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Infrared Multiphoton Dissociation in Quadrupole Time-of-Flight Mass Spectrometry: Top-Down Characterization of Proteins

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The first implementation of infrared multiphoton dissociation (IRMPD) for a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer is reported. Ions were trapped in the radio frequency-only quadrupole (q2), which normally serves as a collision cell, and irradiated by a continuous CO_2 IR laser. The laser beam was introduced coaxially with the quadrupoles in order to maximize overlap with the ion path. The resolution of the TOF mass analyzer allowed direct charge state determination for fragments smaller than 7 kDa. For larger fragments, the charge state could be assigned using the multiple losses of water, characteristic for IRMPD of proteins. The analytical performance is demonstrated by top-down sequencing of several representative proteins (equine myoglobin, bovine casein, and human insulin and chaperonin 10). Various post-translational modifications such as phosphorylation, acetylation, formation of disulfide bridges, and removal of N-terminal methionine followed by acetylation are detected and characterized. The utility of IRMPD for the analysis of biological samples is demonstrated in a study of a recently identified potential marker for endometrial cancer, chaperonin 10.

Infrared multiphoton dissociation (IRMPD) has played major roles in mechanistic investigations and isotope fractionation of small molecules for many years.[1−4] In the past decade, pioneering efforts of McLafferty and others[5−8] showed that IRMPD is also effective for large polypeptides as well as protein ions in the gas phase. Central to IRMPD is the absorption of multiple infrared photons, resulting in a gradual buildup of internal energy by the protein ions. Fragmentation occurs when the internal energy exceeds the barrier against dissociation. Collisional deactivation or cooling is a major pathway for reduction of internal energy; as a result, for effective IRMPD, high-vacuum conditions are preferred.

Collision-induced dissociation (CID) of tryptic peptides has become the cornerstone of gas-phase protein sequencing and protein identification in proteomics.[9] This technology makes use of the relatively predictable and reproducible fragmentation of doubly protonated tryptic peptides to permit computerized matching of the fragment ions in CID spectra with a list of theoretical fragments accounting for the most probable dissociations. The best peptide and protein matches that exceed a given confidence limit are considered positive identifications. This "bottom-up" strategy, relying on sequencing of enzymatically cleaved peptides, has been very effective for protein identification and has fueled the technological revolution in proteomics. Powerful as this strategy may be, it is not without shortcomings: under CID conditions, post-translational modifications (PTMs) are often easily lost and leave no or little signature. In addition, a peptide that carries PTMs has a different mass from the unmodified peptide. The tryptic digest, even from a gel band that comprises only a few proteins, is a mixture of peptides, most of which will match the expected masses of tryptic peptides. Unless PTMs are suspected, those peptides that do not match the expected masses are often misidentified (mismatched) as peptides from a different protein.[10,11] CID of protein ions are inefficient, as internal excitation is minimal due to the large difference between ion and neutral masses, despite the large collisional cross section of protein ions, and hence the large number of collisions. Smith et al.[12] first showed that CID of large multiply charged ions from proteins,

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e.g., myoglobin, can be obtained in the collision cell of a triple quadrupole mass spectrometer. Nemeth-Cawley and Rouse[13] used a hybrid quadrupole time-of-flight mass spectrometer (QqTOF) to perform CID of protein ions. Both studies showed that CID produces a limited number of sequence ions because the precursor and product ions lose energy as they traverse the collision cell. McLuckey and co-workers[14,15] demonstrated that sequence information can be obtained by CID in an ion trap using radial excitation. However, only the precursor ion is resonantly excited; first-generation product ions are not excited to fragment further, thus limiting the extent of dissociation.

