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C-terminal peptide sequencing using acetylated peptides with MS^n in a quadrupole ion trap

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MS/MS has been used to sequence peptides and small proteins for a number of years. This method allows one to isolate the peptide of interest, which makes it possible to analyze impure samples and unseparated mixtures, such as protein digests. Collision-induced dissociation (CID) of the selected peptide ion generates the product ions that provide sequence information. However, often the MS/MS spectrum does not provide adequate information for complete sequence determination. The quadrupole ion trap has the capability to do multiple stages of mass spectrometry, MS^n, which can increase the information available to determine the peptide sequence. A regular and predictable dissociation pattern for peptides further simplifies this analysis. By forming predominantly one type of ion, ambiguity is removed as to whether the ion is N- or C-terminal. This pattern can also be advantageous in that ion intensity remains concentrated for the next stage of MS/MS. In this work, a method to take advantage of the MS^n capabilities of the quadrupole ion trap by controlling the dissociation pathways is explored. Dissociation is altered by acetylating the N-terminus of the peptide. MS^n of a variety of acetylated peptides is used to determine the effects of the identity of the C-terminal residue and the length of the peptide on the dissociation pathways observed.

Introduction

The primary sequence of a protein, the order of amino acids, has long been known to determine the three-dimensional structure of that protein.[1] This sequence can also serve to identify a protein from a cell extract, which assists researchers in discovering that protein's function.[2–4] The growing field of proteomics requires fast, reliable methods to determine the primary sequence from small amounts of precious sample.[5–8] Edman degradation, the most common sequencing method, requires at least 1 pmol of protein and takes approximately 30 min per residue, in addition to stringent purification protocols.[9] Recent advances in sequencing with mass spectrometry allow significantly reduced sample consumption and analysis time, requiring femtomoles to attomoles of sample and times as short as a few minutes for analysis.[10–16]

Tandem mass spectrometry (MS/MS) provides sequence information *via* dissociation of a mass-selected peptide ion. The selected peptide ions are typically activated and induced to dissociate by collisions with a collision gas introduced into the instrument.[17–19] Fortunately, certain bonds along the peptide backbone are particularly susceptible to dissociation when the peptide ion is activated. A nomenclature with two main categories describes the product ions formed: a_n, b_n, and c_n contain the N-terminus, while x_n, y_n, and z_n contain the C-terminus, with n referring to the number of residues in the product ion.[20,21] The mass differences between the parent ion and the resulting product ions of a given product ion series (*e.g.* b ions) correspond to the masses of the residues that dissociate from the peptide, allowing the sequence of the peptide to be determined.

Unfortunately, sequence information is often incomplete, and internal fragmentation, along with the formation of both N- and C-terminal product ions, can make interpretation challenging. The degree of dissociation and types of products that are formed vary depending on factors such as the collision energy and the time frame for analysis of the product ions. Dissociation in ion trapping instruments involves lower energy collisions, which limit the dissociation to only low energy pathways. However, ions with low activation energies, which do not have sufficient time to dissociate in a beam instrument, do undergo dissociation in an ion trap due to the longer time frame for analysis. Thus, fewer types of product ions are seen, but a greater fraction of the parent ion is converted to the observed product ions. The MS^n capabilities of the ion trap can provide additional sequence information by allowing further steps of dissociation, which produces genealogical information that helps to identify the product ions with more certainty. MS^n can be a very useful tool for sequencing, but a predictable dissociation pattern would simplify analysis further. Additionally, care has to be taken to ensure that rearrangements which might lead to erroneous interpretation are minimized or at least recognized.[22]

