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Improving fragmentation of poorly fragmenting peptides and phosphopeptides during collision-induced dissociation by malondialdehyde modification of arginine residues

Alexander Leitner,[1]* Alexandra Foettinger[1,2] and Wolfgang Lindner[1]

[1] Department of Analytical Chemistry and Food Chemistry, University of Vienna, 1090 Vienna, Austria
[2] Baxter AG, Biomedical Research Center, 2304 Orth/Donau, Austria

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Despite significant technological and methodological advancements in peptide sequencing by mass spectrometry, analyzing peptides that exhibit only poor fragmentation upon collision-induced dissociation (CID) remains a challenge. A major cause for unfavorable fragmentation is insufficient proton 'mobility' due to charge localization at strongly basic sites, in particular, the guanidine group of arginine. We have recently demonstrated that the conversion of the guanidine group of the arginine side chain by malondialdehyde (MDA) is a convenient tool to reduce the basicity of arginine residues and can have beneficial effects for peptide fragmentation. In the present work, we have focused on peptides that typically yield incomplete sequence information in CID-MS/MS experiments. Energy-resolved tandem MS experiments were carried out on angiotensins and arginine-containing phosphopeptides to study in detail the influence of the modification step on the fragmentation process. MDA modification dramatically improved the fragmentation behavior of peptides that exhibited only one or two dominant cleavages in their unmodified form. Neutral loss of phosphoric acid from phosphopeptides carrying phosphoserine and threonine residues was significantly reduced in favor of a higher abundance of fragment ions. Complementary experiments were carried out on three different instrumental platforms (triple-quadrupole, 3D ion trap, quadrupole–linear ion trap hybrid) to ascertain that the observation is a general effect. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: arginine modification; malondialdehyde; peptide fragmentation; phosphorylation; tandem mass spectrometry

INTRODUCTION

Peptide sequencing by tandem mass spectrometry is the central technique for protein identification. Regardless of the approach being taken – automated database searches comparing experimental spectra with theoretical ones generated *in silico*[1–5] or *de novo* sequencing without the help of sequence databases[6] – sufficient quality of the MS/MS spectra is necessary. For database searches, this usually means a significant number of the b- or y-type ions most commonly observed when collision-induced dissociation (CID) is employed as the fragmentation technique. *De novo* sequencing demands almost a continuous series of b- and y-ions for complete elucidation of the peptide sequence with reasonable accuracy.

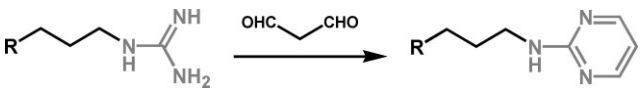
However, peptide fragmentation is frequently far from the ideal 'textbook' case where all amide bonds are cleaved, yielding perfect product ion spectra. In this context, diverse factors play a role including those related to the peptide itself, such as the amino acid sequence, length and molar mass, charge state and the presence of labile modifications, but also characteristics of the MS analyzer, as for example the low-mass cutoff observed in ion trap instruments or the specific signal-to-noise ratio in a spectrum.[7–9] To overcome some of the limitations, improvements have been made or suggested on several stages of the analytical workflow. Alternative fragmentation techniques, in particular electron capture dissociation (ECD)[9,10] and electron transfer dissociation (ETD),[11,12] are increasingly being used as complementary methods, as they lead to other types of amide bond cleavages (c/z-type) and usually more extensive fragmentation because of the more randomized nature of the cleavage process.[13,14] Recently introduced software for data analysis now allows a more accurate simulation of MS/MS spectra for database searches[15–17] or relies on previously acquired spectra for comparison in spectrum libraries;[18,19] *de novo* sequencing algorithms have become more powerful as well.[20–22] However, a majority of peptide sequencing experiments are being carried out using CID-type fragmentation and the data are most frequently analyzed by common search engines like MASCOT[23] or SEQUEST.[24] Datasets from

*Correspondence to: Alexander Leitner, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringer Strasse 38, 1090 Vienna, Austria.
E-mail: alexander.leitner@univie.ac.at

large-scale studies are being analyzed with varying degrees of success, with several groups reporting that only 20 to 30% of all acquired MS/MS spectra actually yield useful information. This may also be because of the presence of a large number of unexpected modifications in complex samples of biological origin, as recently demonstrated by Zubarev's group.[25,26]

The concept of the 'mobile proton' introduced by Wysocki and coworkers[8,27,28] represents a commonly accepted framework to explain peptide fragmentation pathways. Proton mobility is dependent both on the charge state of a peptide and the nature of the basic groups in the molecule that stabilize the positive charges. With a pK of ~12.5, the guanidine group of arginine is in most cases the most basic functional group in peptides, possessing the highest proton affinity.[29] As a result, optimal peptide fragmentation in arginine-containing peptides typically requires a charge state higher than the number of arginine residues present. Even if this criterion is fulfilled, selected cleavages, e.g. *N*-terminal to proline, or charge-remote fragmentation may still be favored,[8] resulting in relatively uninformative MS/MS spectra with a few dominating fragment ions. In addition, energetically labile side-chain modifications are well known to reduce the information content of MS/MS spectra because of dominant neutral loss as the main fragmentation pathway. This phenomenon complicates the CID-MS/MS analysis of certain phosphorylated peptides, particularly those containing phosphoserine and phosphothreonine residues,[30,31] as well as sulfated peptides,[32] but has also been described for peptides containing methionine sulfoxide as an artificial/incidental modification.[33]

