

Investigation of Neutral Losses and the Citrulline Effect for Modified H4 N-Terminal Pentapeptides

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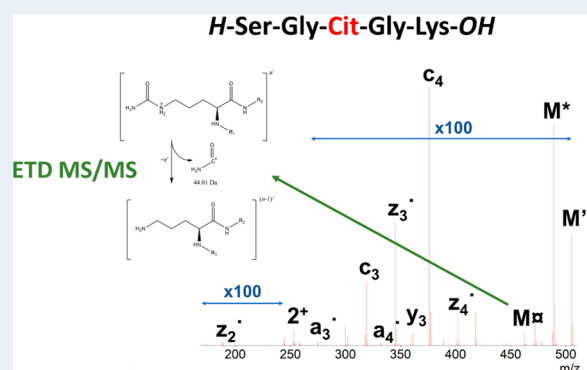
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ABSTRACT: Tandem mass spectrometry is an indispensable tool in proteomics used for protein sequencing and quantitation. On the basis of the sequential fragments usually generated from peptide ions via collision-induced dissociation, electron-transfer dissociation, or a combination of the two, probabilistic database search engines could be used for the identification of the peptides. The correct localization of posttranslational modifications (PTMs) poses a more challenging problem than the general identification of proteins. Histones are involved in the regulation of DNA transcription via the wealth of PTMs on their N-terminal tail. In this study, we analyzed the histone H4 peptide SGRGK incorporating four different posttranslational modifications: citrullination, acetylation, phosphorylation, and arginine methylation at various positions. The pentapeptides model the enzymatic cleavage of the N-terminal tail of human histone H4 protein by LysC protease. Fragmentation of the peptides was investigated using higher-energy collisional dissociation (HCD), electron-transfer dissociation (ETD), and electron-transfer higher-energy collisional dissociation (EThcD) on an ultrahigh resolution and mass accuracy instrument. We found that while all three techniques have their unique characteristics, advantages, and pitfalls, EThcD generated the most fragment ion-rich spectra. Despite potential ambiguities regarding exact fragment identities, full sequence coverage and PTM mapping may also be achievable. We also found novel neutral losses from the charge-reduced precursors characteristic to citrullination in ETD and EThcD which may be used in proteomic applications. N-Terminal acetylation and arginine methylation could also be confirmed by their characteristic neutral losses from the charge-reduced precursors.

KEYWORDS: citrullination, acetylation, arginine methylation, posttranslational modifications, histone code, collision-induced dissociation, electron-transfer dissociation, tandem mass spectrometry



INTRODUCTION

Sequencing of peptides and detailed characterization of their posttranslational modifications (PTMs) could usually be carried out by tandem mass spectrometry using collisional activation (CID), activation by electron-transfer (ETD), or activation by electron transfer followed by collisional activation (EThcD).

Collisional activation (CID, or HCD in Orbitrap instruments) is an activation method that uses collisions with inert gases to impart enough energy to the precursors for fragmentation.¹ It is the best option when peptides are relatively small (<20 amino acids), not highly charged ($z \leq 3$), and devoid of labile PTMs (e.g., phosphorylation). Product ions mostly include y and b type ions² originating from amide bond cleavages as well as neutral losses of small molecules that may or may not be characteristic to modifications present on the peptides. Despite some of its disadvantages, CID is still widely used due to the well-understood mechanism of the processes, reliability, and good sequence coverage of the peptides.

ETD was developed by Syka et al.³ and involves the transfer of electron(s) to multiply charged peptide species to initiate fragmentation. Being only efficient for peptides with higher charge densities, it is usually used for larger peptides or even proteins. Sequence coverage may also be higher than that of CID. ETD is applied as a complementary method to CID as it yields c and z type ions originating from N–C_α bond cleavages. Neutral losses are usually less frequent in ETD spectra. PTMs are preferentially preserved in ETD as opposed to CID. There is no fragmentation at the N-terminus of proline residues in ETD.

EThcD was first introduced by Frese et al.⁴ In EThcD, the transfer of electrons is followed by the collisional activation of all the products generated in the first step. The overall process

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yields b, y, c, and z ions and numerous neutral losses. It has been reported as the best method of choice for the analysis of phosphopeptides and glycopeptides⁵ due to the wealth of fragments generated, which considerably facilitate the localization of PTMs.