Historically, protonated peptides have been used in mass spectrometry almost exclusively. However, it has been shown that sodium-cationized peptides dissociate in a quadrupole ion trap in a very simple and predictable pattern.[23] Sodium-cationized peptides tend to favor the formation of the $[b_{n-1} + Na + OH]^+$ product ion upon collision-induced dissociation.[24–29] This ion is usually the dominant product ion with low abundances of other product ions formed. Thus, the C-terminal residue can be unambiguously identified (with the exception of differentiating leucine and isoleucine). Because there are few other product ions, most of the intensity of the $[M + Na]^+$ peak is transferred to the $[b_{n-1} + Na + OH]^+$ peak. With MS/MS efficiencies of 60–100% and conversion efficiencies of 50–100%, multiple stages of MS/MS are usually possible.[30] Because the initial parent ion $[M + Na]^+$ can also be considered as $[b_n + Na + OH]^+$, the MS/MS product $[b_{n-1} + Na + OH]^+$ is equivalent to a peptide ion one residue shorter than the initial parent ion.[31] Thus, the $[b_{n-1} + Na + OH]^+$ also follows the same dissociation pattern, and the product $[b_{n-2} + Na + OH]^+$ can be expected (and is seen) from MS^3.[27,31] In this way, sequencing from the C-terminus can be accomplished in a regular, stepwise fashion. Singly charged, sodium-cationized peptides up to ten residues in length have been generated for this method of sequencing using electrospray ionization. Longer peptides typically form multiply charged ions when using

electrospray, and these types of ions have yet to be fully characterized to determine if the same dissociation pathways will be observed.

This type of sequencing can be useful for identifying proteins isolated from cell extracts. The protein can be digested with a proteolytic enzyme, such as trypsin. A few stages of MS/MS can easily reveal the identity of a few C-terminal residues of some of the peptides. Searches of protein or DNA databases with this information then identify the protein. Many proteins can be identified simply by searching with the masses of 4–5 of the peptides from a trypsin digest. By adding MS^n sequencing of two or more of the residues, proteins can be identified more confidently from larger databases using lower mass accuracy.[32]

Unfortunately, the C-terminal dissociation pattern is not universal and depends upon the identity of the C-terminal residue.[23] The residues can be divided into two main categories based on their dissociation patterns (Table 1). Category I residues dissociate to form predominantly the $[b_{n-1} + Na + OH]^+$ ion, as described above. Category II residues do not produce the $[b_{n-1} + Na + OH]^+$ ion as the predominant product ion, rather dissociating to more standard N-terminal ions (*e.g.* the sodiated analog of a_n and b_n) and by side-chain losses. Category Ia residues behave as Category I residues when at the C-terminus of longer peptides (> 3 residues) and as Category II on short peptides ($\leqslant$3 residues). The Category II-type dissociation impedes the stepwise C-terminal sequencing by dispersing the parent ion intensity over a number of product ions. This lowers the intensity of the parent ion for the next stage of MS/MS, which reduces the sensitivity and decreases the number of stages of MS/MS that can be achieved.

Controversy exists regarding the location of the sodium ion interaction with the peptide, so it is difficult to speculate as to how the Category I and II interactions differ. Several previous experimental results indicate that the sodium ion directs the C-terminal dissociation through interactions with the C-terminus.[26,27,31] Other results suggest that the sodium ion may associate with backbone carbonyl oxygens or side chains.[29,33] Computer modeling studies indicate that there may be a variety of sites where sodium ions can coordinate. Calculations by both Leary's and Beauchamp's groups indicate that alkali metal ions can interact with internal carbonyls or the N-terminus more favorably than with the C-terminus. Leary and co-workers found that the metal ion coordinated either to the internal carbonyls or to the N-terminus should be significantly more stable than when coordinated to the C-terminus.[34] Beauchamp and co-workers determined that the metal ion associated with the ion pair of a carboxylate side chain and a protonated backbone amide is a likely intermediate in the dissociation of alkali-cationized peptides.[33] It is interesting that in both cases the peptides modeled would be expected to dissociate as Category II peptides. However, the experimental results from Gross and co-workers, mostly with Category I peptides, indicate coordination to the C-terminus.[31]

It is probable that there are a number of sites where the metal ion can coordinate to a peptide, and different peptide sequences may prefer different sites of interaction. This paper describes an attempt to facilitate Category I-type dissociation by acetylating the N-terminus, to make coordination to the N-terminus less favorable and thereby induce dissociation to the $[b_{n-1} + Na + OH]^+$ ion. This would make dissociation more predictable and allow simple, stepwise C-terminal sequencing using MS^n.