Recently, we have demonstrated that the chemical modification of arginine residues, based on a classical reaction with malondialdehyde (MDA),[34] is able to influence fragmentation of arginine-containing peptides favorably.[35] Conversion of the guanidine group into a pyrimidinyl moiety (Scheme 1) results in a significant decrease in basicity of the guanidine side chain. Ideally, the concomitant reduction in proton affinity of the arginine residue causes increased mobility of the proton, opening up additional fragmentation pathways. We have previously presented proof-of-principle results of LC-MS/MS analyses of model peptides and protein digests on an ion trap instrument.[35] In order to obtain a more comprehensive data set, we have now carried out an extensive energy-resolved MS/MS study on a triple-quadrupole instrument, which allowed us to examine the effects of MDA modification on poorly fragmenting peptides in more detail. Phosphopeptides were also included in the data set to study the influence on side-chain neutral loss, and significant findings were corroborated on different instrumental platforms (ion trap, quadrupole-linear ion trap hybrid).



Scheme 1. Reaction of arginine residues with malondialdehyde.

EXPERIMENTAL

Materials

All unphosphorylated model peptides were obtained from Bachem (Weil am Rhein, Germany) or Sigma (Deisenhofen, Germany) and used without further purification. The phosphopeptides NpSVEQGRRL (**pS**), SVENLPEAGIpTHEQR (**pT**) and LIEDNEpYTAR (**pY**) were synthesized using standard Fmoc chemistry and Fmoc-Xxx(PO(OBzl)OH)-OH phosphoamino acids. The peptide NpSVEQGR (**pS′**) was obtained by tryptic digestion of **pS** as described below.

Tryptic digestion of pS

To obtain the phosphopeptide **pS′**, 500 µg of the peptide were dissolved in 500 µl of a 50 mM aqueous ammonium bicarbonate solution containing 10 µg trypsin (proteomics grade, Sigma). The solution was kept at 37 °C for 4 h. After this period, progress of the reaction was monitored by ESI-MS in infusion mode and no signal corresponding to the intact peptide was observed. Trypsin was then removed by ultrafiltration using Microcon YM-3 devices (3 kDa cutoff; Millipore, Bedford, MA, USA) and the filtrate (containing the peptides NpSVEQGR and RL) was directly used for MDA labeling.

Derivatization procedure

MDA-labeled peptides were obtained as described previously[35] with minor modifications. Typically, 100 µg of the peptide was dissolved in 100 µl concentrated hydrochloric acid (Merck, Darmstadt, Germany) and 2.4 µl of tetraethoxypropane (MDA-tetraethyl acetal, Sigma) was added. After 1 h at room temperature, samples were diluted 100-fold with water and desalted by solid-phase extraction (SPE) using C_{18} cartridges as described previously. Because the SPE eluent was directly used for (tandem) mass spectrometric analysis, formic acid was preferably used as the additive for SPE elution instead of TFA to avoid signal suppression effects.

Tandem mass spectrometry

Tandem MS experiments were performed on three different instruments: A PESciex API 365 triple-quadrupole (QqQ, Applied Biosystems/MDS Sciex, Concord, Canada), an Agilent 1100 MSD Trap SL quadrupole ion trap (IT, Agilent Technologies, Waldbronn, Germany) and an Applied Biosystems 4000 Q TRAP quadrupole-linear ion trap (QqLIT) hybrid instrument. All mass spectrometers were equipped with standard, pneumatically assisted electrospray sources.

Sample solutions were infused with syringe pumps at 5 µl min^{-1} (QqQ, IT) or 10 µl min^{-1} (QqLIT). Generally, unmodified peptides were dissolved at 100 µg ml^{-1} in water/acetonitrile/formic acid (50/50/0.1, v/v/v). For MDA-labeled peptides, the eluent from the SPE clean-up step (concentration approximately 100 µg ml^{-1}) was directly used for MS/MS analysis. For control experiments on the more sensitive instruments, samples were diluted accordingly (typically 1 : 10 for the IT system and 1 : 100 for the QqLIT system) with the same solvents.

For QqQ-MS/MS, precursor ion signal intensity was first optimized for each charge state, and peptides were subjected