Histone-mediated gene regulation is one of the main fields where PTMs are of high importance.⁶ Acetylation of the N-terminus⁷ or lysine residues,⁸ methylation of arginine⁹ and lysine residues,¹⁰ and phosphorylation of serine residues¹¹ have been known a long time along with the citrullination site at Arg-3¹² of the histone H4 N-terminal tail. While acetylation on the side chains in CID results in a mass increment of the corresponding fragments carrying the modification and may result in characteristic iminium ions, an N-terminal acetylation may give rise to b₁ ions which are not stable in their original forms.¹³ No characteristic side chain loss attributed to acetylation has been previously described. On the other hand, neutral losses of methylamine, N-methylcarbodiimide, and N-methylguanidine as well as some iminium ion related fragments are reported to be characteristic to methylated arginine residues in CID MS/MS.¹⁴ Phosphorylated residues are highly prone to lose phosphoric acid—or even worse, transfer it to other unmodified hydroxyl side chains upon CID.^{15,16} In ETD, these side chain modifications remain preferentially intact.¹⁷ The presence of fragments corresponding to the neutral loss of isocyanic acid is selective for (homo)citrullinated residues in CID,^{18,19} although no distinctive side chain loss has been reported for citrullination in ETD. Moreover, we previously reported a cleavage preference at the C-termini of citrulline residues in the collision-induced tandem mass spectra of citrullinated peptides (citrulline effect).^{20,21}

Histone modifications have been extensively studied in the past decade. Bottom-up, middle-down, and top-down approaches using CID/HCD and ETD techniques have all been applied to investigate the histone code.^{22–28} However, little work has explored the detailed and systematic fragmentation of small model peptides containing citrulline residues as well. Furthermore, we hypothesized that there should be additional uncharacterized neutral loss ions that may be useful for PTM localization or confirmation.

In this study, we synthesized the human histone H4 N-terminal pentapeptide SGRGK and its combinatorial variants bearing various PTMs: Ac-SGRGK, pSGRGK, Ac-pSGRGK, SGXGK, Ac-SGXGK, pSGXGK, Ac-pSGXGK, SGR(Me)GK, Ac-SGR(Me)GK, pSGR(Me)GK, Ac-pSGR(Me)GK, where X stands for the one-letter abbreviation for citrulline residues. Utilizing the current, commercially available activation techniques—namely, HCD, ETD, and EThcD—our aim was to examine the possible differences of the various tandem mass spectra. We took care to observe the neutral losses that could be used for improving PTM localization.

EXPERIMENTAL PROCEDURES

Model pentapeptides were manually synthesized using the standard Fmoc/tBu strategy of solid phase peptide synthesis on Wang-resin resulting in peptides with free carboxy termini. PTMs—except for acetylation—were introduced by incorporating modified residues (Fmoc-Arg(Me), Pbf)-OH and Fmoc-Ser(PO(OBzl)OH)-OH) into the sequence during synthesis. Acetylation was performed with acetic anhydride in basic conditions. All amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany). After cleavage by trifluoro-

acetic acid in the presence of phenol, anisole, triisopropylsilane, and distilled water as scavengers, the resulting peptides were purified by HPLC-UV and were freeze-dried.

Prior to MS analysis, the freeze-dried samples were dissolved in acetonitrile–water (1:1, v/v), containing 0.1 v/v% formic acid. Final concentration of the peptides was 10 μM. These solutions were directly injected to the electrospray source of an Orbitrap Fusion Tribrid instrument (Thermo Scientific, Waltham, MA, USA) at a flow rate of 5 μL/min. Resolution was set to 120 000. An isolation width of 2 m/z was applied for MS/MS. For ETD and EThcD experiments, the ion activation time was set to 50 ms. Normalized collision energies (NCE) were set between 15 and 35% for HCD and EThcD. Collision energy dependence studies were carried out on a Q-Exactive Focus Hybrid Quadrupole-Orbitrap instrument (Thermo Scientific, Bremen, Germany) in the range of 10–48 eV with 2 eV steps at a resolution of 70 000. Raw data were visualized and annotated by mMass.²⁹

RESULTS

HCD Experiments. We previously demonstrated that the citrulline effect may substantially alter fragment ion distributions in CID.^{20,21} Therefore, in these experiments, we acquired tandem spectra at different collision energies to examine the effect of the various PTMs. Single stage MS data are summarized in Table 1. The results of collision energy