By contrast, the efficiency of IRMPD is not diminished with ion size. Excitation (and eventual dissociation) by absorption of multiple IR photons is characterized as a "slow-heating" method, as many excitation steps are involved, and typically requires trapping and irradiating the precursor ions for a period of milliseconds.[5,10] First-generation product ions are also continuously being excited, which results in a rich fragmentation pattern. Because of trapping and irradiating requirements, IRMPD experiments are typically implemented in Fourier transform-ion cyclotron resonance (FTICR) or ion trap mass spectrometers. The former is more effective, not only because of higher mass resolution which helps reducing ambiguity in ion assignments but also because of its lower operating pressure which reduces collisional deactivation.[16] Ion traps are typically operated at $\sim 10^{-3}$ Torr of pressure; to overcome efficient collision deactivation, thermally assisted IRMPD has been implemented.[17] Typically, in an FTICR experiment, multiply protonated proteins are directly introduced by nanospray, after exhaustive purification and isolation; the use of nanospray for sample infusion typically affords the analyst a longer spectral accumulation time to better counting statistics for low-abundance product ions that, in turn, improve percent sequence coverage. Aside from obviating the trypsin digestion step, important advantages in this "top-down" strategy are that protein molecular masses are determined and that PTMs are more frequently retained by the ionic fragments. Typically, in a mass spectrometric analysis, both the mass and the charge of a given protein ion fragment are unknown. To determine the charge, the spacing between peaks bearing different carbon isotopes is commonly determined, which requires high resolving power achievable only in FTICR instrumentation.[9,18−21] An alternative approach, charge state manipulation through gas-phase ion chemistry, has been demonstrated on ion traps.[22,23]

Here we report the first implementation of IRMPD on a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer. IR laser irradiation of protein ions was performed in the collision cell (q2), making use of the trapping capacity of a linear (two-dimensional) ion trap. For maximum overlapping between the laser and the ion beams, the former was introduced coaxially with the latter.[24] The reflective hybrid mass spectrometer that we used has a routine operating resolution of approximately 7000 for peptide analysis and is a prototype of the commercially available Applied Biosystems/MDS SCIEX QSTAR. We will demonstrate the analytical performance of this system by analyzing a number of standard proteins and a recently identified and verified potential biomarker for endometrial cancer.

EXPERIMENTAL SECTION

Chemicals. Equine myoglobin, human insulin, and bovine α-casein were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. All solvents were of HPLC quality; water was purified using a Milli-Q filtration system (Millipore, Billerica, MA). The sample solutions were 10 μM protein in 50/50 water/methanol for insulin and myoglobin, and in water for casein, acidified to 0.1% formic acid. These were subjected to electrospray at 6 kV and a flow rate of 2 μL/min using a syringe pump. Digestion of insulin was performed in 100 mM ammonium bicarbonate using the enzyme Glu-C (Sigma-Aldrich, enzyme/protein ratio 1:30) at 37 °C for 10 h. Human chaperonin 10 was harvested from HeLa cell culture, purified by size-exclusion chromatography, and concentrated using a ProteoSpin sample preparation kit (CBED, MDS SCIEX, Concord, ON, Canada). The analytical solution was 0.1% formic acid in 50/50 water/methanol and was introduced via a capillary (Proxeon Biosystems AG, Odense, Denmark) for nanospray.

Experimental Setup. All experiments were performed using a hybrid QqTOF mass spectrometer (Centaur, a prototype of the commercially available QSTAR, MDS SCIEX). Figure 1 shows a schematic diagram of the important optical and ion components. The reflectron TOF mass analyzer was calibrated at 132.905 and 829.54 Da. To reduce collisional cooling of irradiated ions, no collision gas was introduced; the q2 pressure was maintained at the background pressure of 2×10^{-6} Torr in the surrounding chamber by machining a set of 5-mm vent holes in the ceramic end cap of the collision cell. To minimize obstruction of the axial laser beam, the grid originally installed between the detector and the pusher/puller stack to the TOF inlet was removed. A second grid located downstream from the focusing lens IQ3 was replaced by a steel plate with a 2.5 mm orifice.