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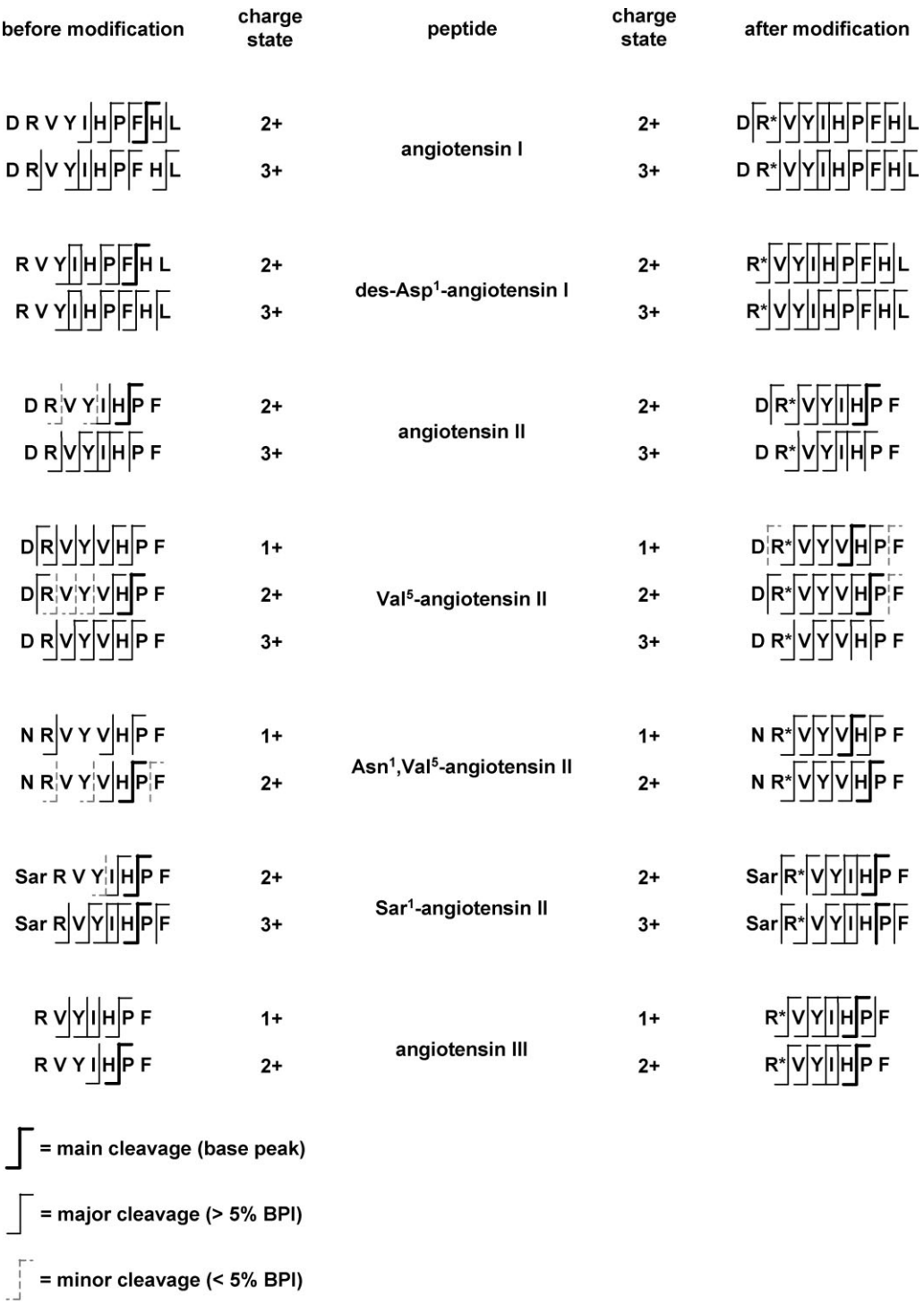

Figure 1. Fragmentation pathways of model angiotensins before (left column) and after (right column) MDA modification. Cleavage events are grouped into main cleavages (either a b- or a y-ion is the base peak), major cleavages (either b- or y-ion >5% BPI) and minor cleavages (b- or y-ion 1–5% BPI). If no main cleavage is labeled, the base peak is the neutral loss from a b- or y-ion or an immonium ion. Modified arginine residues are marked with an asterisk (*). BPI, base peak intensity, Sar = sarcosine.

to CID using nitrogen as the collision gas. Collision energies (given as laboratory frame energies throughout the text) were ramped in six to ten steps from the onset of fragmentation (or the lowest value for which a stable ion current could be obtained) until the precursor ion intensity was reduced >95%. This corresponded to 2 to 5 V intervals (2 min each) for collision energy. All experiments were carried out in triplicate.

Control experiments on IT and QqLIT instruments were carried out on unmodified and modified forms of angiotensin III and the phosphopeptide **pS** under similar conditions as described above.

Data analysis

MS/MS spectra were manually evaluated using the respective instrument software and the tool massXpert (Version

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1.0,[36]) to generate lists of potential fragment ions. Unless mentioned otherwise, only the following ion types were considered for quantitative evaluations: b, y, neutral loss of H_3PO_4 from phosphopeptides; all possible charge states of fragment ions were evaluated.

For qualitative and quantitative evaluation, only signals with an abundance of >1% of the base peak in the respective spectrum were considered. The figures in the 'Results' section list only the fragment ions that could be assigned on the basis of their unique m/z ratios and that were observed with moderate collision energies (<90% attenuation of the precursor ion signal).

RESULTS

Tandem mass spectrometry of angiotensins before and after MDA modification

Angiotensins are a group of neuropeptides that are frequently used for fundamental mass spectrometric studies (see, for example, Refs 37–39). A core motif common to all derivatives with the exception of Ile → Val substituted peptides is the partial sequence Arg-Val-Tyr-Ile-His-Pro-Phe. Overall, we investigated the fragmentation behavior of seven angiotensins (Fig. 1). For every model peptide, at least two different charge states were examined (three for Val^5-angiotensin II). Figure 1 lists all observed bond cleavages for the unmodified peptides and those treated with MDA, and cleavages are grouped into main, major and minor cleavages according to the relative abundances of fragment ions. The spectra were manually interpreted as described in the 'Experimental' section, and only fragment ions that could be unambiguously assigned on the basis of their m/z ratio were included.

As can be seen from Fig. 1, cleavage of the His–Pro bond is energetically most favorable in these peptides so that fragment ions resulting from this cleavage are frequently the most abundant ones, particularly for the 2+ charge state. Predominant cleavage at His–Pro bonds is a well-known phenomenon. Recently, it was found to represent one of the most abundant motifs for cleavage in CID of tryptic peptides in studies by Huang *et al*.[28,40] To illustrate the effect of arginine modification on fragmentation in a more quantitative manner, angiotensin III is chosen as a representative example. The MS/MS spectrum of unmodified, doubly charged angiotensin III (Fig. 2(a)) is dominated by the b_5/y_2 pair, while only one additional cleavage (Ile–His) is observed with significant abundance, even at high collision energies. Clearly, spectral quality is insufficient for *de novo* sequencing in this case, as only three fragment ions (not counting the two a-ions related to the b_4/b_5 ions) are present. Similarly, although in different extents, His–Pro bond cleavage always leads to the most prominent signals in the MS/MS spectra of doubly charged angiotensins. For triply charged ions, this was not the case; the additional mobile proton results in less bias towards preferential cleavages.

