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The exploitation of selective cleavage of singly protonated peptide ions adjacent to aspartic acid residues using a quadrupole orthogonal time-of-flight mass spectrometer equipped with a matrix-assisted laser desorption/ionization source

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Abstract

A series of singly charged tryptic peptide ions were analyzed by tandem mass spectrometry using a quadrupole/time-of-flight instrument equipped with a matrix-assisted laser desorption/ionization source. Highly selective cleavage C-terminal to aspartate, but not glutamate, residues was observed for C-terminal arginine-containing peptides, consistent with earlier findings. Increasing the basicity of C-terminal lysine residues by conversion to homoarginine promoted selective cleavage adjacent to aspartate residues. In contrast, reducing the basicity of C-terminal arginine residues by conversion to dimethylpyrimidylornithine abolished selective backbone cleavage and allowed the formation of multiple sequence ions. The increase in database search selectivity incorporating the single-residue sequence tag information revealed by aspartate-specific cleavage was determined by simulations using the yeast proteome, it was shown that an average of 83% of proteins can be identified on the basis of the mass of a single tryptic peptide together with knowledge of the presence and location of an aspartate residue. (Int J Mass Spectrom 210/211 (2001) 665–676) © 2001 Elsevier Science B.V.

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1. Introduction

The combination of matrix-assisted laser desorption/ionization (MALDI) with hybrid tandem instruments of quadrupole/time-of-flight design provides new opportunities for the study of the collisionally activated decomposition (CAD) of ions produced by MALDI [1,2]. The MALDI technique is now very widely used in the context of proteomics, that is, the analysis of multiple constituents of the full protein complement a cell type or organism. Typically, protein isolation is followed by enzymatic (usually tryptic) digestion followed by mass profiling of the digest and automated comparison with pre-

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Dedicated to Professor Nico Nibbering on the occasion of his retirement.

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dicted mass profiles of proteolytic peptides, constructed by applying the rules of enzyme cleavage to each protein sequence in a database. In this way, previously identified (or predicted) protein sequences may be recognized. MALDI/time-of-flight analyses are of particular value by virtue of the high sensitivities achieved and the simplification (in comparison with electrospray analyses) associated with the concentration of signal in ions of a single charge state for each constituent [3,4]. The database search specificity may, however, be insufficient when based on tryptic masses alone (or the search may be confounded if the digest is derived from multiple proteins), in which case it is beneficial to obtain full or partial sequence information for the proteolytic peptides. Most commonly this is done using the combination of electrospray ionization and tandem mass spectrometry; in this instance the generation of multiply charged ions (usually $[M+2H]^{2+}$ for tryptic peptides) is beneficial because the efficiency of generation of fragment ions indicative of sequence is markedly enhanced by the location of an ionizing proton on the peptide backbone [5–13]. The ionizing proton in singly charged arginine-containing tryptic peptides is expected to be sequestered on the basic side-chain; an equivalent, though less pronounced, effect is expected for lysine-containing peptides. In doubly protonated peptide ions, the second proton is "mobile", thereby generating a precursor ion population that is heterogeneous with respect to site of charge and promoting multiple fragmentation pathways.

An alternative approach, using MALDI/time-of-flight with post-source decay (PSD) analysis of fragment ions, may suffer from a poor yield of sequence ions from singly charged precursors, in addition to the limitations associated with modest sensitivity and extended acquisition times. In general, however, the analysis of the fragmentations of singly charged peptides may have value if the distinction is made between the requirements of de novo sequencing (where, by definition, complete structural information is required) and those of proteomics analysis (where additional diagnostic search parameters are needed to supplement proteolytic peptide masses). Thus, the observation of a single, highly selective fragmentation process may provide informative structural parameters, if that process is sufficiently well understood and its specificity appreciated.

Several previous studies have highlighted the observation of preferential cleavage of peptide ions C-terminal to acidic residues. Yu and coworkers [14] reported fragmentation adjacent to aspartate residues, observed in MALDI/PSD analyses of singly protonated peptides. They proposed the transfer of the side-chain carboxyl proton to the nitrogen of the backbone amide moiety. A similar preference for cleavage adjacent to aspartate was reported by Smith and coworkers in studies of the fragmentations of multiply charged protein ions using sustained off-resonance irradiation in a Fourier-transform ion cyclotron resonance spectrometer. In previous studies from this laboratory, we have shown that doubly protonated peptide ions incorporating both N-and C-terminal arginine residues (and therefore lacking a mobile proton) fragment preferentially adjacent to aspartate, but not glutamate residues [9]. Equivalent findings, for both singly and doubly charged ions lacking a mobile proton, have been reported by Tsaprailis and coworkers, using both CAD with gaseous targets and surface induced dissociation [15,16]. These authors elaborated upon earlier mechanistic discussions, and these are considered in detail in the discussion Sec. 3 of the present paper.

Qin and coworkers have examined selective cleavage adjacent to aspartate residues of singly protonated tryptic peptide ions generated by MALDI in a quadrupole ion trap, noting also the concomitant requirement for a C-terminal arginine, rather than lysine, residue [17]. These researchers recognized the utility of this selective cleavage in informing a database search for the purpose of protein identification [18,19]. In previous studies from our laboratories, we have used the predicted yeast proteome (available by virtue of the complete sequencing of the genome) to evaluate database search strategies and explore the interdependence of such strategies and the design of tandem mass spectrometric analyses of tryptic peptide digests [20,21]. In the predicted yeast proteome, of the more than 347 000 possible tryptic peptides, there are in excess of 210 000 peptides with C-terminal lysine compared to about 130 000 with C-terminal

arginine. Furthermore, approximately 85 000 of the 347 000 tryptic peptides contain one or more internal aspartic acid residues and of these 62% have a C-terminal lysine. Thus, selective cleavage of singly protonated yeast-derived peptides is predicted for a minority of aspartate-containing species.