IRMPD was performed using a chassis-mounted, air-cooled, 25-W continuous wave CO_2 laser (10.6 μm, Synrad-J48-2, Synrad Inc., Mukilteo, WA) equipped with a laser diode pointer. The laser beam was introduced into the mass spectrometer using a set of beam pipes (Umicore Laser Optics, Raleigh, NC) and a ZnSe window (10ZWA60, Umicore Laser Optics) mounted on a flange. Following the design of Hofstadler for IRMPD in an external ion reservoir of an FTICR,[24] the flange/window combination was positioned to allow the laser beam to coincide with the quadrupole axis inside the instrument. Coaxial alignment was simplified by

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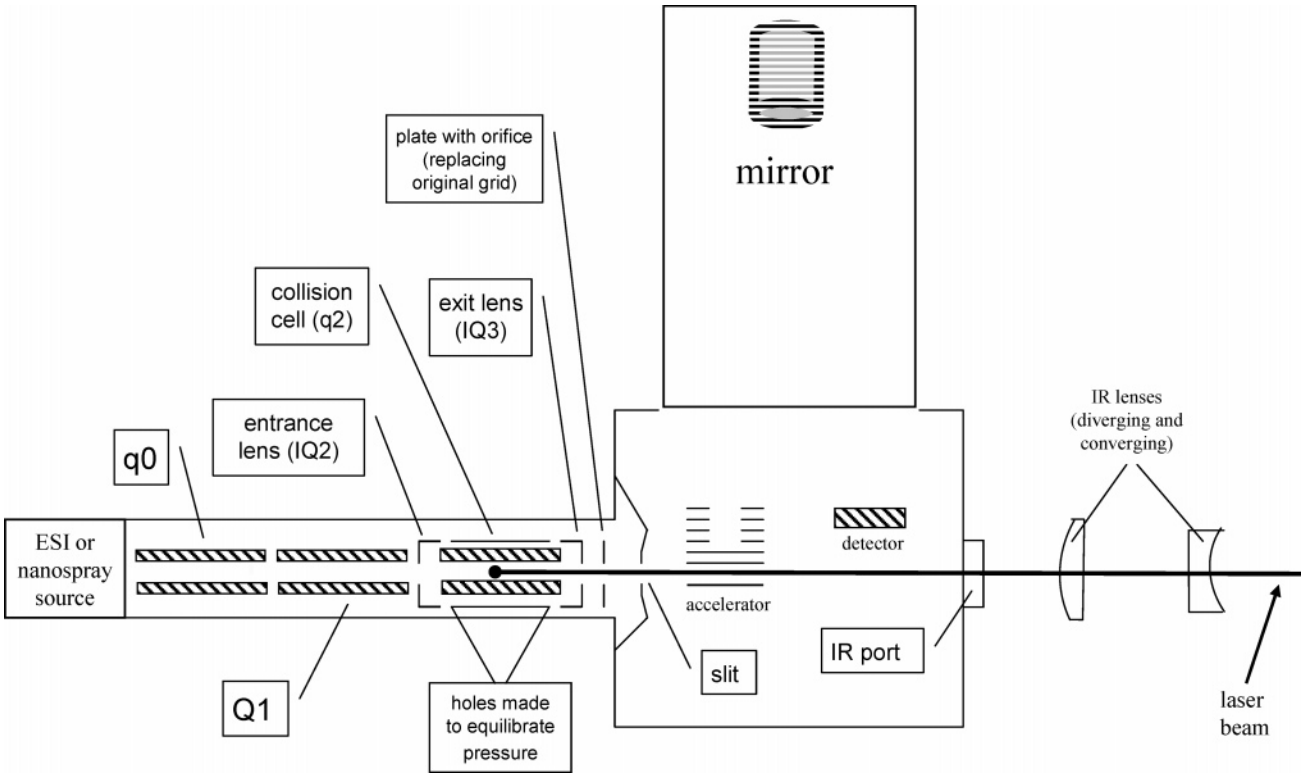

Figure 1. Schematic diagram of the modified QqTOF mass spectrometer.