Table 1. List of the Twelve Pentapeptide Sequences Originated from Human H4 Histone Protein

peptide sequence	measured [M + H] ⁺	calculated [M + H] ⁺	measured [M + 2H] ²⁺	calculated [M + 2H] ²⁺
SGRGK	504.2889	504.2889	252.6480	252.6481
Ac-SGRGK	546.2997	546.2994	273.6530	273.6534
pSGRGK	584.2552	584.2552	292.6307	292.6312
Ac-pSGRGK	626.2653	626.2658	313.6362	313.6365
SGR(Me)GK	518.3042	518.3045	259.6557	259.6559
Ac-SGR(Me)GK	560.3147	560.3151	280.6608	280.6612
pSGR(Me)GK	598.2703	598.2709	299.6385	299.6391
Ac-pSGR(Me)GK	640.2808	640.2814	320.6437	320.6443
SGXGK	505.2723	505.2729	253.1394	253.1401
Ac-SGXGK	547.2831	547.2835	274.1447	274.1454
pSGXGK	585.2383	585.2392	293.1231	293.1232
Ac-pSGXGK	627.2493	627.2498	314.1282	314.1285

dependence studies are summarized for doubly protonated precursor ions in Figure 1 and Supplementary Figures S1a–l in the case of all the 12 histone peptides. For ease of interpretation, only b and y type ions are depicted, other noncharacteristic fragments (peaks corresponding to an ammonia or water loss) are omitted. Singly and doubly protonated fragments of the same type are cumulated. Intact b ions could not be detected in the case of phosphopeptides, only fragments with a phosphoric acid loss. These are shown as b₁P in the figures.

Our results show that the γ₂ ion intensity is significantly higher for citrulline containing peptides compared to the arginine containing variants. This phenomenon can be explained by the citrulline effect. In addition, γ₂ intensity is