We have previously reported the conversion of lysine to homoarginine residues for MALDI mass spectrometry (MS) analyses of tryptic digests for the improvement of mass spectrometric response [20,21]. This strategy was devised in recognition of the generally superior detection of arginine-, rather than lysine-containing peptides during MALDI MS of tryptic digests species [22]. The effect is attributable to the differences in basicity between arginine and lysine, which may affect both ionization efficiency and ion stability [21]. The same benefits of the lysine/homoarginine derivatization have subsequently been reported by other workers [23–25]. The conversion of C-terminal lysine to homoarginine residues is expected also of course, to result in significant modification of fragmentation properties during tandem MS.

In the present paper, we report tandem MS analyses of aspartate- and glutamate-containing tryptic peptides using a MALDI-quadrupole/time-of-flight instrument. We note the striking structural specificity of cleavage adjacent to aspartate residues and demonstrate the "tuning" of fragmentation behavior that may be achieved by derivatization of C-terminal arginine residues. Finally, we quantify the benefits to database searching derived from the additional search parameters derived from the supplementary structural information derived from selective cleavages.

2. Experimental

2.1. Materials and methods

Synthetic peptides AFLDASR and AFLDASK were obtained from the University of Texas Southwestern Medical Center (Dallas, Texas). TPCK-treated trypsin, the yeast proteins enolase, hexokinase, and alcohol dehydrogenase, and other synthetic peptides were supplied by Sigma (Dorset, Poole, UK).

2.2. Mass spectrometry

An experimental MALDI source was fitted to a tandem quadrupole/time-of-flight instrument (Q-Tof II; Micromass, Manchester, UK) instrument. The standard electrospray source housing was replaced (Fig. 1). The new housing allowed a target plate mounted on a probe to be introduced into the housing via a vacuum lock. The housing also included a window to provide direct line-of-sight laser access to the sample plate. A nitrogen UV laser (337 nm; VSL-337ND, Laser Science, Inc., Franklin, MA) was mounted to allow reflection of the UV light off an adjustable mirror and through the window onto the target. The mirror was adjusted to optimize the positioning of the laser beam on the surface. A lens was included in the optical path to focus the laser beam on the target plate surface to a spot approximately 300 μm in diameter. The housing included provision for introducing gas via a capillary line and adjustable needle valve. Reduction of the velocities of the desorbed ions on the ion optical axis was achieved by collision with gas molecules in an rf-only hexapole ion guide [2]. This was done by leaking air via the needle valve into the source housing so that the pressure was raised to 0.25 mbar. The MALDI probe allowed a target plate or strip to be introduced. The target strip accommodated ten 2.5 mm diameter target spots in a line. The probe was moved from spot to spot using a stepping motor drive continuously scanning across each target spot. In this arrangement there was no provision for moving the target plate in any direction other than along one axis. Inspection of the target plate after analysis revealed a faint track in a straight line across the sample spot indicating the area that had been irradiated by the laser beam. Ions from the MALDI target plate were accelerated directly into the RF-only hexapole transport lens. From this point the instrument was essentially unmodified from the standard Q-Tof configuration [26] with the ions then being transported sequentially through a small orifice and into the quadrupole mass filter, a hexapole gas

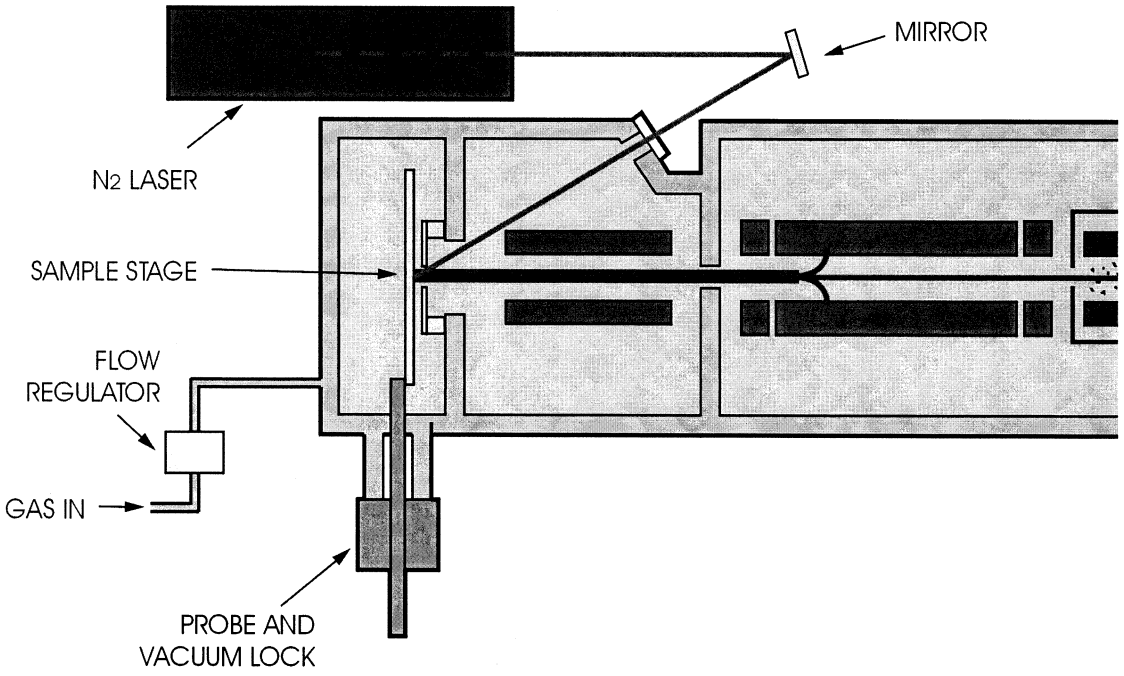

Fig. 1. The source region of the MALDI Q-Tof mass spectrometer.