monitoring the pointer light exiting the orifice plate. The laser beam has a 4 mrad divergence, resulting in a calculated beam diameter of 10 mm at the collision cell. To counteract the beam expansion and focus the beam inside the collision cell,[25] a pair of anti-reflection-coated ZnSe lenses (diverging, focal length −50 mm; converging, focal length +110 mm, Umicore Laser Optics) were mounted in kinematic optical mounts (Newport Instruments, Irvine, CA) outside the vacuum chamber and upstream from the IR port. The lens combination was adjusted to yield optimum fragmentation under IRMPD conditions. The laser intensity, expressed as % of the maximum (25 W) power, was adjusted by increasing or decreasing the pulse-width-modulated duty cycle using a UC-2000 Laser Controller (Synrad Inc). No attempt was made to measure the actual beam intensity inside the collision cell.

In a typical IRMPD experiment, ions were accumulated in the collision cell (q2) for a period of 50 ms by first raising the exit lens voltage (IQ3, 22 V) and then closing the entrance by raising the entrance lens voltage (IQ2, 22V). Trapping efficiency was above 75%. After a controlled delay period, ions were released into the TOF stage by lowering the exit lens voltage (IQ3, 5.5 V) for 50 ms. The laser was on continuously so that ions were exposed to the beam during all time periods. Data were collected by repeating the cycle until sufficient fragment-ion intensity was obtained (typically 1−10 min) under optimized laser power (between 4 and 60%) and exposure time (between 50 and 500 ms). Excessive laser power or irradiation time resulted in extensive internal fragmentation of proteins, which was detrimental for data analysis.

Data Analysis. For protein fragments smaller than 7 kDa, the resolved isotopic envelope allowed charge state assignment. For larger fragments, spacing between peaks due to water loss was used to determine the charge (see Results and Discussion). With use of experimental m/z and charge state values, masses of singly charged fragment ions were calculated. A list of these peaks and their intensities were compiled. These data were then used for searching with "Absolute Mass" retriever and the "Human Test" database in the ProsightPTM[26] proteomics tool. In addition, the MSTag searching tool[27] of the SwissProt[28] database was used to generate a more extensive sequence pool. Fragment mass tolerance of 250 ppm or less was used.

RESULTS AND DISCUSSION

Analytical Performance. Relatively low laser power was sufficient to induce appropriate fragmentation in our setup. An example is shown in Figure 2 for the 15+ charge state of equine myoglobin: Little fragmentation was evident at (a) 20% laser power at an irradiation time of 0.2 s; fragmentation was extensive at (b) 22.5% (also at 0.2 s). At 30−35% power, fragmentation was excessive, and few sequence-rich fragments were obtained. Experimentally, optimizing the laser power was straightforward and fast; typically, the setting was varied in steps of 0.5% power, increasing or decreasing from the initial value.

The removal and modification of the various ion optical components did not substantially alter the specifications of the instrument, for which a routine operating resolution of ap-

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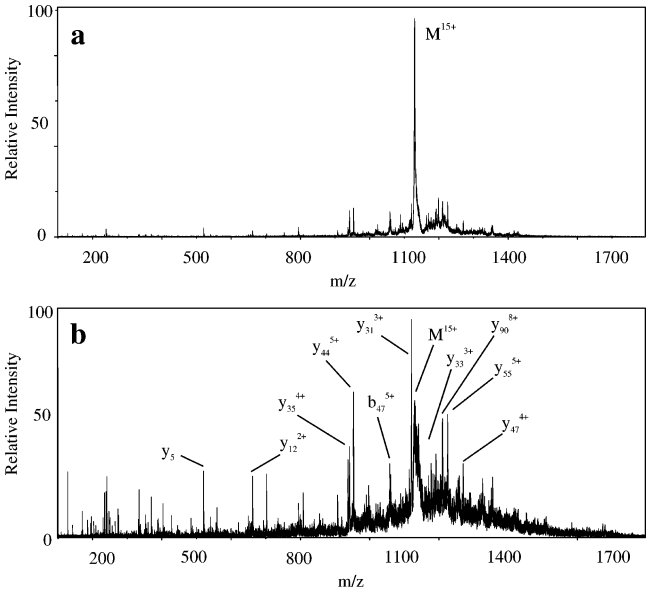

Figure 2. IRMPD spectra of the 15+ charge state of apo-myoglobin at (a, top) 20% laser power and (b, bottom) 22.5% laser power, irradiation time 0.2 s.

