage. MSAD, ion threshing, and external IRMPD can be accomplished at this stage at the location indicated. During ion injection stage, ions are transferred through the quadrupole and are trapped in the FTICR cell. Conventional CAD, IRMPD, ECD, etc. can be induced at this stage inside the FTICR cell. Voltage settings are listed directly above the appropriate electrostatic component.

to be a minor variation of the established CAD techniques listed above, in fact, these multipole dissociation methods afford a number of advantages, including eliminating the need for a collision gas in the high vacuum region of the FTICR instrument and the ability to conduct multiplexed fragmentation at relatively high energies.

MSAD was first observed by accumulating ions in the multipole for an extended time frame.[7] It was postulated that once the ion density reaches the space charge limit in the multipole, the Coulomb force will push the ion ensemble to spread out radially, enabling the ions to oscillate at higher amplitude. This would allow coupling of the rf energy in the hexapole rods to the ions, effectively accelerating them to higher kinetic energy.[8,10,16] Fragmentation then would result from the collisions of excited ions with the background gas molecules in the hexapole (typically air at $\sim 10^{-5}$ Torr) and thus is generally regarded as a form of CAD. Like nozzle−skimmer collisional activated dissociation, MSAD is also an in-source fragmentation. Compared with SORI-CAD, MSAD obviates the need for introduction of collisional gas into the analyzer cell and subsequent pump down. However, in an rf-only multipole, no parent ion selection is possible; thus, MSAD fragments all species present, which limits its use in a targeted fragmentation experiment.

We have undertaken a systematic investigation of ways to control the collision energy and fragmentation pattern for intact proteins to evaluate this MSAD process. In particular, we have focused on examination of the hexapole dc offset voltage and accumulation time, which are the two key parameters in controlling the ion population. Seven representative proteins covering a molecular mass range of 8.5−116 kDa were employed to study the fragmentation pattern of intact proteins under a variety of MSAD conditions. In addition, the ability to conduct MSAD experiments on simple protein mixtures was also investigated.

To extend the capabilities of MSAD, we have devised an experimental method in which selected MSAD fragment ions were subjected to a further stage of tandem mass spectrometry in the FTICR analyzer cell. This MS^3-type experiment enables coupling of the efficient, relatively high energy MSAD process with the more selective SORI-CAD. The goal of this approach was to generate sequence tag information by dissociation of the MSAD fragment peptide for protein identification, in a manner analogous to generating sequence tags by dissociation of peptides from enzymatic digestion.[17] McLafferty and co-workers have employed a similar approach to directly generate sequence tag from intact proteins.[18,19] They have shown that a sequence tag and an intact protein mass were sufficient to identify a protein from a protein database. Although this approach appears to be quite promising, there are at least three major challenges that complicate this method. First, the fragments of intact proteins usually are still very large and exhibit a wide isotopic package. When comparing the masses of two adjacent fragment ions in an effort to identify the residual amino acid, the difficulty in accurately choosing the correct isotopic mass in each packet can lead to the so-called "1 Da error".[20] Because these high-resolution measurements do not directly determine the average molecular masses, transposing the measured isotopic masses into an average value has some inherent uncertainty due to the variation in peak height abundances (which can skew the calculated average mass value and thus degrade the resolution of the mass measurement). Either way of calculating the mass difference will compromise the reliability of obtaining sequence tag information from an unknown protein. Second, due to the large size of intact proteins and their residual tertiary structures, it is very difficult to establish a standard dissociation energy that can induce substantial fragmentation at multiple consecutive peptide bonds, which prevents implementing this approach in a robust fashion to most proteins. Third, the standard dissociation techniques (SORI-CAD, ECD, IRMPD) are virtually ineffective for dissociating very large proteins (M_r > 100 kDa). In contrast, obtaining sequence tag information from peptides that are generated by proteolytic digestion is relatively straightforward to measure and interpret. In fact, the MS^3 approach consisting of an in-source dissociation step and a conventional dissociation step has been used for deriving sequence information from oligonucleotides, oligosaccharides, peptides, and intact proteins.[21−24] Re-

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cently, a new sequence tagging approach for intact protein with in-source dissociation has been shown by using a class of "mass defect" tags incorporating the element ^{35}Br.[25] While the objectives of this study are similar to those aforementioned techniques, we feel that the capabilities of the MSAD technique, in particular for efficient high-energy dissociation, make this uniquely suited for this approach. We propose that MSAD can be used to efficiently generate small fragment ions from intact proteins with molecular masses exceeding 100 kDa, and these fragment ions can be further dissociated to give sequence tag information.

MATERIALS AND METHODS

All protein standards were acquired from Sigma-Aldrich (St. Louis, MO) and used as received with no additional purification. Samples for mass spectrometry were prepared at formal concentrations of 5−20 μM in 50:50 (v/v) acetonitrile:water, with 0.1% acetic acid added. All mass spectrometry experiments were conducted with a HiResESI Fourier transform ion cyclotron resonance mass spectrometer (IonSpec, Lake Forest, CA) equipped with a 9.4 T magnet (Cryomagnetics Inc., Oak Ridge, TN). Samples were introduced to an electrospray source (Analytica of Branford, CT) by direct infusion at 2−3 μL/min. Ions were accumulated in an external hexapole situated between the skimmer cone on one end and an exit lens and mechanical shutter (Figure 1) on the other. The static pressure in this region of the instrument was typically around 2×10^{-5} Torr. At the end of the accumulation time period, the exit lens voltage was dropped to zero and a mechanical shutter was pulsed open to allow ion transfer into an rf-only quadrupole ion transfer device and down to the ICR cell. In SORI-CAD experiments, ion accumulation (typically 0.5−3 s) was followed by ion isolation, which was accomplished with a SWIFT pulse. Off-resonance ion excitation was achieved with an rf pulse (1−4 V p−p, 1s) at a frequency 1 kHz lower than the parent ion cyclotron frequency, in the presence of nitrogen, which was admitted with a pulsed valve to a transient pressure of 5×10^{-6} Torr. An 8−10 s pump-down delay was inserted to allow the base pressure to be reestablished ($\sim 3 \times 10^{-10}$ Torr) prior to ion detection. For normal ESI-FTICR-MS experiments, ion accumulation was usually performed for 0.5−3 s at a hexapole dc offset voltage of −3.5 V, as shown in the top line of Figure 1. This yielded multiply charged molecular ions with virtually no fragmentation. To achieve MSAD, ion accumulation/activation was accomplished by lengthening the accumulation times (2−8 s) and adjusting the hexapole dc offset voltage (−7 to −12 V). This condition creates a deeper axial potential well than the standard offset setting (−3.5 V) and promotes ion fragmentation during the accumulation period. Discrete parent ion isolation and collision gas pump-down delay times were not necessary, so overall scan times for MSAD were determined solely by the accumulation times (∼2−6 s per scan). Each spectrum was comprised of 10 co-added scans acquired at 512K data points/transient, and external calibration was performed with ubiquitin; these conditions typically result in mass accuracy of ±5 ppm and resolutions of 150 000 (fwhm) for intact proteins. Product ion spectra were deconvoluted to zero charge state with the IonSpec software deconvolution tool. Sequence tags were identified by manual inspection of the deconvoluted spectra in the following steps. First, the mass difference between two fragment ions masses and between fragment ion and parent ion mass were calculated, and an amino acid was assigned if this mass difference corresponded to an amino acid mass (denoted |↔|). Second, the mass differences between the parent ion mass and the sum of two fragment ion masses was calculated. If the two fragments are complementary ion types (e.g. y- and b-ion type) and have an amino acid between them, then the mass difference calculated would give the identity of this amino acid, denoted by →| |← in tandem mass spectra. If this mass difference corresponded to an amino acid mass, these two fragments may have arisen from two complementary ion fragment species (e.g. y- or b-ions) with this amino acid situated between them. Third, contiguous identified amino acids constituted a sequence tag.