Table 1 Categorization of amino acids by dissociation patterns

Category I	Category Ia	Category II
A, V, L, I, M, P, F, Y, K, H, C, E, R	G, D, W	S, T, N, Q

Experimental

Peptides and proteins were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Solutions of 1 : 3 mole ratio mixtures of 100 μM peptides with NaCl dissolved in methanol–water (80 + 20) were used to generate sodium-cationized peptide ions. Protonated peptide ions were generated from 100 μM solutions in methanol–water–acetic acid (75 + 20 + 5). Cytochrome c from horse heart (1 mg mL^{-1}) was digested with sequencing grade trypsin in a 50 : 1 ratio in a 100 mM ammonium acetate solution at 37 °C for 24 h. Methanol was added to the digest for a final composition of 60 + 40 methanol–water. The concentrations used are well above the detection limits of the instrument to ensure a more than adequate signal while this method was being investigated. A practical application of this method could easily be done with much lower concentrations.

Acetylated peptides were prepared *via* one of three procedures. In the first, 1 mg of peptide was dissolved in 1–2 mL of dichloromethane, and 100 μL of acetic anhydride were added. This mixture was vortexed for 1–2 h and then dried using a rotary evaporator. The second method combined 0.5 mg of peptide with 50 μL each of methanol and acetic anhydride. This reaction was allowed to proceed for approximately 5 min before being diluted to a 100 μM concentration as described above. Peptides from the tryptic digest were in a water solution and were acetylated with acetic anhydride by keeping the pH above 8. This was achieved by mixing 250 μL each of protein digest and saturated ammonium acetate. While keeping this solution at 0 °C, 25 μL of acetic anhydride were added in five portions over 1 h.[35] No evidence was seen of acetylation at sites other than the N-terminus. Methanol was added to bring the composition to 60 + 40 methanol–water. Less than 1 μL of each sample is consumed during analysis.

Experiments were performed on a modified Finnigan-MAT (San Jose, CA, USA) ITMS controlled with ICMS software.[36] An external calibration was used to ensure correct mass assignments. Helium buffer gas was added to approximately 1 mTorr. Collision-induced dissociation (CID) was achieved *via* resonant excitation as described previously.[37] Generation of peptide ions in the gas phase was achieved using a custom-built nanoelectrospray source. This source consumes approximately 6 nL min^{-1}, which allows attomole detection limits.

Modified neglect of differential overlap (MNDO) calculations were performed with the MOPAC package, version 6.0. PCMODEL (Serena Software) was used to generate a starting structure for MOPAC. Sodium ions were initially coordinated at various sites along the peptide, then the calculation optimized the structure to the local energetically minimized stable conformation. The heats of formation and bond lengths were obtained from these calculations.

Results and discussion

Dissociation patterns for both the protonated and sodiated acetylated peptides were investigated. Because the $[b_{n-1} + Na + OH]^+$ ions have the same structure as an $[M + Na]^+$ ion and only the identity of the C-terminal residue is under consideration, each $[b_{n-1} + Na + OH]^+$ ion is treated as a new $[M + Na]^+$ ion. For example, MS/MS of [GGY + Na] is actually MS^3 of [GGYR + Na]. Thus, to avoid confusion as to which residue is being investigated as the C-terminal residue, all the experiments will be referred to as MS/MS. The desired result would be to induce Category I-type dissociation for all residues. Identification of the C-terminal residue would then be unambiguous (except for the isomers leucine and isoleucine), and the product ion intensity would be concentrated in one peak, $[b_{n-1} + Na + OH]^+$, for the next stage of MS/MS. The dissociation of each acetylated peptide was compared with the dissociation of the

corresponding non-acetylated peptide to determine whether the derivatization offers these advantages for sequencing.