collision cell, and into the orthogonal acceleration time-of-flight (TOF) analyzer. Conventional mass spectra were recorded using the TOF analyzer, with the quadrupole device operated in rf-only mode. The resolution was 4000–5000 (full width at half maximum height definition). For tandem MS analyses, precursor ions were selected using MS1 in resolving (rf/dc) mode and were fragmented in the enclosed rf-only hexapole gas collision cell using a collision energy (CE, eV) that was set such that the CE=0.05× precursor ion mass (Da). Argon was the collision gas. The quadrupole resolution was set to achieve transmission of a precursor ion window of 4 Th. Product ion spectra were recorded using the TOF analyzer.

Initial experiments were performed using renin substrate (DRVYIHPFHLVIHN) and Glu-fibrinopeptide B (EGVNDNEEGFFSAR) prepared in water/acetonitrile 1:1 at a concentration of 1 pmol/μL. The MALDI matrix was 2,5-dihydroxybenzoic acid (DHB), prepared as a saturated solution in 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile (3:7 v/v). The tryptic digest solutions and the standards were mixed 1:1 with the matrix solution and applied to the target surface using the "dried droplet" method [27].

All electrospray (ES) tandem mass spectrometry (MS/MS) experiments were performed on a Q-Tof mass spectrometer (Micromass) fitted with a dual orthogonal microelectrospray ion source. Tryptic digest solutions were infused at a flow rate of 0.5 μL/min. The resolution of the mass-analyzing quadrupole was set to transmit the full isotopic envelope corresponding to the selected precursor ion. Argon was used as the collision gas. The collision energy was varied between 55 and 100 eV. The resolution of the TOF analyzer was 4500 (full-width at half maximum height definition.)

MALDI PSD experiments were performed on a Voyager DE-STR mass spectrometer (Applied Biosystems, Warrington, UK). PSD spectra were acquired using an extraction delay of 170 ns and a 20 kV accelerating voltage. For the recording of PSD spectra, the reflectron potential/accelerating potential ratio was reduced by a factor of 0.8 for each successive acquisition, which was the sum of 50 shots. Stored

spectra were the sum of ten acquisitions. The matrix used was 2,5-DHB, prepared as a saturated solution in 0.1% (v/v) TFA and acetonitrile (1:1 v/v). Tryptic digest solutions were mixed 1:1 with the matrix solution and applied to the target surface using the dried droplet method.

2.3. Conversion of arginine residues to dimethylpyrimidylornithine

The procedure used was essentially unchanged from that described by Summerfield and Gaskell [7]. The tryptic digest mixture (containing approximately 20 μg of peptides) was placed in a tube and taken to dryness under nitrogen. Water (50 μL), ethanol (100 μL), and triethylamine (50 μL) were added. The tube was capped under nitrogen and vortex mixed for 10 min. Acetylacetone (100 μL) was added, the mixture was vortex mixed, and then heated at 60°C for three hours. The mixture was dried using a vacuum centrifuge (Savant, Holbrook, NY) leaving a brown oily residue. To this, water (100 μL) and glacial acetic acid (60 μL) were added; the mixture was heated to 70°C and held at that temperature for 30 min. When cooled, the mixture was extracted with three 500 μL-portions of diethyl ether. The aqueous portion was retained for MALDI-Q-Tof analysis.

2.4. Guanidination of lysine residues

The procedure followed was that of Brancia et al. [21]. An aqueous solution of tryptic digest mixture was mixed with an equal volume of 0.5M O-methylisourea, and adjusted to pH 10.5 with NaOH. The reaction was allowed to proceed overnight and was stopped by addition of an equal volume of 1% (v/v) aqueous TFA. Sample aliquots were mixed with an equal volume of matrix solution and loaded onto the target, without further purification.

3. Results and discussion

Striking examples of the preferential fragmentation of tryptic Asp-containing peptides are shown in Fig. 2. Figs. 2(a) and 2(b) show the MALDI- Q-Tof product ion spectra of a tryptic fragment of yeast enolase with $[M+H]^+$ at *m/z* 2552 and a tryptic fragment of hexokinase with $[M+H]^+$ at *m/z* 1823, respectively. In both cases the fragment ion distributions are dominated by y-ions corresponding to peptide bond cleavage C-terminal to the Asp residues. The product ion spectrum of $[M+H]^+$ of the peptide INEGILQR [Fig. 2(c)] shows an abundant y_5 ion resulting from fragmentation C-terminal to the Glu residue, but its prominence does not compare with the equivalent ions derived from the Asp-containing peptides shown in Figs. 2(a) and 2(b). The generalization of predominant y-ions arising from cleavage adjacent to Asp, but not Glu, residues in C-terminal Arg-containing peptides was substantiated by analysis of other tryptic peptides derived from enolase and hexokinase (data not shown).