RESULTS AND DISCUSSION

Experimental Parameters for Controlling MSAD in a Hexapole Storage Trap. To optimize the MSAD technique, a systematic examination of the experimental parameters governing this dissociation method was undertaken. The two key factors involved in MSAD (at similar protein concentrations) were observed to be the ion accumulation time in the hexapole and dc offset voltage. The dependencies on accumulation time, rf amplitude, skimmer and exit lens voltages, and target gas pressure in the hexapole have been discussed previously,[8,10] but the effect of the dc offset voltage has not been reported in the literature.

The dc offset voltage controls the depth of the electrostatic axial well. To probe the effect of dc offset voltage on fragmentation, we examined the MSAD of the protein apomyoglobin with the accumulation time maintained at 4 s and all other parameters kept constant. When dc voltage is between −3.5 and −6 V, mass spectra revealed no fragmentation for most protein ions, as shown in Figure 2A for apomyoglobin. A sharp threshold for dissociation is observed at dc offset voltages between −6 and −7 V. For example, at −6.5 V, apomyoglobin dissociates into two types of fragments, a few abundant multiply charged fragments and many low-abundance singly charged fragments (Figure 2B). At −7 V, low-mass, singly charged fragments dominate the mass spectra (Figure 2C). From −7 to −11 V, no noticeable differences in fragment ion species are observed. However, a more negative dc voltage will induce fragmentation at much shorter accumulation times (Figure 2D). These results indicate that even at a fixed ion accumulation time in the hexapole, the magnitude of the dc offset voltage has a dramatic effect on ion fragmentation.

The information obtained above suggests that a combination of dc offset voltage and ion accumulation time can be used to effectively control the degree of fragmentation in a MSAD experiment. Empirically, the lower boundary for fragmentation to occur involves accumulation times of at least 1200 ms and for dc voltages of at least −6.5 V for the protein samples (approximately micromolar concentration) that were examined in this study.

The ion storage/accumulation capability of a hexapole is controlled by the confining forces of the multipole device. In

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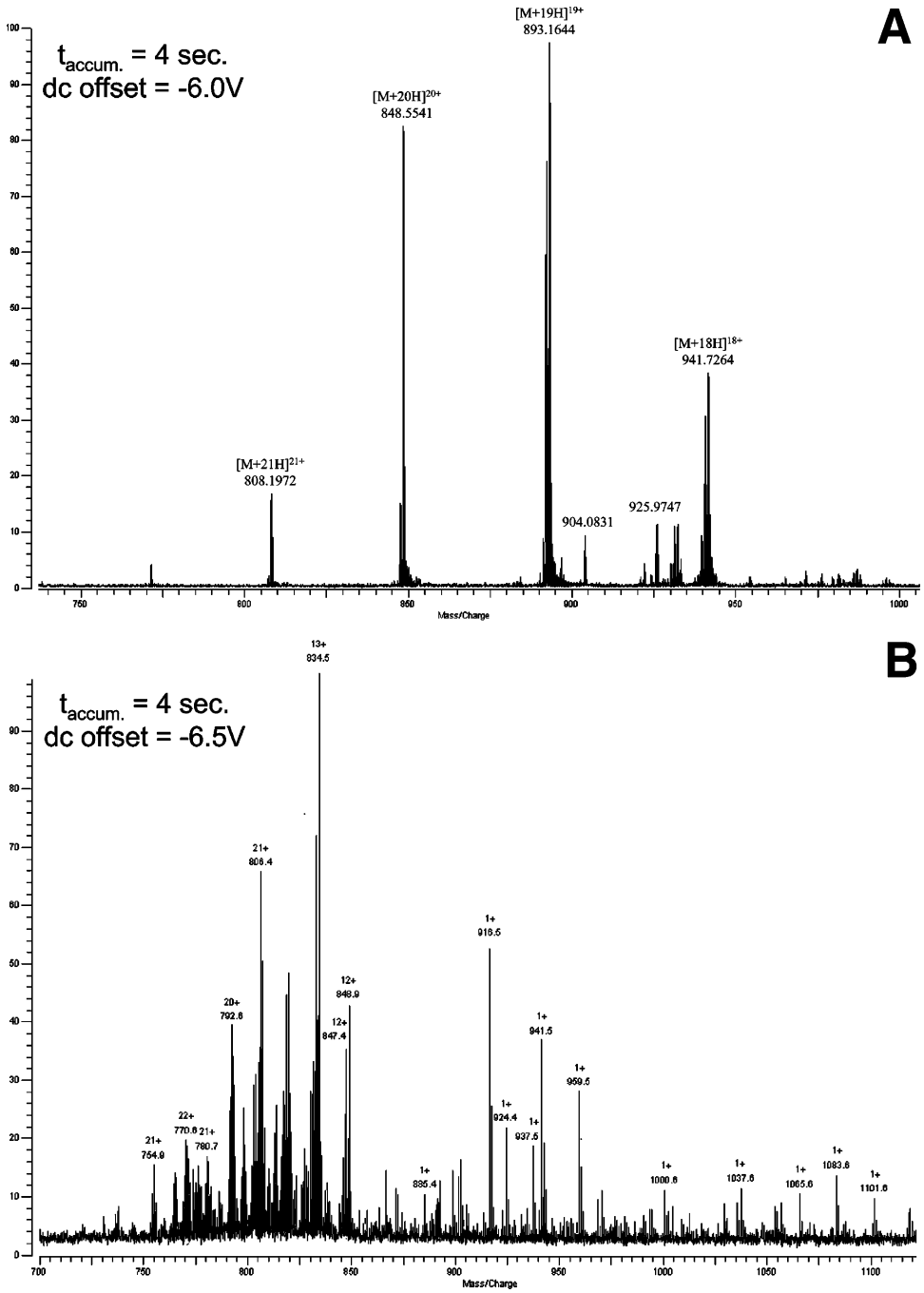