After modification with MDA, there is a clear trend towards the appearance of additional fragment ions in the spectra. In every case, more fragment ions were observed

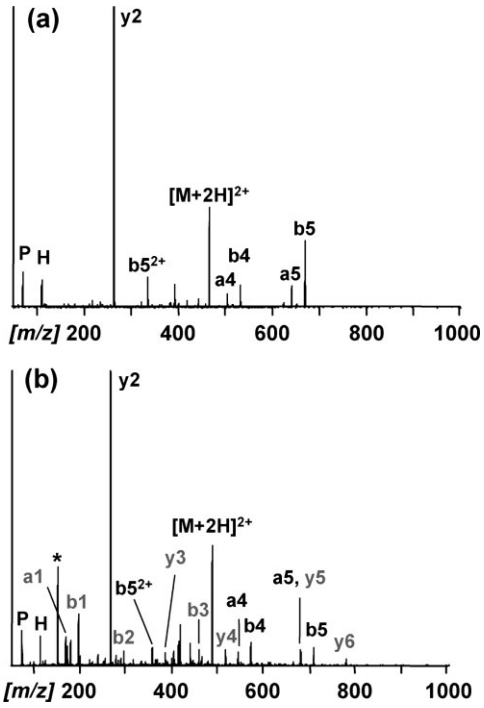

Figure 2. QqQ-MS/MS spectra of doubly charged angiotensin III (RVYIHPF) before (a) and after (b) MDA modification. Additionally observed fragment ions in (b) are highlighted in gray. Immonium ions of proline and histidine are abbreviated as P and H, respectively. a_5 and y_5 ions are only separated by 1 Da in mass, but clearly resolved. The origin of the signal marked with an asterisk is discussed later in the text.

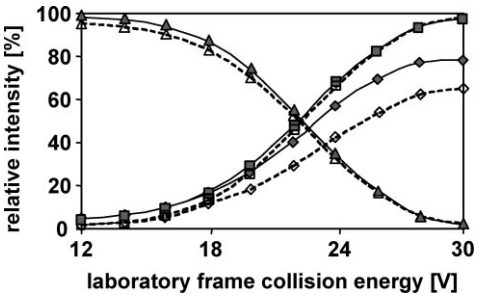

Figure 3. Energy-resolved tandem mass spectrometry of doubly charged angiotensin III (RVYIHPF) before and after MDA modification. Relative abundances of precursor ions (triangles), total b/y ions (squares) and the b_5/y_2 pair (diamonds; main fragmentation pathway resulting from the His–Pro bond cleavage) are compared for the peptide before (solid lines) and after (broken lines) MDA modification.

for doubly charged ions carrying a modified arginine residue. The most dramatic improvement was observed for angiotensin III, in which the number of fragment ions increased from three to ten (Fig. 2(b)). Effects were less clear for the other charge states (1+, 3+). A very interesting observation was that b_1 ions were observed for peptides carrying a modified arginine at their *N*-terminus (angiotensin III and des-Asp^1-angiotensin II), whereas they were absent in the unmodified counterparts and all other peptides regardless of whether they were modified or not. This peculiarity is discussed in more detail below.

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Table 1. Fragment ion abundances for the spectra of angiotensin III shown in Fig. 2. Shown are the relative intensities of the major ions that were assigned (in % relative to the base peak) and relative contribution of different ion types to the total ion current (in % of total signal intensity). Only ions with abundance of ≥1% were considered. BPI, base peak intensity

Ion	Rel. int. (% BPI) − MDA	Rel. int. (% BPI) + MDA	Ion series	Total rel. int. (%), −MDA	Total rel. int. (%), +MDA
Remaining precursor	33.1	40.6	Precursor	14.0	10.4
y_2	100.0	100.0	a-ions	5.5	4.8
y_3	<1% BPI	4.7	b-ions	16.6	11.9
y_4	<1% BPI	5.1	y-ions	42.4	30.1
y_5	<1% BPI	5.3	Neutral loss from b/y and precursor	1.1	16.3
y_6	<1% BPI	2.2	Immonium ions	8.8	5.9
b_1	<1% BPI	17.1	Unassigned	11.7	20.5
b_2	<1% BPI	4.6	(of which ≥2% BPI)	(6.3)	(12.7)
b_3	<1% BPI	5.4	–	–	–
b_4	7.3	7.4	–	–	–
b_5	22.1	6.2	–	–	–
b_5, 2+	9.8	5.9	–	–	–

In addition to the quantitative improvements in terms of fragment ions or bond cleavages, we noticed a significant reduction of the preferred His–Pro cleavage after modification. Energy-resolved tandem MS experiments allowed us to compare the relative contribution of the b_2/y_5 pathway *vs* other backbone cleavages. As can be seen from the graphs in Fig. 3 – again using angiotensin III as the example – cleavages other than those *N*-terminal to the proline residue contribute significantly more signal abundance to the MS/MS spectrum, as is also reflected in the increased number of fragment ions observed. As an example, at a collision energy of 22 V (corresponding to about 50% reduction of the precursor ion abundance), backbone ions other than b_2 and y_5 contribute about 7% of the total ion current in the spectrum of the unmodified peptide. After modification, this contribution rises to almost 17%. At 26 V collision energy, the respective values are 14 and 29%; thus, the modification roughly leads to a doubling of the abundances of additional fragment ions. Similar improvements were obtained for other peptides (data not shown). A detailed list of the relative ion abundances for the spectra depicted in Fig. 2 is given in Table 1.