The differences in fragmentation properties of singly and doubly charged ions was illustrated by analyses of the synthetic peptide AFLDASR using ES- Q-Tof MS/MS with precursors in both charge states. The doubly charged AFLDASR ion [Fig. 3(a)] produces a range of fragment ions, both N- and C-terminal, without specific preference for the formation of the y_3 ion; this behavior reflects the influence of the mobile proton not sequestered by the arginine residue. The products of the singly charged precursor (generated by raising the nozzle/skimmer potential in the ES interface) are dominated by the y_3 ion [Fig. 3(b)], consistent with the earlier MALDI MS/MS observations for Asp-containing C-terminal-Arg peptides. There is no significant difference between the product ion spectra of the $[M+H]^+$ ions generated by ES and MALDI [Figs. 3(b) and 3(c)]. The product ion spectrum of singly charged AFLDASR obtained by MALDI-PSD [Fig. 3(d)] is similar to that obtained in the other modes, though lower mass fragments assume somewhat greater prominence. This may be attributable to more effective collisional cooling of precursor ions in the MALDI source on the Q-Tof instrument than the Voyager [2]. The product ion spectrum of singly charged AFLDASK was also recorded using the MALDI-Q-Tof [Fig. 3(e)]. The substitution of Lys for Arg at the C-terminus abol-

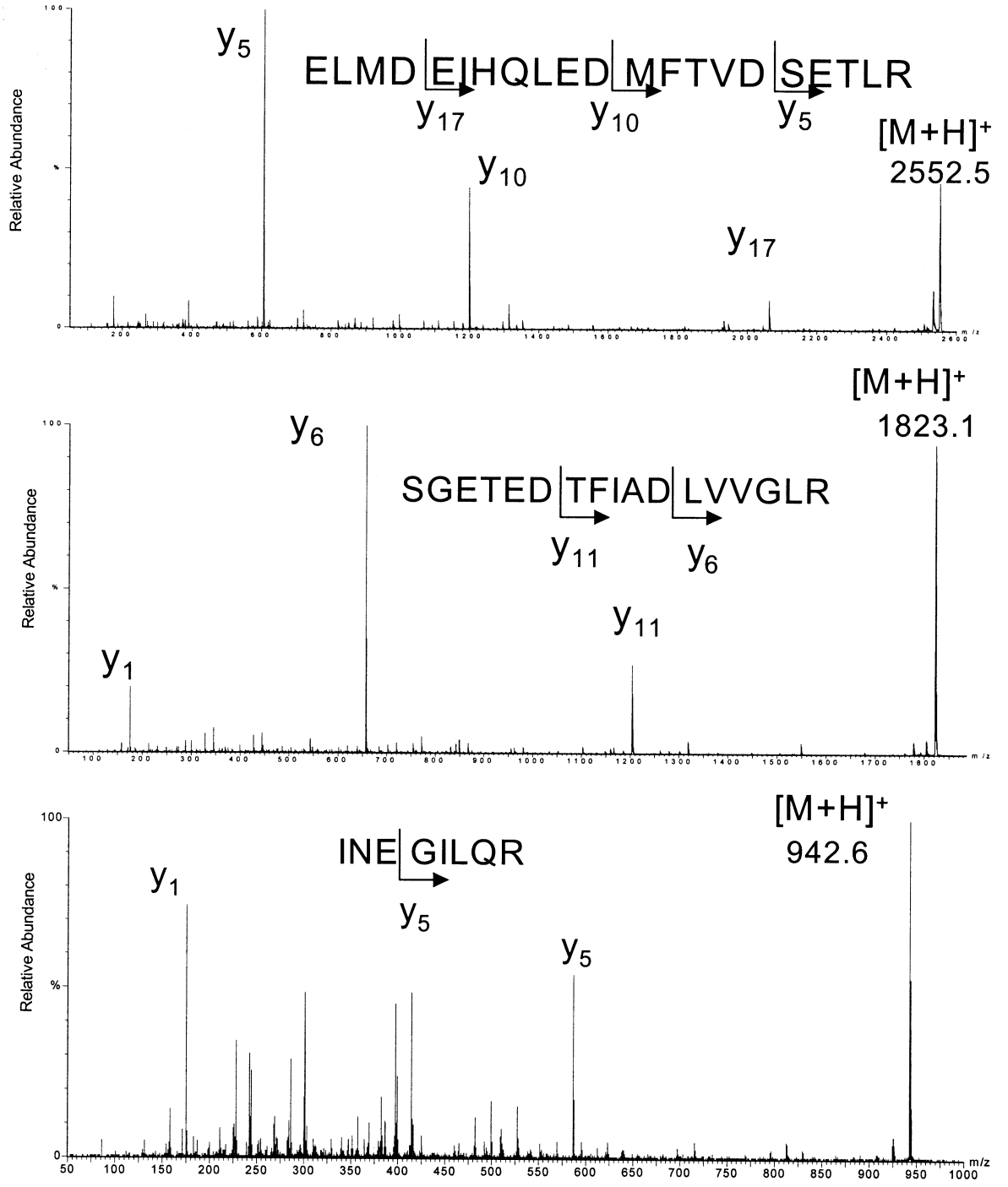

Fig. 2. Product ion spectra of $[M+H]^+$ ions recorded using the MALDI Q-Tof mass spectrometer. (a) Precursor ion *m/z* 2552.2, derived from the tryptic digest of hexokinase; (b) Precursor ion *m/z* 1823.1, derived from the tryptic digest of enolase; (c) Precursor ion *m/z* 942.6, derived from the tryptic digest of hexokinase.