particular, the electrostatic potential created by the rf-only mode of operation of a hexapole provides extensive ion confinement in the $x-y$ direction (i.e. perpendicular to the hexapole rods) and somewhat more limited ion confinement in the z-direction (parallel to the hexapole rods). By employing electrostatic voltage confinement at the ends of the hexapole, it is possible to accumulate and store ions for an extended period of time in the hexapole device. Previous reports have suggested that extended ion accumulation results in a sufficiently large ion population for which space charge pushes the ions outward radially and allows energy coupling with the rf-only hexapole rods. However, our experiments on the dc offset voltage and previous reports on the skimmer and exit lens voltage[10,16] have revealed that the depth of the axial potential well is critically important and can induce fragmentation. These results suggest an alternative fragmentation process. Because the voltage at the entrance of the hexapole is static (usually held at 25 V in our experiments), the lowering of the dc offset on the hexapole to more negative voltages will induce a translational energy component to the ions as they enter the hexapole. Since the ions are confined in the hexapole in a multiple pass configuration along the z-direction, even a modest amount of translational energy added as the ions enter the multipole device will result in substantial fragmentation. Note that the higher charged parent ions will pick up a proportionately higher translational energy as they enter the multipole. Therefore, while we cannot rule out the possibility of rf-coupling with the hexapole rods as the energy source for fragmentation, we believe that we have identified an additional MSAD fragmentation mechanism, in which a translation energy component can be exploited to produce substantial ion fragmentation in the multipole device.

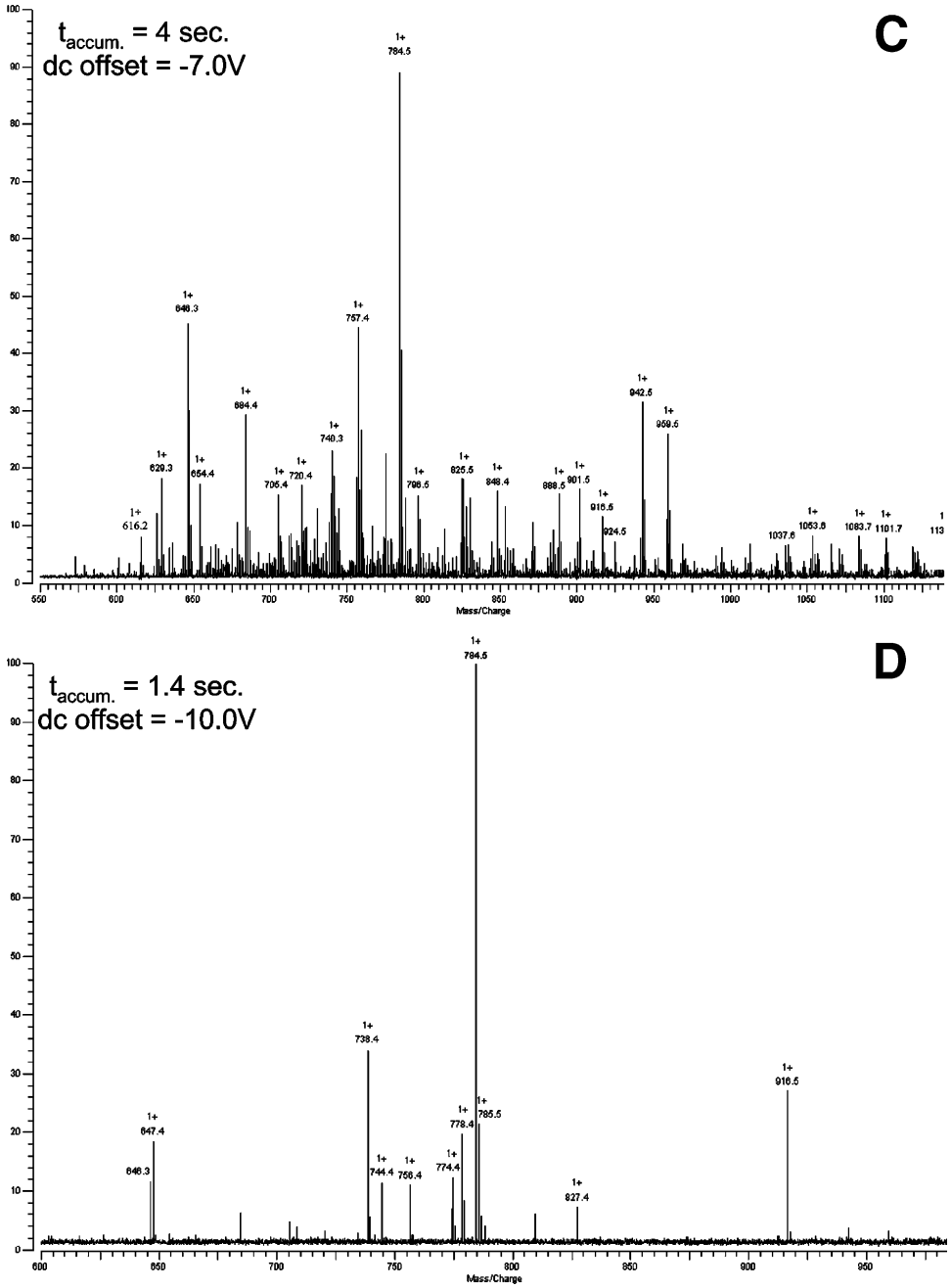

Figure 2. Apomyoglobin MS^2 from MSAD with ion accumulation time and hexapole dc offset voltage offset respectively at 4 s and −6.0 V (A), 4 s and −6.5 V (B), 4 s and −7 V (C), and 1.4 s and −10 V (D). A sharp threshold of hexapole dc offset voltage for MSAD from no dissociation (A) to extensive sequential dissociation (C) was observed. Similar fragmentation can be achieved by long accumulation time and high dc offset voltage (A) or short accumulation time and very high dc offset voltage (D). The intermediate condition for MSAD yielded two distinctive population of fragments, singly charged small fragments and highly charged large fragments (B).