To further support our findings, we attempted to reproduce the results on two different instrumental platforms, a QqLIT instrument and a 'classic' 3D IT. Apart from system-specific effects like the low-mass cutoff observed on the IT instrument, the appearance of the spectra was very comparable. Summarizing our results on angiotensins, improvements in the fragmentation pattern were observed in all cases and for most charge states, resulting in both larger numbers of fragment ions observed and increased abundances.

Tandem mass spectrometry of arginine-containing phosphopeptides before and after MDA modification

As noted in the introduction, peptides with phosphorylated serine or threonine residues frequently show a dominant neutral loss of 98 Da upon CID, corresponding to the cleavage of H_3PO_4 from the phosphorylated side chain. Such a neutral loss prevents efficient sequencing of phosphopeptides, as signals of backbone ions are often of low abundance in the MS/MS spectra and therefore little sequence information may be extracted. Alternative fragmentation techniques such as ECD and ETD are increasingly used; however, these technologies require multiply (ideally triply) charged peptide ions, which are not formed for every peptide. As of now, a large majority of MS/MS-capable instruments still use CID and we reasoned that MDA modification of arginine-containing phosphopeptides should lead to improved fragmentation, as was observed for angiotensins.

Four phosphorylated model peptides (Fig. 4), carrying arginine and either phosphorylated serine, threonine or tyrosine residues, were selected and subjected to fragmentation prior to and after modification with MDA. The observed cleavages in unmodified phosphopeptides (Fig. 4) show significant differences. The two phosphoserine-containing peptides **pS** (NpSVEQGRRL) and **pS′** (NpSVEQGR, obtained from tryptic digestion of **pS**) exhibited neutral loss of H_3PO_4 as the dominant fragmentation pathway for singly and doubly charged unmodified precursor ions over the whole range of activation energies examined. For moderate collision energies, backbone ions were only present in very low abundance for **pS′** and almost completely absent in the case of **pS** as shown in Fig. 5(a). Singly charged ions gave similar results. **pS**, but not **pS′**, was also observed as a triply charged ion, for which the neutral loss of phosphoric acid was still a prominent fragmentation pathway, although not as dominant as in the lower charge states. Despite the fact that an additional proton is available for the 3+ ion, the number of bond cleavages observed was similar to those of the 2+ ion (the presence of the y_6^{2+} ion could not be confirmed in the spectrum of unmodified **pS** owing to overlapping with the precursor ion).

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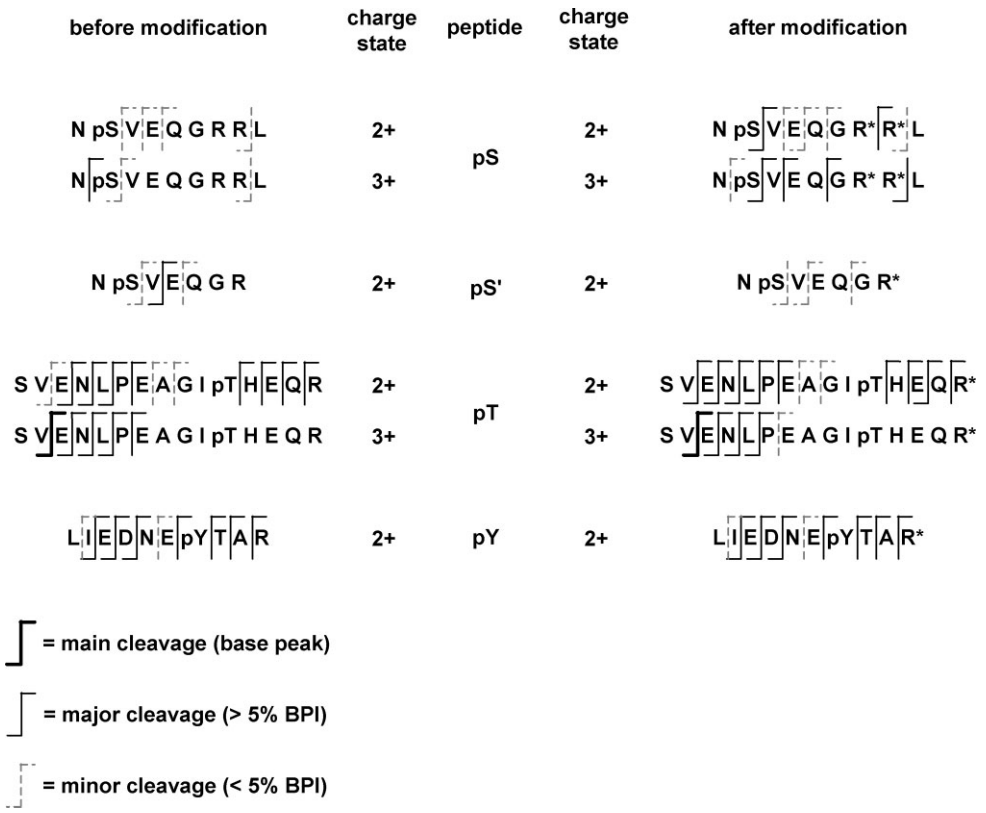

Figure 4. Fragmentation pathways of model phosphopeptides before (left column) and after (right column) MDA modification. Cleavage events are grouped into main cleavages (either a b- or a y-ion is base peak), major cleavages (either b- or y-ion >5% BPI) and minor cleavages (b- or y-ion 1–5% BPI). If no main cleavage is labeled, the base peak is the remaining precursor or the result of neutral loss of phosphoric acid or water. Modified arginine residues are marked with an asterisk (*). BPI, base peak intensity.