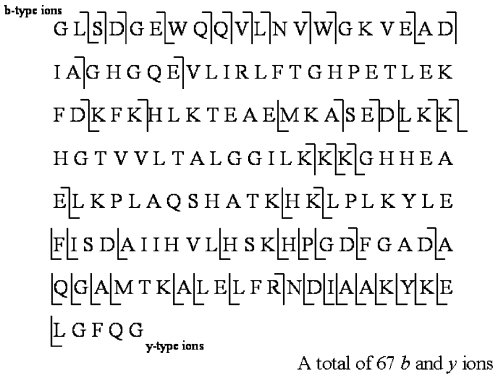

Figure 3. Fragment map of equine apo-Myoglobin: 9+, 15+, 18+, and 21+ charge states.

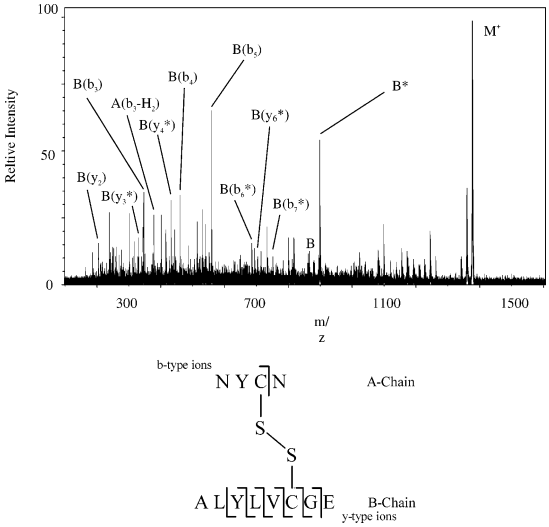

Figure 4. IRMPD spectrum of the singly charged human insulin fragment, 1377.6 Da, at 35% laser power, irradiation time 0.2 s. Fragment ions with asterisks are modified residues as described in the text. Minor fragment ions with intact disulfide bond are not labeled for readability.

proximately 7000 (fwhm) was routinely measured. The TOF analyzer was calibrated daily: a mass accuracy of 100 ppm was achievable with external calibrants. At a mass resolution of 7000, direct charge-state assignment for protein fragments smaller than 7 kDa was possible, using the resolved isotopic envelope. For larger fragments, the elimination of water was used to determine the fragment charge. As loss of ammonia was also possible, the spacing of the parent ion series was first measured and the calculated mass loss applied to the fragment ions of unknown charge. With the exception of human insulin (vide infra), all proteins studied showed a mass loss of 18 Da under IRMPD conditions.

As stated earlier, we used a data accumulation time of 1−10 min. To obtain just a few fragment ions for protein identification, a much shorter period could be used. However, as we typically saw efficient fragmentation and preferred wide sequence coverage to short analysis time, the 1−10 min time frame was optimal. (Time was not a factor as the samples were continuously infused, the preferred sample introduction mode for top-down analysis. To conserve samples, we used nanospray for introducing the extract from a HeLa cell culture (vide infra)). The protein standard concentrations we used, 10 μM, and spectral accumulation time are comparable to those used by Nemeth-Cawley and Rouse[13] for top-down analysis of proteins using CID on a QqTOF instrument. Experiments with increasing collision-cell pressure and increasing laser power did not result in sensitivity improvement, in accordance with modeling that showed typically cooling and collisional focusing of m/z 16000 ions at a background pressure of $\sim 5 \times 10^{-5}$ Torr requiring less than 40 ms.