Although variation of sample concentration, rf amplitude, skimmer and exit lens voltages, and collision gas pressure in the hexapole undoubtedly also would affect the MSAD experiment, those parameters were not examined in this study. Control of the collisional energy was based only on the dc offset voltage and accumulation time.

Single Protein MSAD: Identification of Fragmentation Extent and Ion Types. A range of proteins was examined with MSAD, to determine the general utility of the technique as well as to investigate any sequence dependent fragmentation. Previously, only limited research had been conducted on pure small- to medium-sized proteins over a narrow low-energy range.[7−12] In this study, we have conducted MSAD over a wide collisional energy range on seven individual proteins whose molecular masses range from 8 to 115 kDa (ubiquitin, lysozyme, apomyoglobin, β-lactoglobulin B, carbonic dehydrogenase, serum albumin, and β-galatosidase). The proteins examined in this study exhibit substantial diversity in their amino acid sequence, molecular weight, and number of disulfide bonds present and thus should represent a general case for other proteins. All protein samples were prepared by directly solubilizing the protein into the ESI solution (see Materials and Methods). Due to the preservation of disulfide bonds and the gentle experimental conditions, these proteins may have a large amount of residual tertiary structure.

It has been demonstrated that proteins have similar fragmentation behavior in low-energy MSAD experiments and in SORI-CAD experiments.[8] We also have verified this trend under our experimental conditions.[12,26] In our study, MSAD with accumulation times ranging from 2000 to 3000 ms and dc voltages ranging from −6.0 to −6.5 V were regarded as low-energy conditions. This rather empirical range is defined as low energy based on the experimental observation of a small amount of fairly large fragment ions for most of the proteins examined. In these typical low-energy MSAD experiments, the mass spectra consisted of a few large y- and b-type ions derived from the parent molecular species. The observed fragmentation is less extensive in low-energy MSAD experiments than in SORI-CAD experiments. However, the types of fragment ions common to MSAD (i.e., y- and b-type species) are quite similar to SORI-CAD experiments and differ substantially from ECD (which is predominantly c- and *z*-type ions). In particular, both MSAD and SORI-CAD not only reveal similar types of fragment ions[12] but also a common preference for dissociation at residues such as proline, asparatic acid, and glutamic acid in some cases. These results suggest that MSAD may serve as an alternative gas-phase CAD method with a higher duty cycle than SORI-CAD (provided that ion isolation is not required). A large amount of undissociated parent ion is present in the low-energy MSAD experiment, which indicates that there is no clear cutoff between the normal MS experiment and the low-energy MSAD experiment.

Even though low-energy MSAD is attractive due to its similar fragmentation with SORI-CAD, high-energy dissociation makes MSAD unique among SORI-CAD and nozzle−skimmer CAD in terms of the amount of collisional energy that can be put into protein ions. High-energy collisions can be achieved simply by elongating the accumulation time and/or adjusting the magnitude of the dc offset voltage. In this study, we found that a 4 s accumulation time and −7 V dc offset is a generic high-energy MSAD condition that can be employed to dissociate most proteins. For illustration, MSAD tandem mass spectra under such conditions of apomyoglobin, β-lactoglobulin B, and β-galatosidase are shown in Figures 2, 3, and 4, respectively.

This MSAD process technique is illustrated for the protein β-lactoglobulin B in Figure 3. "Normal" electrospray mass spectra can be acquired easily with a modest accumulation time (2 s) and dc offset (−3.5 V) and reveal multiply charged ions corresponding to the protonated molecule with no fragmentation (Figure 3A). The inset reveals the isotopic resolution of the deconvoluted molecular ion region, illustrating the high-resolution capabilities of the FTICR-MS technique. By altering the hexapole conditions to those listed above for high-energy MSAD (i.e. 4 s accumulation time with −7 V dc offset), it was possible to completely dissociate the protein into small, singly charged fragment ions, as shown in Figure 3B. Similar fragmentation results from high-energy MSAD experiment were observed in all other examined proteins. The MSAD fragments are generally small, singly charged, abundant, and quite distinct for different proteins. When longer accumulation times and more negative dc offset voltages were used, the types of fragment ions remain basically the same, although their relative abundances vary and the overall signal/noise for the spectra decreases. The small size of the fragments suggests they may come from sequential fragmentation. This is further supported by the identity of fragments determined by the sequence tag technique, as will be discussed below. Thus, these MSAD fragments correspond to not only classical terminal fragment species, such as y- and b-ions, but also internal fragment species such as y/b ions from parent ion. In our proposed MSAD mechanism, the sequential fragmentation may be a result of ions' multiple pass in the *z*-direction in the hexapole. Alternatively, this could be explained with the space-charge repulsion mechanism by considering the continuous deposition of energy through rf-coupling with the hexapole. Thus, high-energy MSAD tandem mass spectra for large proteins, while complex, are not completely intractable. Many of the initial fragment ions are not stable enough to survive the multiple high-energy collisions. The most stable fragments, corresponding to the abundant peaks in the spectrum, are undoubtedly dictated by their sequence, the sequence surrounding them, the residual protein tertiary structure, and the distribution of positive charges. Because the stable fragments are fairly characteristic for each protein (due to the complex factors involved), we refer to them as the "MSAD signature", which potentially could provide identification of a protein.

As a remarkable demonstration of the high collisional energy, MSAD was used to fragment β-galatosidase, which has a molecular mass of 116 351 Da (Figure 4A). Note that because the translational energy gained scales proportionally to the protein's charge state, the accumulation time and dc offset voltage of the MSAD experiments for β-galatosidase are identical with those for smaller proteins. To our knowledge, this may be the most extensive fragmentation of a protein whose molecular mass is over 100 kDa. With conventional CAD, IRMPD, or ECD experiments, even if fragmentation could be achieved, the fragments of this protein most likely would still be too large to be easily resolved in FTICR-MS. In contrast, the sequential fragmentation under MSAD was able to dissociate the intact protein to fragment ions that are easily measured.