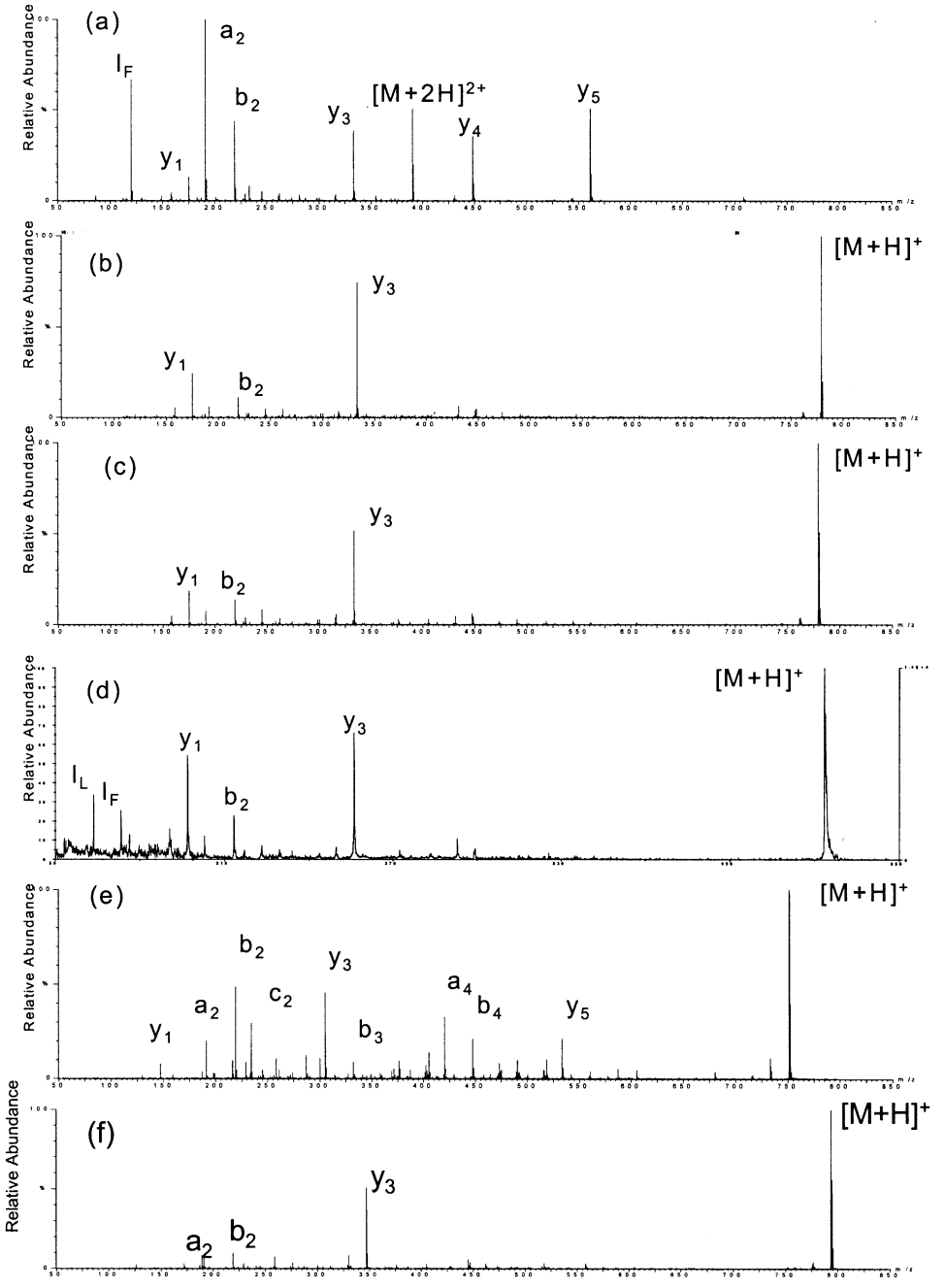

Fig. 3. Product ion spectra of protonated peptide ions: (a) Products of $[M+2H]^{2+}$ ions of AFLDASR, produced by electrospray and recorded using a Q-Tof instrument; (b) Products of $[M+H]^+$ ions of AFLDASR, produced by electrospray and recorded using a Q-Tof instrument; (c) Products of $[M+H]^+$ ions of AFLDASR, produced by MALDI and recorded using a Q-Tof instrument; (d) Products of $[M+H]^+$ ions of AFLDASR, produced by MALDI and recorded using a time-of-flight instrument (post-source decay); (e) Products of $[M+H]^+$ ions of AFLDASK, produced by MALDI and recorded using a Q-Tof instrument; (f) Products of $[M+H]^+$ ions of AFLDASK* (where K* represents the homoarginine residue), produced by MALDI and recorded using a Q-Tof instrument.

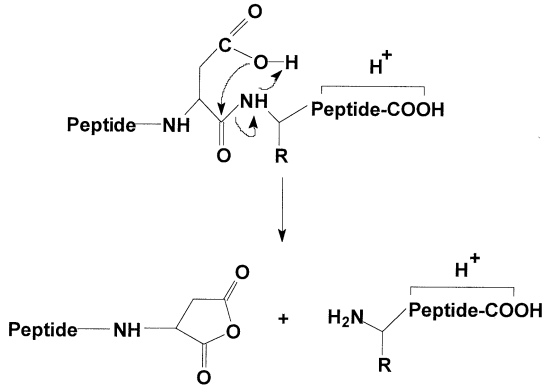

Fig. 4. A mechanism for peptide backbone fragmentation adjacent to an aspartate residue.

ishes the preference for y_3 formation. Thus, the prevalence of this low-energy fragmentation pathway is dependent on the presence of both Asp and Arg residues, in confirmation of earlier findings [17,28].