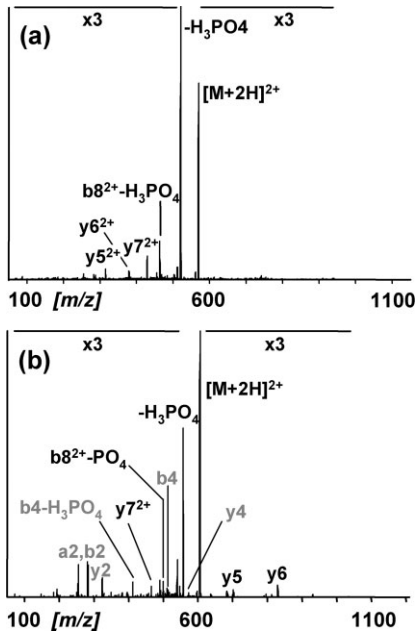

Figure 5. QqQ-MS/MS spectra of the doubly charged phosphopeptide **pS** (NpSVEQGRRL) before (a) and after (b) MDA modification. Additionally observed fragment ions in (b) are highlighted in gray.

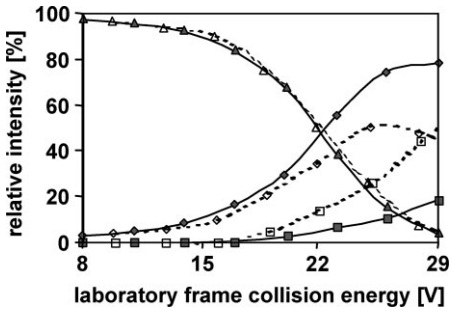

Figure 6. Energy-resolved tandem mass spectrometry of the doubly charged phosphopeptide **pS** (NpSVEQGRRL) before and after MDA modification. Relative abundances of precursor ions (triangles), total b/y ions (squares) and neutral loss of phosphoric acid (diamonds) are compared for the peptide before (solid lines) and after (broken lines) MDA modification.

After MDA modification, backbone fragmentation improved both qualitatively and quantitatively. The relative intensity of backbone fragment ions relative to cleavage of H_3PO_4 increased as shown in Fig. 5(b), while fragment ions corresponding to cleavages of six of the eight amide bonds were observed for the doubly charged peptide **pS** carrying two tagged arginine residues (Fig. 4). An improved fragmentation was also observed for the 3+ ion of **pS**, overall supporting the results obtained from the unphosphorylated peptides shown above. Energy-resolved MS/MS experiments gave a more detailed, quantitative picture of the effects of modification: For the peptide **pS** in the 2+ charge state, the proportion of backbone ions was continuously higher by at least a factor of 3 or more from

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Table 2. Fragment ion abundances for the spectra of **pS** shown in Fig. 5. Shown are the relative intensities of the major ions that were assigned (in % relative to the base peak) and relative contribution of different ion types to the total ion current (in % of total signal intensity). Only ions with abundance of ≥1% were considered. BPI, base peak intensity

Ion	Rel. int. (% BPI) − MDA	Rel. int. (% BPI) + MDA	Ion series	Total rel. int. (%), −MDA	Total rel. int. (%), +MDA
Remaining precursor	69.1	48.2	Precursor	36.9	14.0
Precursor − H_3PO_4	100.0	100.0	a-ions	0.0	3.5
b_2	<1% BPI	14.3	b-ions	0.0	4.8
b_4	<1% BPI	2.2	y-ions	3.0	5.4
b_4 − H_3PO_4	<1% BPI	3.7	Neutral loss (−H_3PO_4)	55.9	30.1
b_8 − H_3PO_4, 2+	4.8	9.0	Neutral loss (other)	4.2	3.6
y_2	<1% BPI	8.1	Immonium ions	0.0	1.5
y_4	<1% BPI	2.2	Unassigned	0.0	36.9
y_5	<1% BPI	1.3	(of which ≥2% BPI)	(0.0)	(25.1)
y_5, 2+	1.6	<1% BPI	–	–	–
y_6	<1% BPI	2.4	–	–	–
y_6, 2+	1.1	<1% BPI	–	–	–
y_7, 2+	3.0	4.4	–	–	–

the onset of fragmentation up to the highest values examined, as can be seen in Fig. 6. Detailed data on ion abundances for the spectra from Fig. 5 are shown in Table 2. The significant contribution of unassigned signals to the total ion current of the MDA-treated sample is mostly a consequence of the reduced dominance of the neutral loss of phosphoric acid after modification. Thus, a more 'typical' fragment ion spectrum is observed, with a large number of low-intensity signals. Only a few unassigned signals in the spectrum reach more than 3% of the base peak.

To determine whether the effects were specific to one instrumental platform, one peptide, **pS**, was additionally examined on an IT and an QqLIT instrument. Results were comparable, although the extent of backbone fragmentation relative to neutral loss of H_3PO_4 varied to some degree, as could be expected from differences in the activation process. Slow heating in the ion trap is known to be a more 'gentle' activation process compared to CID in a quadrupole collision cell,[41–43] so that low-energy fragmentation processes tend to be more dominant.