Under "normal" hexapole ion accumulation conditions, no fragmentation occurs. In low-energy MSAD, limited dissociation occurs to generate large fragment ions. In high-energy MSAD, sequential fragmentation occurs until only small singly or doubly charged fragments remain. This leaves one to speculate about a protein's fragmentation behavior in the intermediate-energy MSAD experiment. By definition, intermediate-energy MSAD should give medium-sized fragments with multiple charges (more than three). When using experimental conditions intermediate between high-energy MSAD and low-energy MSAD, the simultaneous coexistence of large fragments and small fragments, instead of medium size fragments, was observed (Figure 2B). Such a sharp transition between high- and low-energy MSAD suggests that intermediate-energy MSAD may be difficult to achieve. This is probably because once sufficient collisional energy conditions are achieved in a MSAD experiment, the sequential fragmentation process will continue to reduce midsized fragment ions to the smaller, more stable species.

Sequencing MSAD Fragment Ions by Subsequent SORI-CAD. High-energy MSAD is a very efficient way to generate small-sized, singly charged peptides. When subjected to SORI-CAD experiment, many of these fragment peptides produce an easily

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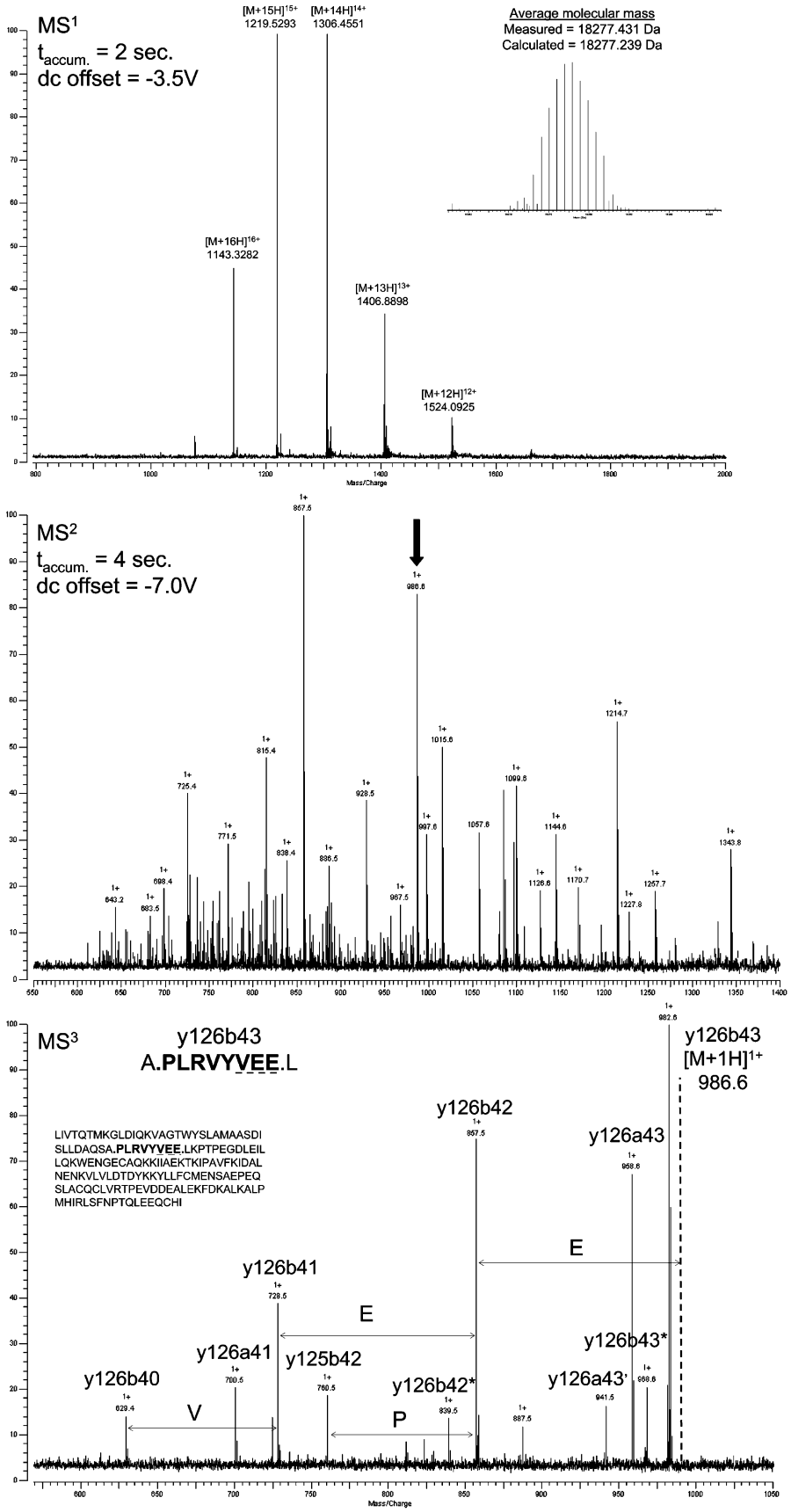

Figure 3. Examination of β-lactoglobulin B MS^3 by MSAD/SORI-CAD for acquiring sequence tag information. A sequence tagging experiment consists of normal MS (A) for determining molecular weight of intact protein, MS^2 (B) from MSAD for identifying fragments, and MS^3 (C) from MSAD/SORI-CAD for acquiring the sequence tag from a MSAD fragment. The deconvoluted mass spectrum and protein sequence is show in the inset of (A). The MSAD fragment indicated with an arrow in (B) is isolated and fragmented, as shown in (C). This fragment is highlighted in the protein sequence with sequence tag underlined. Most ions in MS^3 can be identified using the general rules of peptide CAD fragmentation.