A representation of the aspartate-specific cleavage mechanism is shown in Fig. 4. The key feature is the transfer of the carboxylate proton from the aspartate side chain to a proximal location on the peptide backbone, shown here as part of a concerted process resulting in amide bond cleavage. An alternative (and closely related) representation suggested by Wysocki et al. [13] envisages a two-stage process in which initial proton transfer takes place to the carbonyl oxygen of the amide moiety.

The observation of much greater prevalence of this fragmentation in aspartate-, rather than glutamate-containing peptides can be attributed to the somewhat greater gas-phase acidity of aspartic acid [29] and the lesser favorability, for entropic reasons, of the six-membered ring [30] formed in the equivalent process involving a glutamate residue.

Detailed consideration of the factors that promote proton migration to the peptide backbone remain the subject of debate. In earlier papers [9,31] we argued that acid-base interaction between the strongly basic side chain of arginines located at the N-terminus (in peptides not typical of those obtained by tryptic digestion) and mid-chain aspartic acid side chains releases the ionizing proton to the peptide backbone, enabling the promotion of fragmentation of the backbone. It may be argued, however, that such a mechanism fails to explain the observation of selective promotion of backbone cleavage C-terminal to an aspartate residue, since release of a proton to the peptide backbone would afford a mobile proton (with no singly favored site) with consequent promotion of multiple fragmentation pathways [16]. (Such considerations might also argue against the explicit intermediacy of a zwitterionic structure arising from proton migration from the aspartate residue to the peptide backbone.) Furthermore, Gu et al. [32] have recently demonstrated selective cleavage adjacent to aspartate residues in peptide ions where the charge is derived from a precharged derivative group. The mechanism shown in Fig. 4 (and close analogues) assumes that the aspartate residue at which selective cleavage occurs is *not* involved in charge solvation, implying, for example, an interaction between the protonated arginine side chain in singly protonated AFLDASR and other sites in the molecule. These considerations, as examples of intra-ionic nucleophile-electrophile interactions, are the subject of continuing study in this laboratory and others [33].

Regardless of details of charge solvation, the ionizing proton is envisaged to be sequestered at the arginine residue and is therefore a spectator to the chemistry specific to the cleavage adjacent to aspartate. When the ionizing proton is mobile, as in C-terminal Lys-containing peptides, other low energy fragmentation channels are available and compete with cleavage adjacent to the aspartate residue.

We recently reported the benefit of conversion of lysine to homoarginine residues in tryptic peptides for the purpose of improved detection during MALDI-TOF analyses [21]. A comparison of Figs. 3(e) and 3(f) shows that the same derivatization influences the prevalence of cleavage adjacent to aspartate residues; the properties of the arginine- and homoarginine-containing analogues are closely similar. Equivalent findings have been made for tryptic fragments derived from the yeast proteins, enolase, and hexokinase; an example is shown in Fig. 5 which compares the fragmentation of a singly protonated 15-residue peptide before and after guanidination of the lysine side-chain.