Significant improvements in the quality of MS/MS spectra after MDA tagging were observed in all cases. In addition to MS^2 experiments, MS^3 was carried out on doubly charged **pS** ions in the IT instrument: The product ions corresponding to loss of H_3PO_4 were subjected to further fragmentation. The effect of MDA modification was even more pronounced in this case: Again, six bond cleavages were observed for the modified peptide, while only two cleavages were observed for unmodified **pS** (Fig. 7). No useful MS^3 data could be obtained for **pS′**.

In contrast to the two peptides carrying phosphoserine residues, neutral loss of phosphoric acid was less dominant for the phosphothreonine-containing peptide **pT** (SVENLPEAGIpTHEQR), although the signal of the dephosphorylated peptide was still one of the major signals in the MS/MS spectrum of the 2+ ion. For the 3+ ion, however, neutral loss was almost absent, in accordance with the lower activation energy required for initiating backbone cleavage in this case. Upon modification of the MDA group, the number of backbone cleavages observed was identical for both charge states (2+ and 3+, see Fig. 4), but the loss of H_3PO_4 was again less pronounced (data not shown). Similar fragmentation patterns were obtained in MS^3 experiments of the unlabeled and labeled peptide following neutral loss of phosphoric acid.

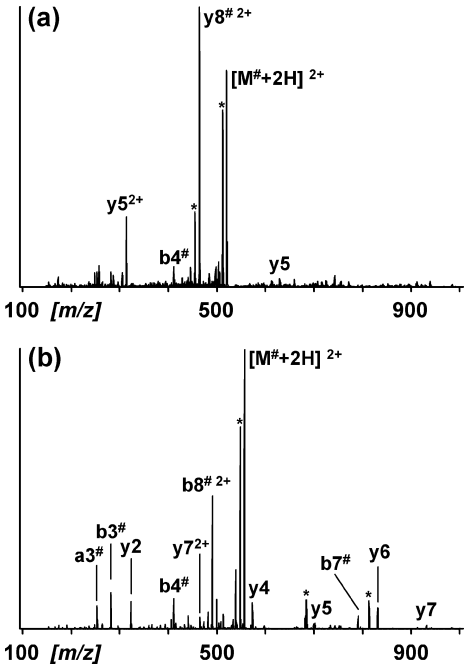

Figure 7. IT-MS^3 spectra of the fragment ion resulting from neutral loss of the phosphate group of the peptide **pS** (NpSVEQGRRL) before (a) and after (b) MDA modification. A hash (#) denotes the parent and fragment ions containing a dephosphorylated serine residue; neutral losses of H_2O or NH_3 are marked with asterisks.

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The last phosphopeptide we examined, **pY** (LIEDNEpY-TAR), reflected the typical higher stability of phosphate groups on tyrosine residues rather than on serines or threonines, as no neutral loss was observed in MS/MS experiments. There was neither a positive nor a negative effect of MDA tagging, as complete y-ion series were obtained for both the unmodified and modified peptide (Fig. 4) in the 2+ charge state. Relative intensities of fragment ions were almost identical, ensuring that there was no negative effect of MDA modification for peptides that already yielded good MS/MS spectra. Summing up, the results from the phosphopeptide experiments were in line with those obtained from unphosphorylated peptides.

DISCUSSION

On the basis of the results of the detailed tandem MS study summarized above, we are able to gain more insight into the effects of MDA modification than from our previous, rather preliminary data.[35] In general, the most significant effects were observed for doubly charged peptides, whereas they were less pronounced for singly and triply charged ions. Obviously, proton mobility is an important factor, as it is strongly affected by the replacement of the strongly basic guanidine group by the less basic pyrimidine group in MDA-treated peptides. Previously, Burlet and Gaskell had determined a reduced proton affinity of dimethylpyrimidylornithine compared to arginine (resulting from the reaction of arginine residues with 2,4-pentanedione),[44] and similar results are expected for the MDA modification.

A general trend that emerged from our experiments is the reduced dominance of fragmentation pathways with low activation energies, i.e. cleavages on the *N*-terminal side of proline residues in angiotensins and the neutral loss of phosphate in phosphoserine-containing peptides. This can be explained by a higher degree of proton mobilization in modified peptides, thereby lowering the energetic barriers for other bond cleavages. In addition to a more homogeneous distribution of fragment ion intensities, this frequently resulted in a larger number of backbone cleavages (Fig. 1). For angiotensins, the number of backbone cleavages for all peptides and charge states increased from 71 in the unmodified peptides to 96 after MDA modification, roughly an increase of 35%. Furthermore, the number of fragment ions increased by almost 60%, from 103 to 162. This reflects the increased number of complementary fragment ions from b/y ion pairs; upon fragmentation, it is less likely for MDA-modified peptides that the charge(s) are exclusively retained on the fragment ion carrying the arginine residue. Although only a limited number of phosphorylated peptides were evaluated in this study, it is clear that MDA modification also facilitates backbone cleavages in the presence of dominant fragmentation pathways for this set of analytes. In this case, the relative contribution of neutral loss of the phosphate group from phosphoserine or threonine residues was reduced in favor of b- and y-ions appearing in larger number and/or with higher abundance. Figures 5, 6 and 7 highlight the improvements in fragmentation behavior that are obtainable with the arginine modification step. Thus, MDA modification of phosphopeptides carrying both Arg and pSer or pThr residues is considered of particular interest. As mentioned above, ECD or ETD as alternative fragmentation techniques work most efficiently with triply charged precursor ions. However, the negatively charged phosphate group reduces the likelihood that high charge states are observed. In our case, **pT** was the only peptide where the 3+ charge state was of comparable intensity to the 2+ charge state. For **pS**, the triply charged ion gave a threefold lower signal than the doubly charged ion, both prior to and after MDA modification. For the other two peptides, no triply charged ions were observed in sufficient abundance. A question that needs to be investigated in further studies is whether peptides with multiple arginine residues benefit the most from the modification.