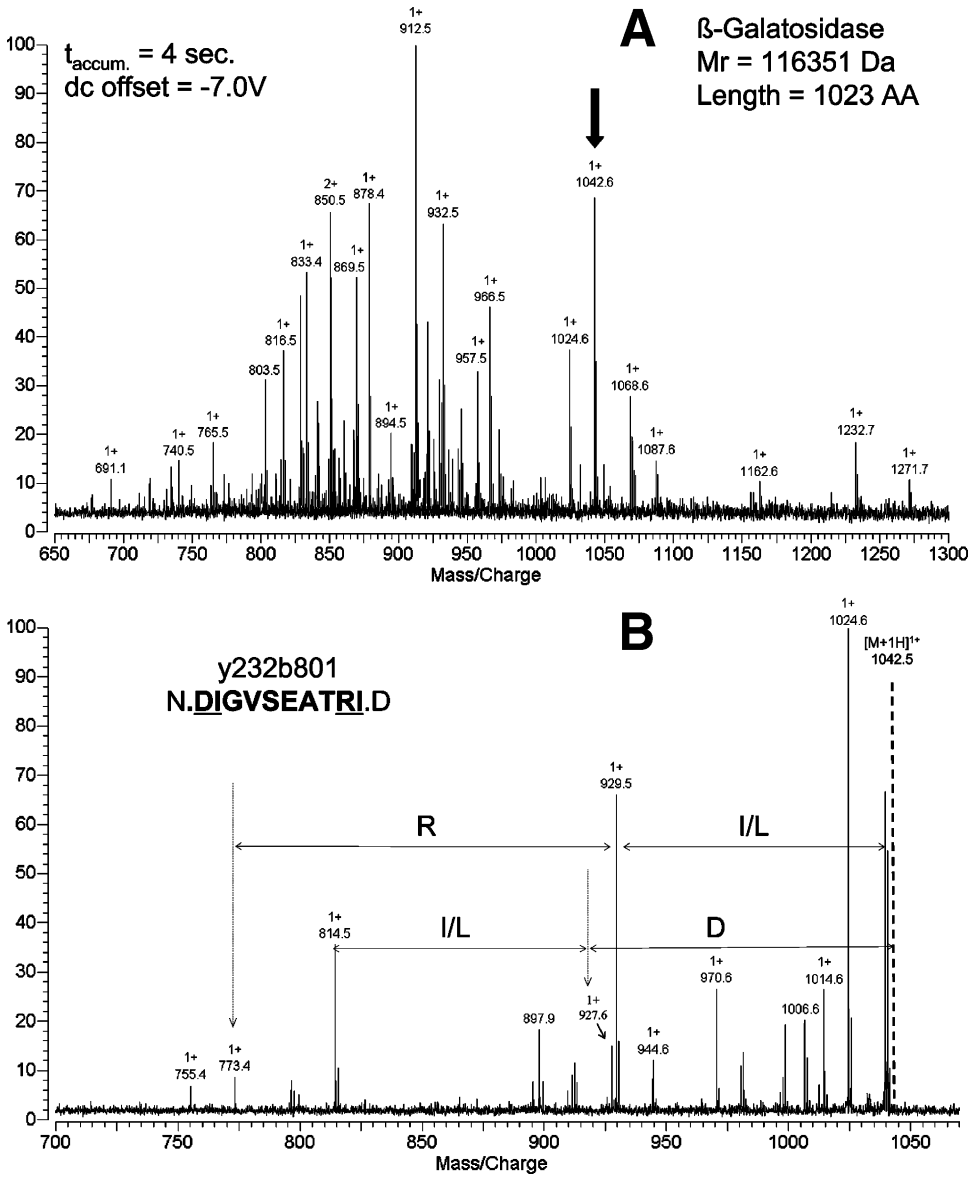

Figure 4. Examination of β-galatosidase MS^3 by MSAD/SORI-CAD for acquiring sequence tag information. Despite this protein's large size (~116 kDa), MSAD with 4 s ion accumulation and −7.0 V dc offset voltage generated a complex pattern of small fragments (A). Its MSAD condition and fragmentation pattern were similar to other medium-sized proteins. Sequence tag information was then derived (B) with MSAD/SORI-CAD from a MSAD fragment. This fragment is a y-/b-ion resulting from sequential fragmentation during MSAD.

interpretable tandem mass spectrum that often yields a sequence tag for the protein. This MS^3 experiment is illustrated with β-lactoglobulin B in Figure 3. The accurate mass of the multiply charged intact protein is first measured with a normal mass spectrum (Figure 3A). Then the accumulation time is extended to 4 s and the dc offset voltage is decreased to −7 V to acquire this protein's MSAD tandem mass spectrum (Figure 3B). Now the high complexity of the fragmentation products is actually advantageous for offering a wide range of peptide fragments for SORI-CAD interrogation. The SORI-CAD tandem mass spectrum of an abundant fragment ion species of m/z 986.6 is shown in Figure 3C. A sequence tag (VEE) can be found and the parent ion of m/z 986.6 can be identified from the β-lactoglobulin B sequence as $y_{126}b_{43}$, which is shown in boldface in the protein sequence in the inset of Figure 3C with the sequence tag underlined. This is direct evidence of the identities of high-energy MSAD fragments to be internal fragments. Most of the fragment ions of the $y_{126}b_{43}$ parent ion can be readily attributed to common y-, b-, and a-type ions, along with internal fragments and ions resulting from loss of water or ammonia. MS^3 experiments from MSAD/SORI-CAD have a comparable S/N level to MS^2 experiments from SORI-CAD alone. This is probably because the dissociation and fragment ion collection efficiencies in the MSAD MS^2 experiment are very high and appear to be superior by an order of magnitude over the in-cell MS^2 experiments.

Identification of large intact proteins (>100 kDa) has been a challenge, as large proteins can neither be easily measured in mass nor be dissociated to give informative tandem mass spectra. We have shown the extensive dissociation of β-galatosidase with high-energy MSAD (Figure 4A). Once large proteins are dissociated into small peptide fragments, these ions are no different from those of small proteins. Thus, we conducted a MSAD/SORI-CAD experiment on the β-galatosidase MSAD fragment ion of m/z 1042.6 with identical MSAD and SORI-CAD conditions as those

Table 1. Summary of the Proteins Examined with MSAD/SORI-CAD

proteins[a]	MW	sequence tag	parent ion (m/z)	identity	sequence
ubiquitin	8 564	X_2 (I/L) (K/Q) (K/Q) E X_n	699.8^{3+}	y_{18}	D.YNIQKESTLHLVLRLRGG
apomyoglobin	16 951	(I/L) H V (I/L) H X_2	784.5^{+}	$y_{42}u_{118}$[b]	I.HVLHSK.H
lactoglobulin	18 281	X_n V E E	986.6^{+}	$y_{126}b_{43}$	A.PLRVYVEE.L
serum albumin	66 433	(I/L) P (K/Q) (I/L) (K/Q) P D	792.5^{+}	$y_{472}b_{118}$	D.LPKLKPD.P
galatosidase	116 351	D (I/L) X_n R (I/L)	1042.5^{+}	$y_{232}b_{801}$	N.DIGVSEATRI.D

[a] Lysozyme (14 kDa) and carbonic dehydrogenase (35 kDa) were not included, since we were unable to generate sequence tags from the major fragment ions of these two proteins. [b] See ref 30.

for β-lactoglobulin B. A sequence tag consisting of two identified residues from each end of this fragment are identified (Figure 4B), verifying that this fragment ion is an $y_{232}b_{801}$ ion. This sequence tag provides enough information to identify this protein from the SWISS-PROT database. Thus MSAD/SORI-CAD presents a unique way to characterizing large proteins.