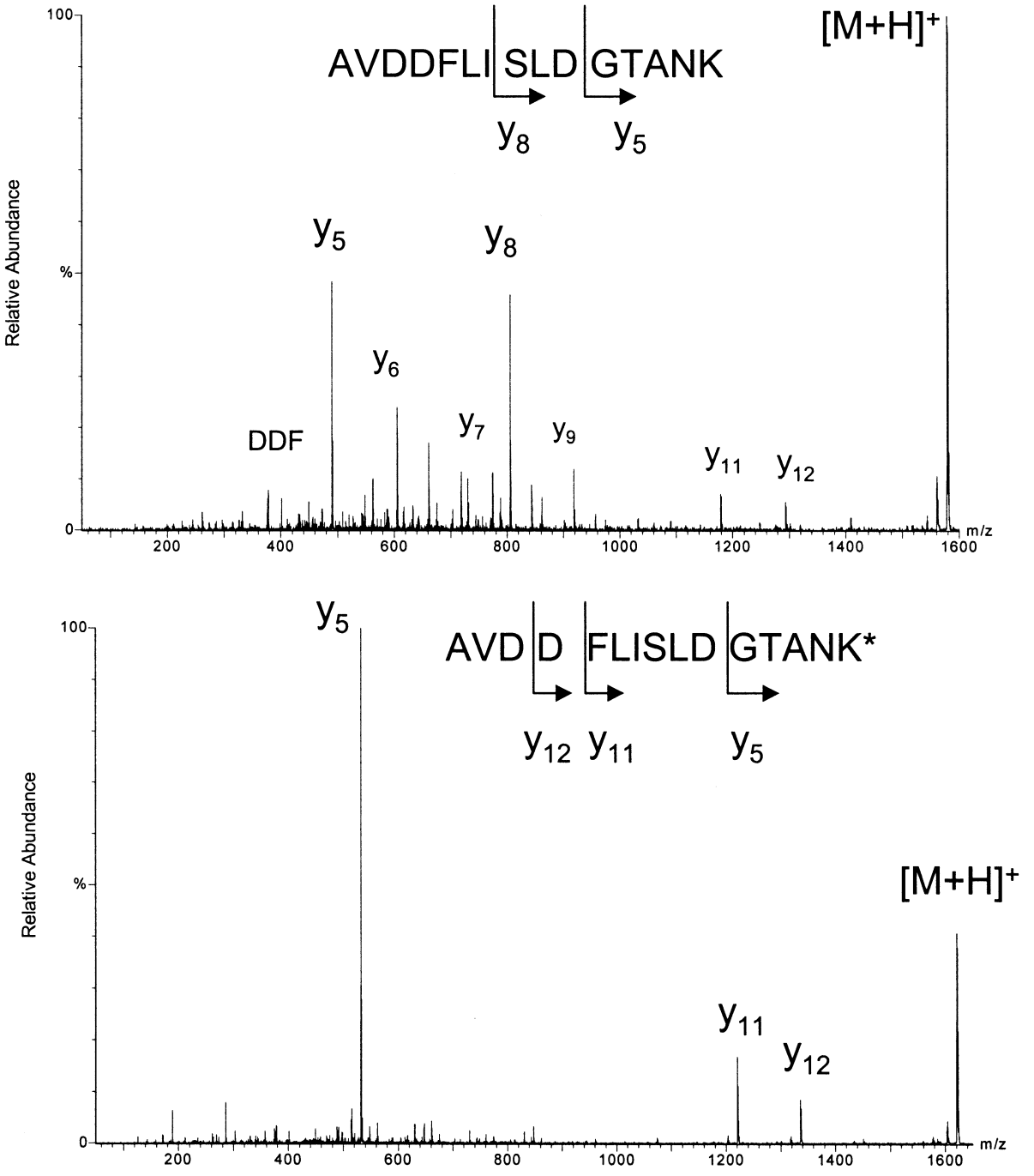

Fig. 5. Product ion spectra of the $[M+H]^+$ ion of AVDDFLISLDGTANK (an enolase tryptic fragment) analyzed by MALDI using a Q-Tof instrument, before (top) and after (bottom) conversion of the lysine residue to homoarginine.

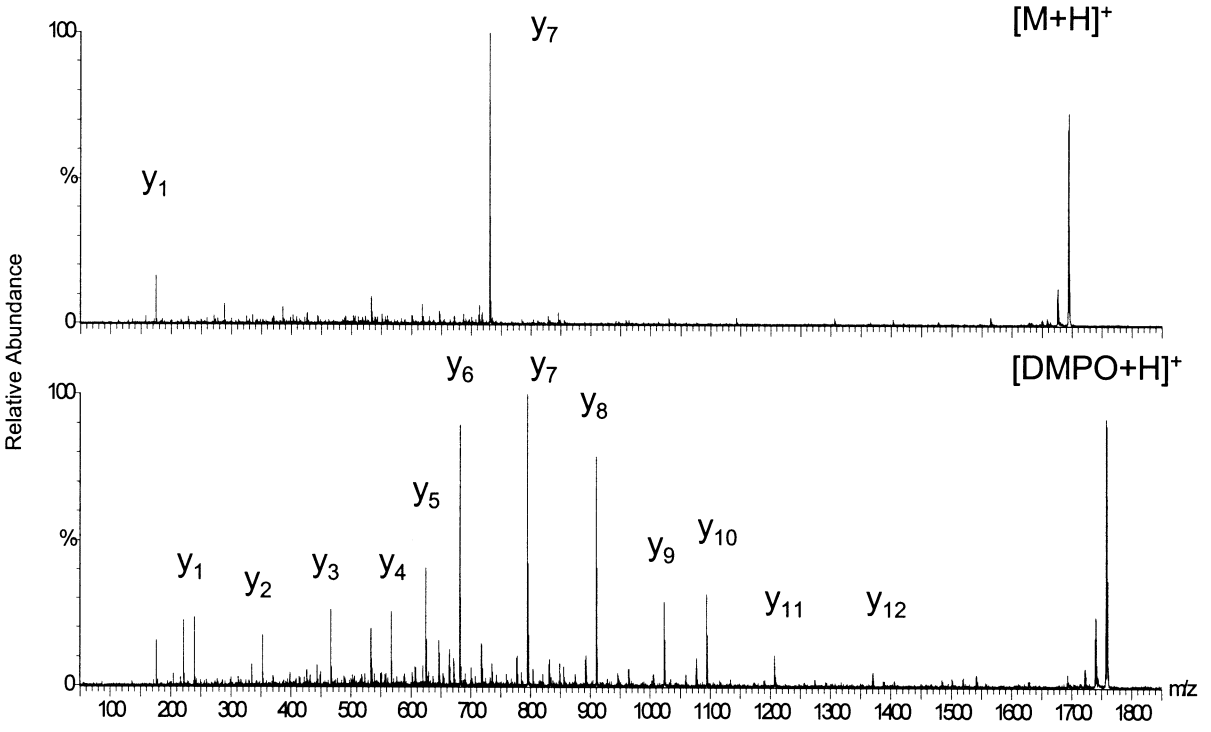

Fig. 6. Product ion spectra of the [M+H]+ ion of ESGNYLAIDLGGTNLR, analyzed by MALDI using a Q-Tof instrument, before (top) and after (bottom) conversion of the arginine residue to dimethylpyrimidylornithine.

Conversely, the properties of arginine-containing peptides can be modified by derivatization to moderate basicity. Conversion of arginine to dimethylpyrimidylornithine residues has been show previously to influence fragmentation [34,35]. This is illustrated in a striking fashion in Fig. 6, which shows the product ion spectra of the hexokinase tryptic peptide ESGNYLAIDLGGTNLR before and after conversion of the arginine to a dimethylpyrimidinylornithine residue.