Individual Protein Analysis. In a test of the instrumental setup the 9+, 15+, 18+, and 21+ charge states of equine myoglobin were isolated, fragmented by IRMPD, and analyzed using the ProsightPTM search engine and database. In total, 67 b and y ions (a score of 67) were observed for all four charge states (Figure 3); this is conceptually similar to the 64-hit score reported for apo-myoglobin with FTICR except we did not apply a multiplier for high-abundance ions.[29] Fragments larger than y_{60} and b_{59}, which could not be resolved isotopically, were correctly identified based on charge-state determination using the multiple loss of water. The IRMPD-induced cleavages are considered typical with prominent cleavages C-terminal to the acidic Asp (D) and Glu (E) residues, yielding abundant y ions.[5,20]

Human insulin was used as a model for disulfide-bridged proteins. Human insulin is a small polypeptide consisting of two peptide chains cross-linked by three disulfide bridges. To elucidate details of the IRMPD dissociation, an insulin fragment (Figure 4) containing a single disulfide bond was prepared by partial digestion of insulin using the endoproteinase Glu-C, which cleaves C-terminal to Glu and Asp residues.[30] Fragmentation of the so-obtained, singly charged, insulin fragment under IRMPD conditions is shown in Figure 4. The IRMPD results were analyzed by searching the data against the separate chain A and B sequences of the digestion product (Figure 4). Four non-cysteine-containing fragment ions of chain B—b_3, b_4, b_5, and y_2—were identified. In

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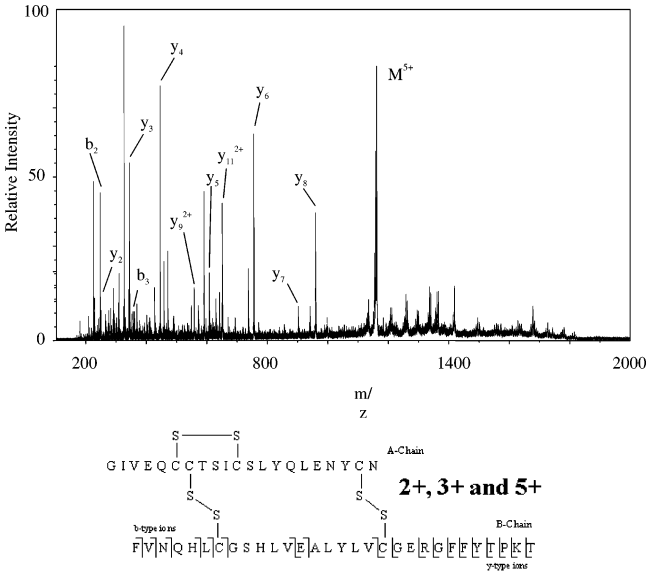

Figure 5. IRMPD spectrum of human insulin, 5+ charge state, at 30% laser power, irradiation time 0.2 s. Only fragments originating from chain B are shown. Fragmentation map shows ions of 2+, 3+, and 5+.

addition, cleavage of the C−S bond on the cysteine residue of chain A plus hydrogen transfer from chain A to B (probably due to the high hydrogen-atom affinity of the disulfide moiety[31]) was also observed, resulting in the following *modified* fragment ions: b_6, b_7, y_3, y_4, y_5, y_6, and the complete chain B (identified with asterisks in Figure 4).

Experience gained in interpreting the dissociation of the insulin fragment was applied to the complete protein. The IRMPD spectrum of the 5+ ion of insulin is shown in Figure 5 along with the dissociation coverage map of the 2+, 3+, and 5+ charge states. Data analysis was more complicated due to the presence of three disulfide linkages, which generated a considerable number of possible combinations of individual cleavages. In addition, there was evidence for the loss of ammonia (17 Da) along with that of water.[32] Abundant fragment ions were only generated from chain B, the most probable location for the added, mobile protons. As observed before for the insulin fragment, fragmentation of a cysteinyl C−S bond on chain A plus hydrogen transfer from chain A to B was commonly observed. Extensive fragmentation was apparent outside the "ring" formed by the interchain disulfide bridges (Figure 5). This fact and the absence of "ring fragmentation" were also noted in an earlier study using a quadrupole ion-trap mass spectrometer.[32]