General classifications of proton mobility have been introduced by Kapp *et al*.[45] and Ulintz *et al*.[46] The latter group proposed a mobile proton factor (MPF) reflecting the normalized basicities of the residues arginine (guanidine group), lysine (primary amino group) and histidine (imidazole group). The MPF is calculated as follows: $MPF = (1.0 \times R + 0.8 \times K + 0.5 \times H)/z$, where R, K and H are the number of Arg, Lys and His residues, respectively, and z is the charge. An interesting observation is that, although only a limited amount of data is available, the most significant increase in the number of fragment ions of the angiotensins was observed for MPFs in the range from 0.67 to 1.00 (an *MPF* of 1 or above classifies the protons as nonmobile[46]). Figure 8 plots the number of observed fragment ions against the calculated MPFs and supports our assumption that proton mobility is enhanced by MDA modification. For peptides with the highest proton mobility prior to modification ($MPF = 0.5$), the number of fragment ions is roughly constant, as would be expected.

As already noted in the 'Results' section, signals corresponding to the m/z ratio of the b_1 ion were observed for the

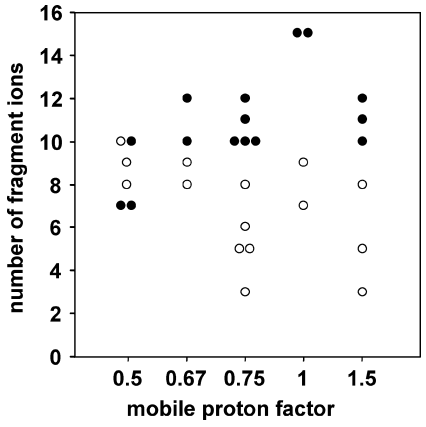

Figure 8. Increase in the number of fragment ions after MDA modification as a function of proton mobility. The number of fragment ions for all charge states of the angiotensin data set (Fig. 1) is plotted against the mobile proton factor (MPF, calculated according to Ref. 46) of the corresponding peptides. In the dot plot, unmodified peptides are shown as empty circles, and modified ones as filled circles.

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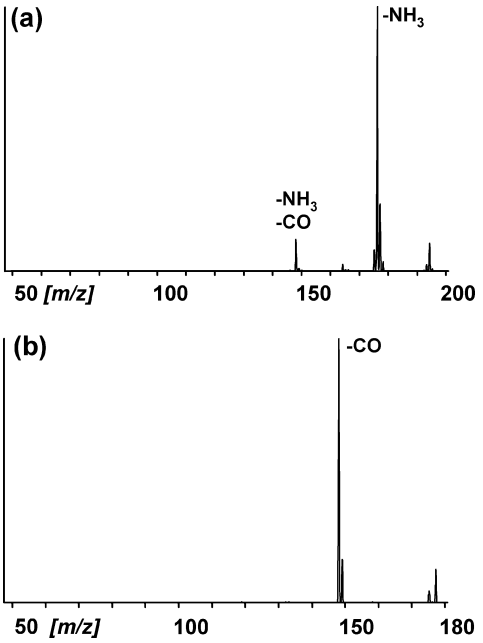

Figure 9. Multiple-stage MS experiments on the b_1 ion of MDA-modified angiotensin III (R*VYIHPF). (a) MS^3 of the fragment ion at 193.1 Da from MS^2, (b) MS^4 of the fragment ion at 176.0 Da from MS^3.

peptides with MDA-modified arginines at their *N*-terminus. Typically, the formation of b_1 ions is not favored, as dissociation of the *N*-terminal amide bond usually leads to the formation of the a_1 ion together with the corresponding y-ion. (For a detailed discussion on the proposed mechanisms, see Ref. 8 and references therein.) As a result, there has been little evidence for the formation of b_1 ions from underivatized peptides in the literature. In contrast, strongly enhanced b_1 ion formation has been observed for peptides with a modified *N*-terminus such as the phenylthiocarbamoylated peptides studied by Gaskell and coworkers.[47,48]

In our case, the modified arginine side chain may serve a similar function to promote b_1 ion formation instead of the *N*-terminal amino group carrying the modification. This is consistent with the fact that the fragment was observed only after MDA modification. In order to validate the assignment, we performed multiple-stage mass spectrometry on an ion trap instrument (Fig. 9). The signal at 193.1 Da was isolated from the product ions of MDA-modified, doubly charged angiotensin III and subjected to further fragmentation. Consecutive losses of NH_3 and CO were observed in MS^3 and MS^4 experiments, respectively. Further fragmentation of the MS^4 product ion at m/z 148.0 was not possible. This is in accordance with our observation that the modified arginine side chain carrying the aminopyrimidine group is very stable during CID, so that even at high collision energies no neutral loss is observed. The same signal at m/z 148 also appears in the low-mass region of some MS/MS spectra of peptides carrying an *N*-terminal-modified arginine (compare Fig. 3). Peptides with internal modified arginines yield this fragment to a much lesser degree and only at higher collision energies; in this case the ion appears to be a result of a further dissociation of the respective immonium ion.

CONCLUSIONS

We have demonstrated that the MDA modification of the guanidine group of arginine residues in peptides has beneficial effects if peptides show unfavorable fragmentation behavior, as exemplified by preferential backbone cleavages or dominant neutral loss. Results were obtained on different instrumental platforms, which allowed the generalization of the effects for low-energy CID regimes. Future studies in our group will address the possible benefits of the labeling technique for samples of biological origin.

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