We have examined the impact of knowledge of the locations of Asp residues in tryptic peptides on the efficacy of database searching by conducting simulations in the context of the yeast proteome. Using our in-house search tool Pepmapper [36], peptides were randomly selected and the number of database hits determined for several hypothetical conditions (Fig. 6). We assumed that fragmentation would occur C-terminal to all aspartate residues, yielding the corresponding y-series product ion masses. A mass accuracy was assumed in the simulations of 50 ppm for the precursor and 20 ppm for product ions. Missed cleavages were not considered. In the yeast proteome (estimated to include approximately 6500 constituents) about 80% of all proteins contain 5 or more possible tryptic Asp-containing peptides. The masses of fragments derived from cleavages C-terminal to Asp residues were used as search parameters, additional to the masses of the intact tryptic peptides. The efficacies of the alternative approaches were assessed by the frequency of unambiguous identification of the parent protein in the simulations, expressed as a percentage. Unambiguous identification considered to be made when only one protein in the yeast proteome was consistent with all the peptide mass and associated search data. As can be seen from Fig. 7, the incorporation of search parameters indicative of Asp locations markedly improved database search selectivity, with a higher level of unambiguous identification compared with standard MALDI mass finger-

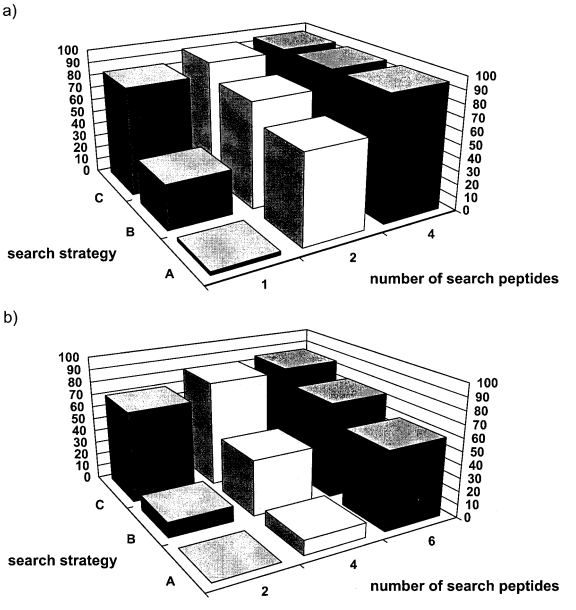

Fig. 7. Percentages of unambiguously identified proteins in a search of the yeast proteome database using parameters derived from tryptic peptides under various simulated conditions. In (a) one protein was randomly selected; in (b) a mixture of two randomly selected proteins were selected. The number of search peptides is shown on one axis, whereas the search strategy (A, B, C) is shown on the second. For the simulation using a random mixture of two proteins, at least one tryptic peptide was selected from each protein. The search parameters were: A: tryptic peptide masses alone; B: tryptic peptide masses and evidence for the presence (or not) and location of an aspartate residue, where the peptides were selected at random; C: tryptic peptide masses and evidence for the presence (or not) and location of an aspartate residue, where only aspartate-containing peptides were selected.

printing. Two simulation series were considered which used the aspartate-specific data. The first picked peptides randomly from the yeast proteome (series B in Fig. 7) whilst the second only considered peptides containing at least one aspartate residue (series C in Fig. 7). The simulations demonstrated that on average 83% of yeast proteins can be identified from only a single peptide and promoted fragment ion data when a peptide contains an aspartate residue. A similar statistic of 79% unambiguous identification was obtained searching the *Caenorhabditis elegans* genome, containing 18576 proteins (data not shown). For the unconstrained simulations, where any peptides may be picked, these values dropped, but 73% of proteins could still be unambiguously identified from two randomly selected peptides per protein. Similar encouraging statistics were observed for simulations on two-protein mixtures [Fig. 7(b)], where at least one peptide was picked from each protein under consideration. In 70% of cases both proteins could be unambiguously identified from only 2 aspartic acid-containing peptides, increasing to 82% when 4 such peptides were determined from the mixture.

4. Conclusions

The combination of MALDI and a tandem quadrupole/time-of-flight mass spectrometer has been used to investigate aspects of the selectivity of fragmentation of singly charged peptides. In confirmation of earlier findings, highly selective cleavage C-terminal to aspartic, but not glutamic, acid residues is observed for tryptic peptides incorporting a C-terminal arginine, but not lysine, residue. The data are consistent with a mechanism involving selective proton transfer to the peptide backbone proximal to the aspartic acid residue; the ionizing proton remains sequestered on the strongly basic arginine side-chain. Simple derivatization of lysine residues to homoarginine (by reaction with O-methylisourea) converts the properties of lysine-containing peptides to those of the arginine-containing analogues. Conversely, reduction of the basicity of arginine residues by derivatization to afford dimethylpyrimidylornithines abolishes the tendency to selective cleavage adjacent to aspartate; extensive sequence information is observed.

The facility to modify fragmentation properties enables the adjustment of analytical strategies to match requirements for de novo sequencing or for recognition of a protein via database searching using parameters derived from analysis of tryptic peptides. The latter application, particularly in the context of analysis of proteins from organisms with sequenced genomes, may require little structural information in addition to the masses of tryptic peptides. Simulation studies with the yeast genome, for example, have shown that an average of 83% of proteins can be identified on the basis of the mass of a single tryptic

peptide together with knowledge of the presence and location of an aspartate residue.

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