Bovine α-casein, a 23.6-kDa protein, contains eight phosphoserine residues. Figure 6 shows the IRMPD spectrum of the 11+ charge state. Although loss of one phosphate group was observed, phosphorylation was largely retained as shown, for example, in the inset. The ProsightPTM database does not contain the sequence of bovine α-casein; our experimental data were therefore manually analyzed in the "single-protein" mode. A search of the mass spectral data against a sequence containing unmodified

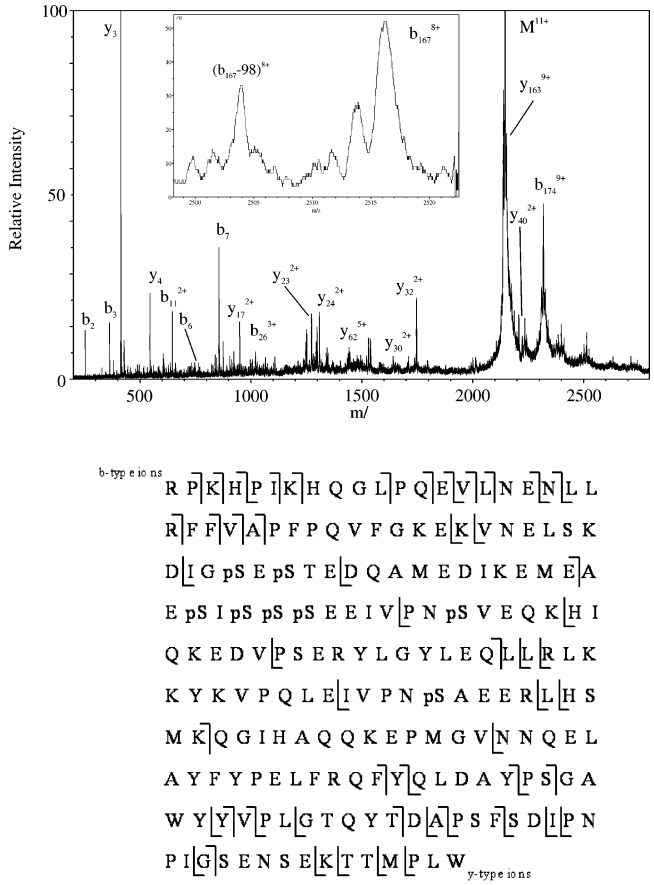

Figure 6. IRMPD spectrum of bovine α-casein, 11+ charge state, at 17% laser power, irradiation time 0.2 s. The inset shows an expansion of *m/z* 2500 to 2520. Fragmentation map shows coverage of 11+, 15+, 20+, and 27+ ions.

serines returned a total score (number of matched b and y ions) of 33. Including serine phosphorylation yielded a score of 61. This illustrates that, in an automatic search, the phosphoserine-containing protein will give a better match with the experimental results. In addition, a search was also performed using the assumption that all phosphate groups had been lost from serine residues through *β*-elimination reaction (−98 Da), which occurs with high efficiency in collision-induced dissociation of phosphopeptides.[33] This search results in a score of only 37, which is consistent with the earlier observation that serine phosphorylation was largely retained. Figure 6 demonstrates that with use of a total of four charge states (11+, 15+, 20+, and 27+), a percent sequence coverage of 22% was achieved.[26] Again, the propensity for fragmentation is evidently residue-dependent: for instance, 3 of the 6 Asp residues, 8 of the 24 Glu residues, 6 of the 14 Lys residues, and 2 of the 6 Arg residues cleaved on the C-terminal side, while 11 of the 16 Pro residues cleaved on the N-terminal side.