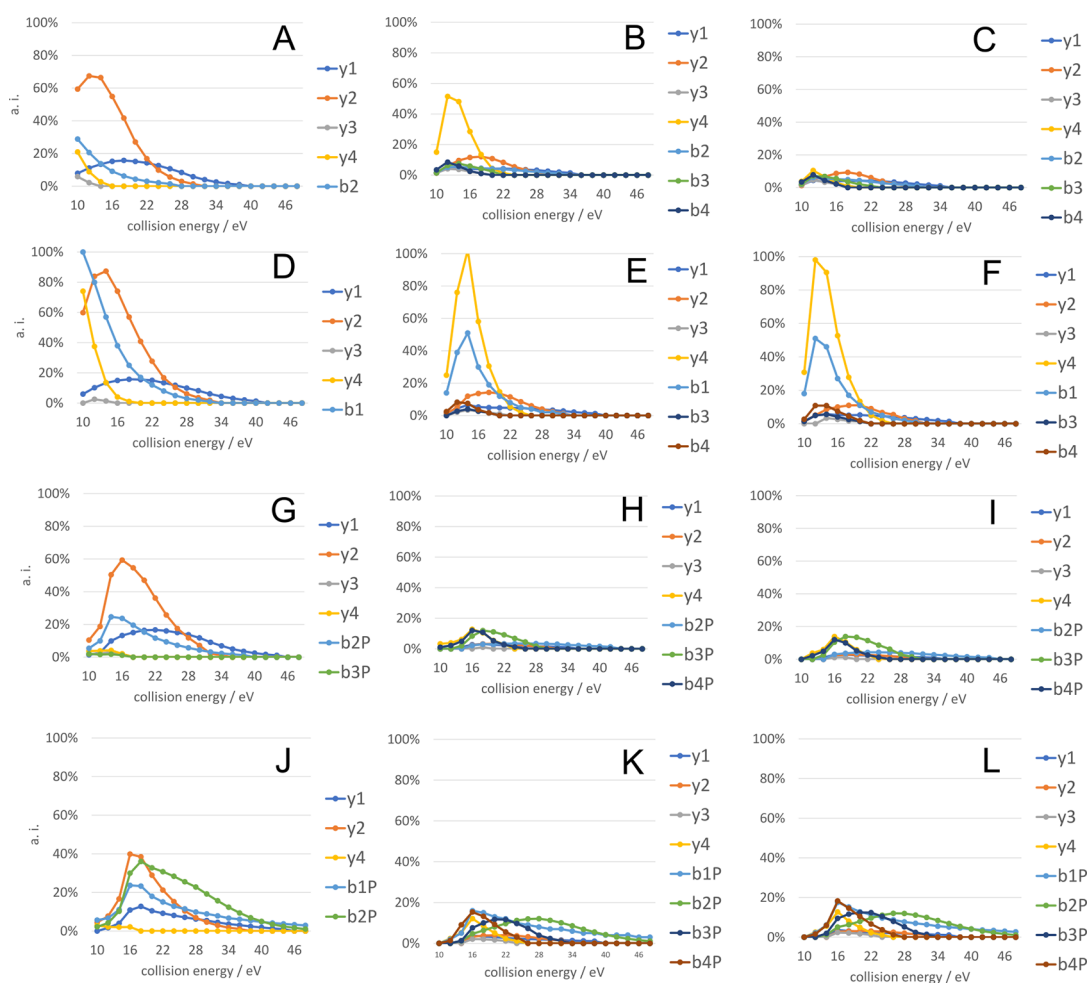


Figure 1. HCD curves of appearance for the fragments originating from doubly protonated precursors of citrulline containing peptides (first column), N^G -methylarginine containing peptides (second column) and arginine containing reference peptides (third column). SGZGK (A–C), Ac-SGZGK (D–F), pSGZGK (G–I), Ac-pSGZGK (J–L). Z = X (first column), Z = R(Me) (second column), and Z = R (third column). B_iP fragments denote orthophosphoric acid loss from the corresponding b_i ions.

usually higher than or as intense as that of other sequential fragments in its own spectrum. Conversely, we could not find any remarkable difference in the cleavage preference between N^G -methylarginine and arginine containing histone peptides besides a more intense y_4 ion for SGR(Me)GK vs SGRGK (Figure 1B–C).

For acetylated peptides, we observed b₁ ions which are otherwise unstable if the N-terminus is unmodified. N^G -Methylarginine containing peptides usually displayed at least two out of three previously described neutral losses¹⁴ at the same time, namely, the loss of methylamine (31.042 Da), *N*-methylguanidine (74.071 Da) and *N*-methylcarbodiimide (57.045). These could be used for confirmation of arginine methylation. By using these characteristic ions, false positive identification rates may also be reduced.

ETD Experiments. Electron-transfer dissociation is usually used for larger or highly charged peptides. For low charges, CID or HCD outperforms ETD in terms of sequence coverage.³⁰ In our experiments, doubly charged ions were selected for fragmentation using ETD. In these cases, charge-reduced precursors could not be detected; their characteristic ammonia and other previously described losses could be observed. As it is expected, however, the most intense peaks in the ETD spectra correspond to precursor ions in the spectra

due to the low efficiency of electron transfer to low-charged species. In these experiments, we mostly observed c and z type fragments with a relatively lower occurrence of y and a ions (Supplementary Figure S2(a–l)). The latter ones denote the radical type a ion that contains an extra hydrogen atom. Interestingly, we detected a neutral loss of 44.01 Da in the ETD MS/MS spectrum of the citrullinated SGXGK peptide possibly originating from the undetected charge-reduced precursor (Figure 2A). In the ETD spectrum of the unmodified SGRGK peptide, we obtained a similar loss that is 43.03 Da (Figure 2B). The difference between the two losses is the same as the increment for citrullinated versus unmodified arginine residues suggesting that these neutral losses can be associated with citrulline and arginine side chains. Arginine side chains may produce relatively strong fragment ion peaks corresponding to neutral losses in ETD. One of these possible reactions is the above-mentioned 43.03 Da loss which is suggested by Hunt et al.³¹ to be carbamidoyl radical ($\text{NH}=\text{C}^{\bullet}\text{-NH}_2$). We propose that the loss of 44.01 Da could be thus identified as the carbamoyl radical ($\text{O}=\text{C}^{\bullet}\text{-NH}_2$) [Scheme 1]. Zolig et al. investigated the neutral losses characteristic to citrullination in HCD and ETD and found none for the latter technique.³² Our results show that the carbamoyl radical loss could, however, be selective for