Protonated acetylated peptides do not appear to provide any advantages for MS^n over the protonated non-acetylated peptides. The acetylated peptides do exhibit a slightly different distribution of dissociation products than the non-acetylated peptides. However, there is no single type of product that is favored and can be expected for either Category I or II residues (Table 2). As seen in Fig. 1, the ion intensity is distributed among many products in both spectra. Both spectra show that the only backbone cleavage site is the peptide amide bond (b-type ions), but that some of these ions also lose NH_3, H_2O, or a side chain fragment (such as the loss of 42 that is seen when arginine is present).[38,39] In fact, simply MS/MS of either the protonated or sodiated non-acetylated peptides usually offers a more easily interpreted dissociation because the b series is less cluttered and more easily distinguished.

MS/MS of acetylated sodium-cationized peptides did show some advantages over the non-acetylated peptides. Sodium-cationized peptides with Category I residues usually dissociate to the $[b_{n-1} + Na + OH]^+$ ion both when acetylated and when not. In addition, some Category II and Category Ia residues dissociate like Category I residues when the peptide is acetylated. This behavior can be seen with the peptide ALILTLVS in Fig. 2. This peptide has serine, a Category II residue, at the C-terminus. The sodium-cationized peptide dissociates to a large number of products, with $[b_{n-1} + Na + OH]^+$ present in very low abundance. It would be desirable to select this ion for the next stage of MS/MS (MS^3), as $[b_{n-2} + Na + OH]^+$ could then be expected, but the dissociation of a low intensity parent ion would severely limit the intensity in the product ion spectrum.

Upon acetylation of the N-terminus, the dissociation pattern of the peptide, ALILTLVS, changes drastically. Category I-type dissociation is now seen. The C-terminal residue can be easily and confidently identified, and the $[b_{n-1}+Na+OH]^+$ ion is available in good abundance to continue the C-terminal sequencing by MS^n. Other Category II peptides also adopted Category I-type dissociation upon acetylation, for example SSS, PPGFS, and YGG, as shown in Table 3. However, the Category I-type dissociation was not observed for every peptide. The effect of acetylation does not appear to depend solely upon the identity of the C-terminal residue or the length of the peptide, as is seen with the sodium-cationized peptides. There is no readily apparent pattern to predict which peptides will be favorably affected by acetylation.

The acetylation technique was also used with a cytochrome c tryptic digest to evaluate the potential usefulness with protein digest analysis. The mass spectra of the digest before and after acetylation are compared in Fig. 3. The complexity of the spectra is seen to increase greatly following acetylation. The acetylation reaction gives a 60% yield at best.[35] Thus, the digest mixture becomes a mixture of both acetylated and non-acetylated peptides. Upon the addition of salt, the mixture