The above data demonstrate that loss of phosphorylation from the protein α-casein was not prominent in our IRMPD experiment. This observation may seem surprising because the tendency to lose a phosphate group is expected to be pronounced for phosphoserine residues.[33−36] Extensive loss of phosphorylation is

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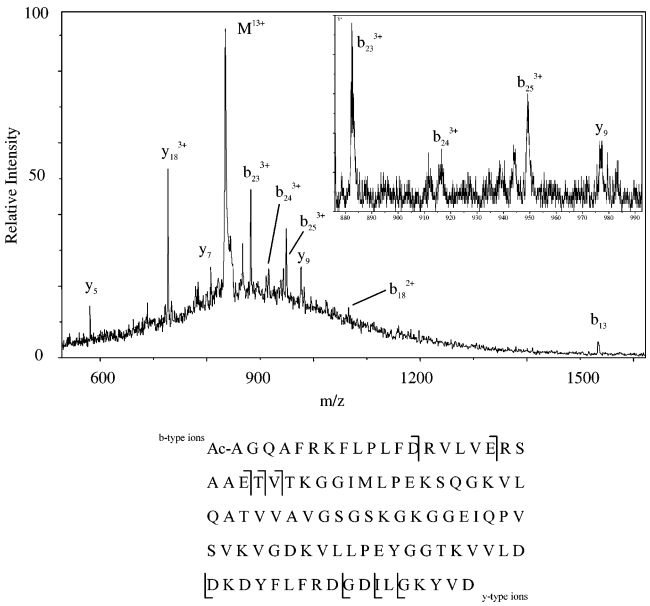

Figure 7. IRMPD spectrum of human chaperonin 10, 13+ charge state, at 20% laser power, irradiation time 0.2 s. The inset shows an expansion of *m/z* 880 to 990. Fragmentation map shows ions of the 13+ charge state.

believed to be a major shortcoming in determining phosphorylation sites of small polypeptides by CID and IRMPD. However, our results strongly suggest that phosphate loss may be much less of a problem for large proteins and is consistent with those in recent reports on IRMPD of phosphorylated polypeptides.[37,38] A decrease in phosphate loss combined with an increase in peptide backbone fragmentation was noted when the phosphoserine residue was in the neighborhood of the basic arginine residue.[33] In addition, phosphotyrosine and threonine show a decrease in phosphate loss with increasing positive charge on the polypeptide.[33] These observations are in accord with the hypothesis that the phosphate group is stabilized by hydrogen bonding with a basic residue (arginine or lysine), which might contribute to the observed difference between the IRMPD of small peptides of low charge and large polypeptides and proteins of higher charge.

The IRMPD of human chaperonin 10 is presented here as an example of biological application. Chaperonin 10 is the first potential cancer marker identified by our group as an overexpressed protein in tissues of endometrial cancer patients.[39] This protein is also highly expressed in HeLa cells (originally donated by a cervical cancer patient). Chaperonin 10 was identified by three internal tryptic peptides; from the molecular weight, we proposed that the protein carried two PTMs: cleavage of the original N-terminal methionine residue and *N*-acetylation of the second, alanine residue. The IRMPD spectrum of the 13+ ion of chaperonin 10 is shown in Figure 7. The ProsightPTM database contains an entry for chaperonin 10, thus permitting an automated analysis. Observation of the b_{23}^{3+}, b_{24}^{3+}, and b_{25}^{3+} ions (Figure 7) plus the b_{13}^{+} and b_{18}^{2+} ions strongly supports our earlier assignment of the two N-terminal PTMs.[39]

CONCLUSIONS

We have demonstrated successful implementation of IRMPD in a QqTOF mass spectrometer. The maximum overlapping of the IR and ion beams plus moderate collision deactivation as a result of a nominal pressure of 2×10^{-6} Torr in q2 result in efficient protein dissociation. Top-down analyses of a number of proteins, including post-translationally modified proteins, illustrate the utility of this instrumental approach. Trapping efficiency of ions during the initial filling period could be improved by temporarily raising the q2 pressure, which will be implemented in the next round of modifications.

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