Table 1 summarizes the information on the sequence tags and their parent MSAD fragments from different proteins. Most of those MSAD fragments are small, singly charged y-/b-ions with high abundance in MSAD tandem mass spectra. They are often the fragmentation products of cleavage next to P or D. The sequence tags generally arise from the cleavage of two or three peptide bonds next to the peptide termini. For all of these sequence tag measurements, a 4 s accumulation time and −7 V dc offset voltage were used for MSAD and 3.7 V excitation voltage was used for SORI-CAD. SORI-CAD tandem mass spectra were interpreted and sequenced manually as described in the experimental methods. High-confidence sequence tag determination can be achieved from the accurate mass measurement of FTICR-MS, the simple isotopic envelope, the small size of the parent ion, and the sparse fragment ions in the tandem mass spectra. In addition to peaks that contribute to the sequence tag, there are other, less informative peaks coming from loss of water, ammonia, a-type ions, or other internal fragmentation. A fraction of MSAD fragments do not generate sequence tags because of limited fragmentation of parent ion due to their small size or amino acid sequences. Because of the standardized MSAD/CAD conditions and a rich diversity of fragment ions, many MSAD ions can be evaluated with reasonable effort and time. In this study, SORI-CAD was conducted only on a few major MSAD fragments. In our initial survey, we were unable to obtain sequence tags from major MSAD fragments of lysozyme and carbonic dehydrogenase; however, there may be other less abundant MSAD fragment ions from these proteins that could yield such information.

There are three unique advantages for this MSAD/SORI-CAD method that make it most promising to be applied in a high throughput manner for obtaining sequence tags. First, unlike other fragmentation methods, MSAD/SORI-CAD exhibits no discrimination against large proteins or certain protein sequences. As demonstrated with several different representative proteins, MSAD dissociates proteins regardless of their size into uniform small peptides, which can then be fragmented to yield sequence tags. Second, generic experimental conditions for MSAD/SORI-CAD experiments work for most proteins. The automation of tandem mass spectra processing is also straightforward, due to the high interpretability of the peptide tandem mass spectra and the continued development of computational tools for processing peptide CAD spectra. Third, MSAD of any protein yields a large number of MSAD fragments that can be surveyed by an additional step of SORI-CAD. This might provide a versatile method for investigation of sequence tag information from proteins.

A current limitation of this MSAD/SORI-CAD method is that the success of obtaining sequence tag from a given MSAD fragment is variable. This is probably because the fragments that survive MSAD are reasonably stable and less likely to be fragmented easily by SORI-CAD again. In addition, the time scale for each MSAD/SORI-CAD scan is between 10 and 15 s, which might prevent its *direct* application in LC/MS^3 measurement. A possible solution to this issue is to employ other faster dissociation methods such as IRMPD to dissociate MSAD fragments.

Simple Protein Mixture MSAD: Identification of Protein Components. Despite the inability of MSAD to isolate a parent ion for subsequent fragmentation, two methods were attempted to identify proteins from mixtures up to four components: inspection of the MSAD signature for each protein and MS^3 to obtain sequence tag information. Experimental approaches that provide multiplexed tandem mass spectrometry capabilities are beginning to appear[27−29] and provide the potential for high-throughput measurements of complex mixtures. Even without parent ion selection, MSAD can be employed for generating multiplexed tandem mass spectra for protein mixtures. The challenge for the application of MSAD in this case, much like the other multiplexed CAD techniques, is the interpretation of the complex fragmentation patterns. Because of the high degree of internal fragmentation for MSAD, it appears that the spectra interpretation for this method will be formidable, especially for de novo sequencing or correlation-related computational techniques to identify proteins. However, the abundant MSAD fragments are quite characteristic for each protein, and, at high resolution and high mass accuracy, could be used to generate a protein's MSAD signature. Thus a protein's presence in a mixture could be suggested by its high-resolution, accurate fragment ion mass MSAD signature. A protein mixture with four components at equal ratio was examined with high-energy MSAD. The tandem mass spectrum was then compared with the previously acquired MSAD tandem mass spectra of each of the proteins. Most of major fragments were confidently attributed to one and only one component protein due to the high mass accuracy and the uniqueness of their MSAD signature (Figure 5A). Thus, ubiquitin,