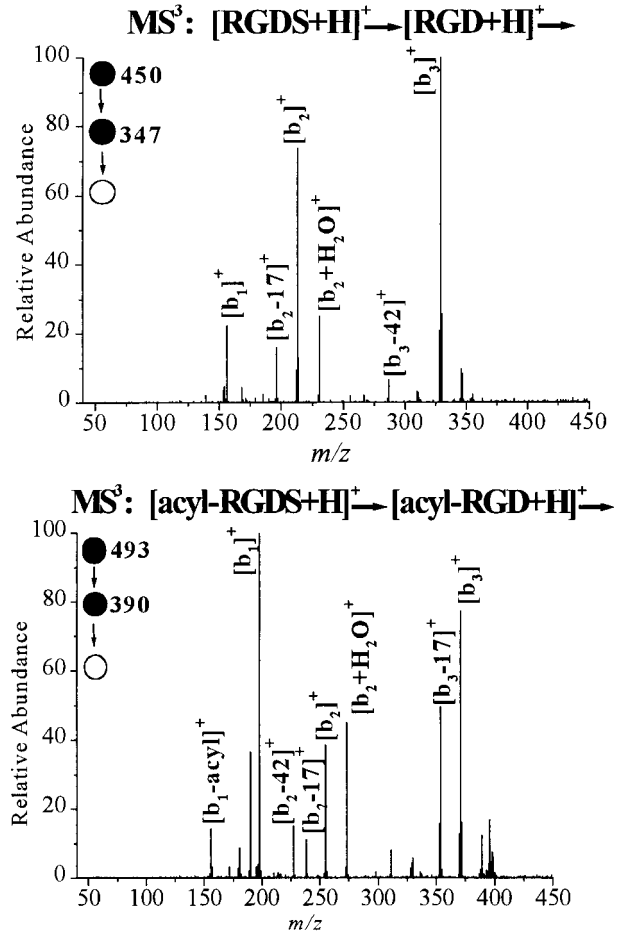

Fig. 1 Comparison of dissociation patterns of acetylated and non-acetylated peptide, RGD. The $[b_n - 42]^+$ ions result from the loss of a portion of the R side chain.

Table 2 Dissociation of protonated peptides[a]

	$[b_n]^+$	$[a_n]^+$	$[b_{n-1} + OH_2]^+$	$[b_{n-1}]^+$	$[a_{n-1}]^+$	$[b_{n-2} + OH_2]^+$	$[b_{n-2}]^+$	Other
GGYR	100							$[b_n\text{-}17]^+$ 19; $[y_{n-3}]^+$ 52; $[y_{n-3}\text{-}17]^+$ 11; $[G^*R]^+$ 21
acyl-GGYR	100			40	80			$[y_{n-1}\text{-}H_2O]^+$ 70
YG(GLF)		24			100			$[b_{n-1}\text{-}17]^+$ 62; $[a_{n-1}\text{-}17]^+$ 9
acyl-YG(GLF)				100	18			$[a_{n-1}\text{-acyl}]^+$ 6
RGD(S)	100		25	74				$[b_{n-1}\text{-}17]^+$ 16, $[b_{n-2}]^+$ 22
acyl-RGD(S)	78		49	42			100	$[b_n\text{-}17]^+$ 52, $[b_{n-1}\text{-}42]^+$ 21 $[b_{n-2}\text{-acyl}]^+$ 13
KWD(NQ)	100		84	36			57	$[b_n\text{-}H_2O]^+$ 26, $[b_{n-1}\text{-}H_2O]^+$ 32
acyl-KWD(NQ)		43		38				$[b_n\text{-}H_2O]^+$ 91, $[b_{n-1}\text{-}H_2O]^+$ 49, $[a_{n-1}\text{-acyl}]^+$ 100, $[a_n\text{-}H_2O]^+$ 30
KWDNQ	100		84	36			57	$[b_n\text{-}H_2O]^+$ 26; $[b_{n-1}\text{-}H_2O]^+$ 32
acyl-KWDNQ	100		9	21	71		95	$[b_n\text{-}H_2O]^+$ 42; $[b_n\text{-}2H_2O]^+$ 26; $[b_{n-1}\text{-}H_2O]^+$ 65; $[a_{n-1}\text{-}H_2O]^+$ 26
RGDS	39		24	74				$[b_n\text{-}17]^+$ 20; $[(b_4y_2)_2]^+$ 21
acyl-RGDS	45		13	100				$[b_{n-3}]^+$ 9
LLFGYPVYV	17			100				$[y_7]^+$ 13
acyl-LLFGYPVYV				100			11	$[y_7\text{-acyl}]^+$ 7

[a] Residues in parentheses were previously dissociated from the original peptide. For example, the MS/MS stage under consideration for YGG(LF) is MS^4, where the original peptide was YGGLF and G is the residue currently at the C-terminus.

becomes one of both sodiated and non-sodiated peptides as well. This complexity would make the analysis of an unknown protein significantly more challenging. Not only has the number of species in the mixture been multiplied, but also the intensity of each of the original peptide fragments has been distributed among four types of ions.

The dissociation of some peptides from this digest was examined. Fig. 4 shows a comparison of a protonated and an acetylated, sodiated version of the digest fragment, TGPNLHGLFGR. The protonated ion dissociates to a large number of products including a b ion series, some y ions, and an internal fragment. The acetylated, sodiated ion has significantly fewer products, with lower intensity y ions and much of the intensity in the $[b_{n-1} + Na + OH]^+$ ion. Identification of the C-terminal ion is easily and confidently made using the acetylated peptide. However, the intensity of the parent ion is reduced by the factors mentioned above. The result is that, although the $[b_{n-1} + Na + OH]^+$ ion is a significant product (about 50% relative abundance), the intensity is insufficient to perform MS^3. (Note that parent peptide ions available for MS/MS exhibit very low intensity in the mass spectrum of the acetylated digest shown in Fig. 3.)

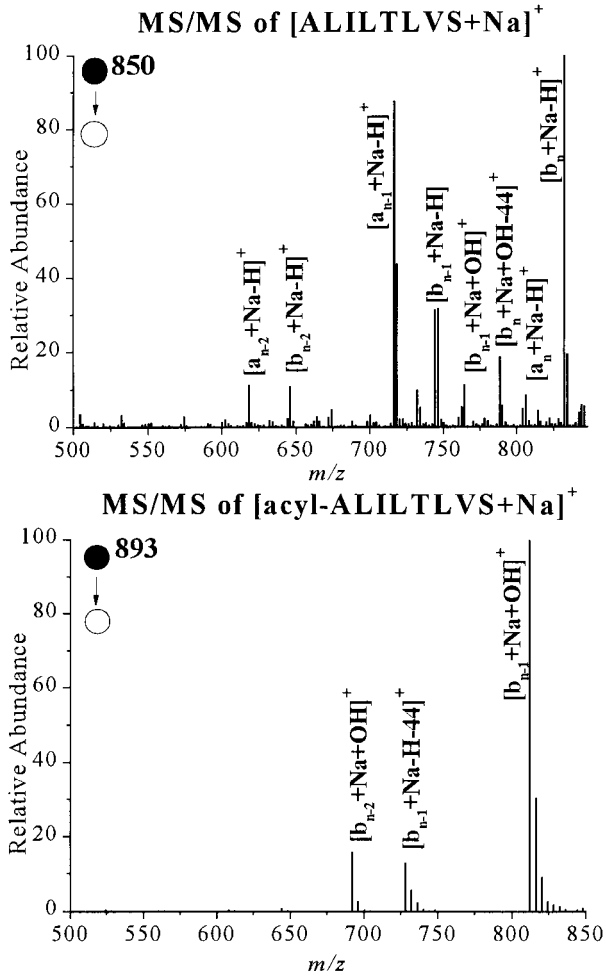

Fig. 2 Comparison of dissociation patterns of acetylated and non-acetylated sodium-cationized peptide, ALILTLVS.