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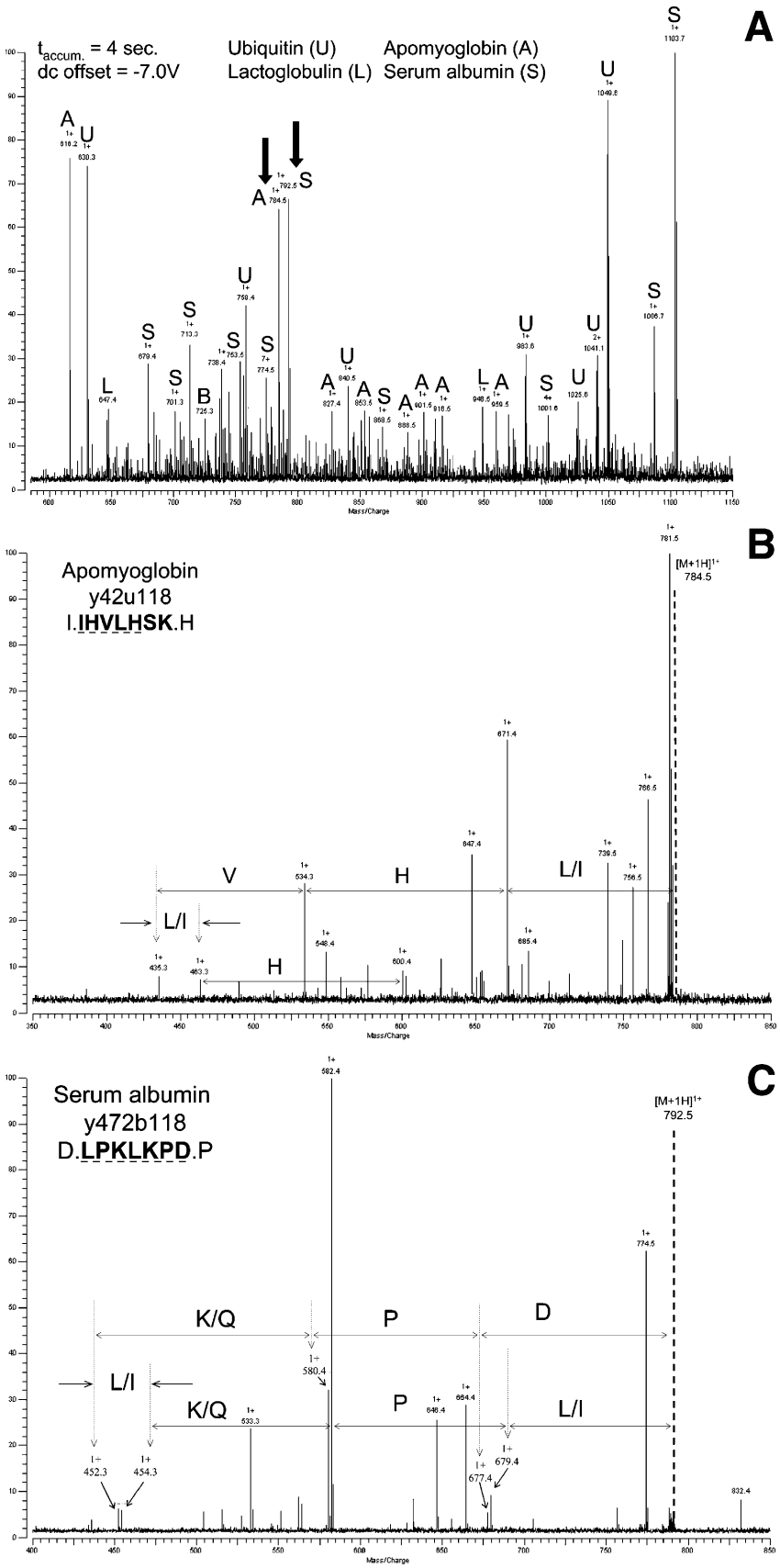

Figure 5. MSAD MS^2 of a four-protein equimolar mixture consisting of ubiquitin, apomyoglobin, lactoglobulin, and serum albumin (A). The origin of the fragments was assigned by matching with individual protein's MSAD fragments at a accuracy of ±5 ppm and labeled with the protein name's initial in the spectrum. The MSAD fragments can be then used to generate a sequence tag of the component proteins (B, C). Note that a pair of single-headed arrows pointing each other (→| |←) denotes that the mass differences between the parent ion mass and the sum of two fragment ion masses corresponds to an amino acid, which wraps sequence tag from y-ion series to b-ion series or vice versa. The sequence tags also validated our assignment of these two fragments in (A).

apomyoglobin, and serum albumin can be identified by their MSAD signature. However, lactoglobin's MSAD signature is not discernible in this mixture, probably due to the discrimination against lactoglubin by electrospray ionization and/or MSAD. In general, we estimate that the dynamic range for protein identification by MSAD in mixtures might range to at least 1:10. This should be tempered with the knowledge that ionization suppression in the ESI source may suppress the observed signal of some species, even before the MSAD process is conducted.

The second identification method attempted is sequence tagging determination of the component proteins by MSAD/SORI-CAD experiments. Similar to what enzymatic digestion does in solution, MSAD transforms a gas-phase protein mixture into a more complex gas-phase peptide mixture. Yet complex peptide mixtures are amenable for sequential examination of constituents with ion isolation. Peptides from MSAD fragmentation of the four-component mixture were surveyed (Figure 5A) and sequentially examined with SORI-CAD. Tandem mass spectra of fragment $y_{42}u_{118}$[30] from apomyoglobin and fragment $y_{472}b_{118}$ from serum albumin are shown in parts B and C of Figure 5, respectively, both of which yield sequence tags for the originating proteins. For comparison, we attempted direct SORI-CAD on the intact proteins in an effort to obtain sequence tag information. However, we were unable to successfully dissociate large proteins such as serum albumin to give informative fragment ions, much less sequence tag information.

Previously, MSAD has only been used to measure individual purified proteins, due to its lack of parent ion isolation capability. In this report, we have demonstrated two MSAD approaches that could potentially identify proteins from simple mixtures. The identification of a protein by its MSAD high-resolution signature is fast and parallel but requires prior information on the protein's fragmentation pattern by MSAD. The identification by sequence tagging would be suitable for singling out proteins from a sequence database, for applications such as top-down intact protein measurements. Exploiting the capability of MSAD for dissociating large proteins enables both methods to identify large proteins from a mixture in a robust and standardized manner. Even though more research would be needed to fully probe the dynamic range and sensitivity of MSAD for protein mixtures, we have demonstrated at least at the basic level that this experimental method has some value for simple mixtures. The results of this study have extended the application of MSAD from individual purified proteins to simple mixtures with a few proteins at similar concentration, which could be a partially purified protein sample or a HPLC eluent fraction.

(30) The cleavage type at the C-terminus of this peptide is nonstandard and is denoted by "u" to indicate a fragment 31.0 Da less than that from b-ion type cleavage, possibly due to side chain cleavages.

CONCLUSIONS

MSAD is a new in-source fragmentation method, initially attributed to hexapole rf-coupling induced by extended accumulation time. In this report, we provide evidence that dc offset voltage of the hexapole can be used to induce fragmentation at shorter accumulation times than previously reported. The data obtained with the dc offset voltage suggested an alternate dissociation mechanism based on ion kinetic energy excitation due to the increased potential difference between skimmer and hexapole.

A variety of proteins were examined with MSAD at a range of collisional energies measured by accumulation time and dc offset voltage. While low-energy MSAD yields y- and b-ions similar to SORI-CAD, high-energy MSAD can fragment proteins up to 116 kDa into small singly charged ions, which are characteristic of the protein and can be referred to as its MSAD signature. This MSAD signature was used as a method to identify a protein's presence in a simple mixture.

The protein fragments from high-energy MSAD can be dissociated further by SORI-CAD (MSAD/SORI-CAD), analogous to enzymatic digestion followed by CAD. From such MS^3 spectra, sequence tags were obtained for five proteins whose sizes range from 8 to 116 kDa. This sequence tagging technique was extended to proteins in a mixture, showing the potential of being applied in top-down characterization of simple protein mixtures in direct infusion mode. Its advantages include standardized experimental conditions, readily interpretable peptide fragmentation mass spectra, and applicability to large proteins.

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