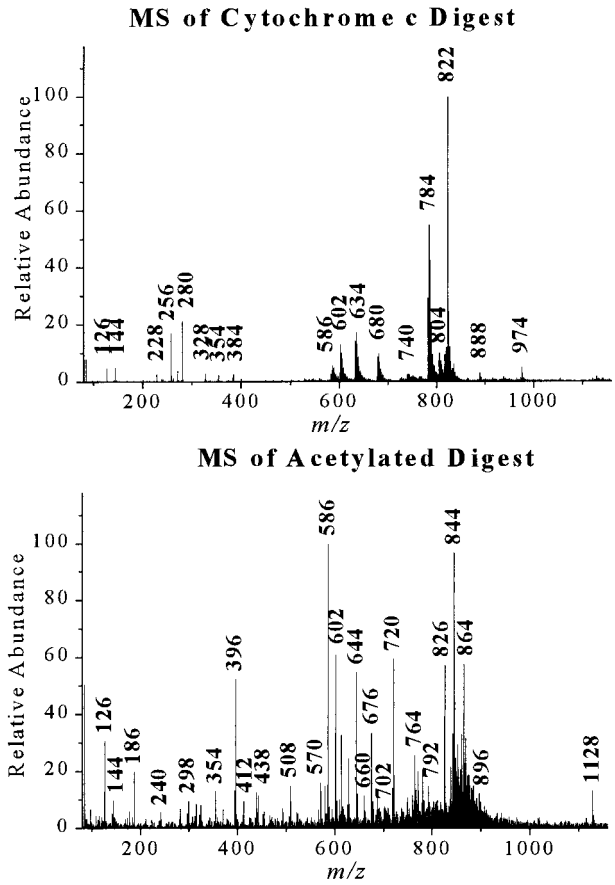

Fig. 3 Comparison of mass spectra of a cytochrome c tryptic digest and the same digest following acetylation.

Table 3 Dissociation of sodium-cationized peptides[a]

	$[b_n + Na - H]^+$	$[a_n + Na - H]^+$	$[b_{n-1} + Na + OH]^+$	$[b_{n-1} + Na - H]^+$	$[a_{n-1} + Na - H]^+$	$[b_{n-2} + Na - H]^+$	Other
SSS	41	9	27	100	4		$[b_{n-2} + Na+OH]^+$ 15
acyl-SSS	77		100	54	20		
PPGFS	64		60	65	100		
acyl-PPGFS	60		100		98		
ALILTLVS	100	5	13	38	45		
acyl-ALILTLVS			100				
RYLPT	100		14				
acyl-RYLPT	20	100	17				
GYR	100		30				$[b_n + Na-H - 42]^+$ 96
acyl-GGYR	100		30				$[b_n + Na - H - 42]^+$ 14
GGY(R)	40						$[(b_3y_2)_1 + Na + OH]^+$ 100
acyl-GGY(R)			100				
RGD(S)			100	11		14	$[b_n + Na - H - 17]^+$ 40
acyl-RGD(S)			18	13		100	$[b_{n-2} + Na - H - 42]^+$ 10
YGG(LF)	70		65	100	20		$[a_{n-1} + Na - H]^+$ 53
acyl-YGG(LF)	46		100				

[a] No significant difference between dissociation patterns of acetylated and non-acetylated: RGDS, RGDT, GAVSTA, YGGLF, YGGFL, KWDNQ, LLFGYPVYV, LFGYPVY.

Semi-empirical MO calculations

Computer modeling of acetylated and non-acetylated ions was performed to investigate the effect of the derivatization on these ions. The peptides YGGFL, YGGL, GGGG, and GVYVH were used as models for Category I peptides, and YGG, YGGS, RGDS and PPGFS represented the Category Ia and II peptides. PPGFS and YGG were observed experimentally to adopt Category I-type dissociation upon acetylation, while RGDS did not. The sodium ion was coordinated to several sites in each peptide, the C-terminus, N-terminus, and internal carbonyls. The heats of formation for each of the coordination sites were compared to try to identify where the sodium ion is most stable. However, the energies for the different coordination sites were very similar, so it was not possible to identify a preferred location for the sodium ion in either the acetylated or non-acetylated peptides.

Bond lengths were also examined to evaluate the effect of coordination site on various bonds. The protonated peptides were also modeled for comparison. In certain cases, the peptide bonds, those that would break to form b ions, were observed to lengthen by approximately 0.15 nm relative to the protonated ions. The lengthening depends on both the location of the sodium ion and the conformation of the peptide. Peptide starting configurations were either in extended form, as the bonds in a strand of a beta sheet, or in a more compact form, as in an alpha helix. In no case were the bond lengths observed to change due to the derivatization.

When the peptides are bonded in a beta sheet conformation, the behaviors of Category I and II peptides are distinct. In Category I peptides, the C-terminal peptide bond length is unchanged regardless of the location of the sodium ion. When the sodium is at the C-terminus, the N-terminal peptide bond is lengthened, but when the sodium is at the N-terminus, the bond lengths are unaffected. Category II peptides behave differently. When the sodium ion is at the C-terminus, each peptide bond is lengthened by approximately 0.15 nm. However, when the sodium ion is at the N-terminus, these bonds are close to the length of the protonated peptides. When the peptides are in an alpha-helical conformation, both Category I and II peptides behave similarly. Here, the C-terminal peptide bond is lengthened when the sodium ion is at the N-terminus.

Because comparison of heats of formation of the various peptides modeled did not reveal a preferred coordination site of the sodium ion, no conclusions could be drawn as to the effect of acetylation on the location of the sodium ion. However, there was a clear effect of the location of the sodium ion on the length of the peptide bonds. Weakening of the peptide bonds would enhance the formation of the b-type ions, and weakening of the C-terminal peptide bond would favor the b_{n-1} ion. This bond is lengthened in Category Ia and II peptides when the sodium is at the C-terminus and the peptide is in a beta-type conformation. Two of the peptides modeled were observed to form predominantly the b_{n-1} ion upon acetylation. Thus, this derivatization may encourage the sodium ion to coordinate to the C-terminus by blocking the N-terminus for some Category Ia and II peptides. However, if a peptide has a more alpha-helical conformation, this effect would not be seen. If the different peptides have more alpha helix-type conformation, this may explain why the dissociation of some Category Ia and II peptides becomes like Category I (PPGFS, YGG), while that of others does not (RGDS).

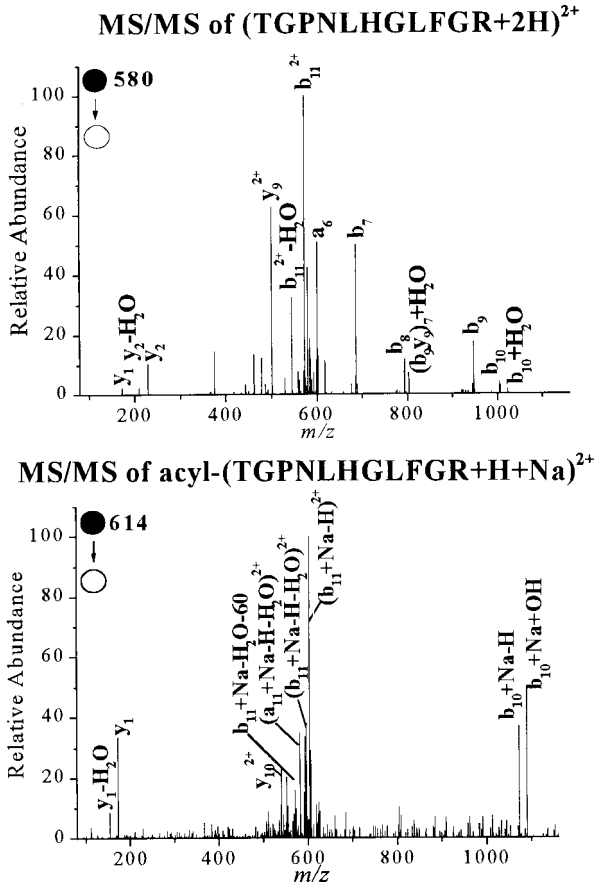

Fig. 4 MS/MS spectra of a peptide, TGPNLHGLFGR, from the tryptic digest comparing the protonated peptide and the acetylated, sodium-cationized peptide. Note the more selective dissociation with the acetylated version.

Conclusions

Because acetylation does not universally affect the dissociation and no clear pattern could be identified, it does not offer sufficient advantages for regular use in sequencing. Acetylation does increase the percentage of peptides that dissociate as Category I, but the reaction lengthens sample preparation time. Also, because the reaction does not go to completion, the solution composition becomes more complex, especially for mixtures (*e.g.* protein digests). Sodium-cationized peptides offer notable advantages for sequencing. Acetylation further improves the proportion of peptides that exhibit Category I dissociation. However, because acetylation would only improve sequencing for a small number of residues, these limited advantages would not outweigh the disadvantages of this derivatization for routine sequencing. Simply using sodium-cationized peptides is therefore the generally recommended method for sequencing using MS^n in a quadrupole ion trap.

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