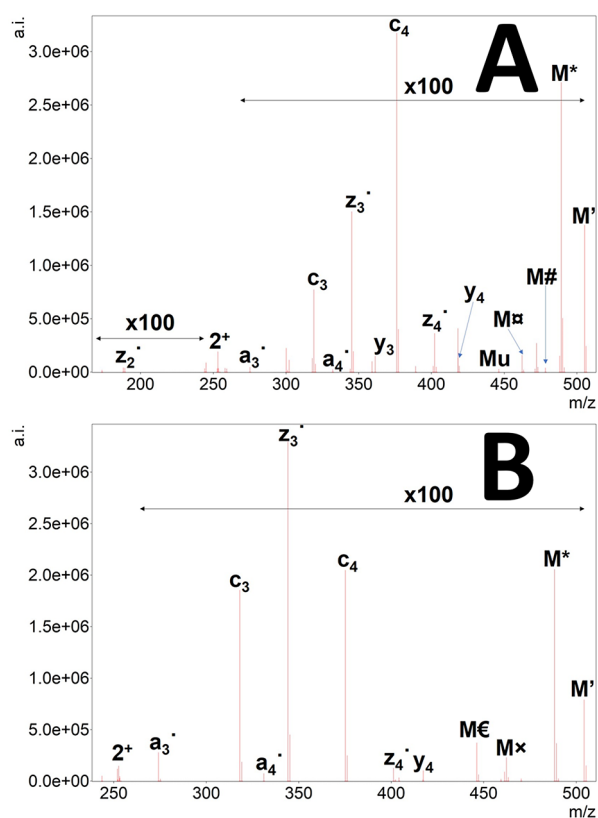


Figure 2. ETD tandem mass spectrum of doubly protonated SGXGK (A) and SGRGK (B). Precursor ions are denoted as “2+”, and the undetected charge-reduced precursors are only signed as M. Hydrogen atom loss is depicted as '. Neutral losses of ammonia (*), CO (#), and the citrulline-selective carbamoyl radical (currency sign) and urea loss (u) are also shown for SGXGK along with arginine-selective carbamimidoyl radical (x) and carbamidine (Euro sign) for SGRGK. For ease of interpretation, precursor intensities are reduced to 1/100 of their original value.

citrulline-containing peptides. The loss of CO₂ which is frequently observed in ETD spectra has a very similar transition (43.990 Da). CO₂ and carbamoyl loss differentiation therefore may be hard for higher charge-state precursors or instruments with lower resolution and mass accuracy. All citrulline-containing histone peptides in this study showed this fragmentation route as opposed to their arginine-containing variants. Therefore, we screened ETD spectra for additional selective losses and found that although being much less intense, the elimination of urea (H₂N-CO-NH₂) could also be indicative of citrullination which is analogous to the arginine-selective carbamidine loss (H₂N-C(=NH)-NH₂) that was previously described only with a formula of CH₅N₃.³³ However, urea loss is less frequently observed for citrullinated peptides. As expected, phosphate groups remained mostly intact in our ETD experiments. Almost all acetylated peptides displayed a relatively strong loss of 59.04 Da which could be attributed to the elimination of H₃C-C(-OH)=NH (ethanimidic acid) from the N-terminus (Figure 3). This could be elucidated by the original fragmentation mechanism proposed for electron-transfer dissociation by Syka et al.,³ if it is applied for the amide group of the acetylated N-terminus. A neutral loss with the same formula has already been reported for asparagine and glutamine side chains³³ as well. N^G-methylarginine residues also produced selective elimination

Scheme 1. Suggested Mechanism for the Elimination of Carbamoyl Radical from Citrulline Side Chains in ETD Fragmentation

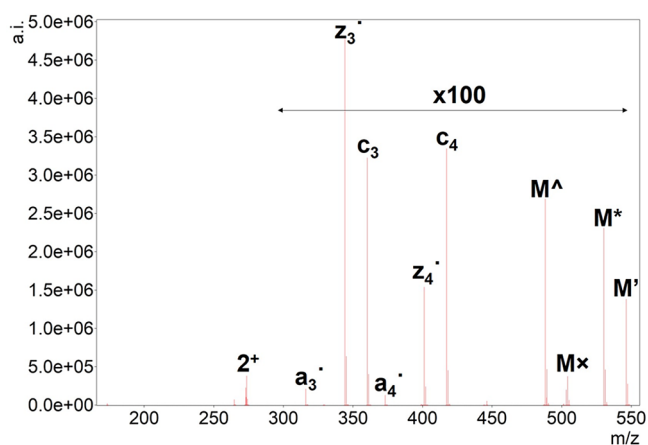
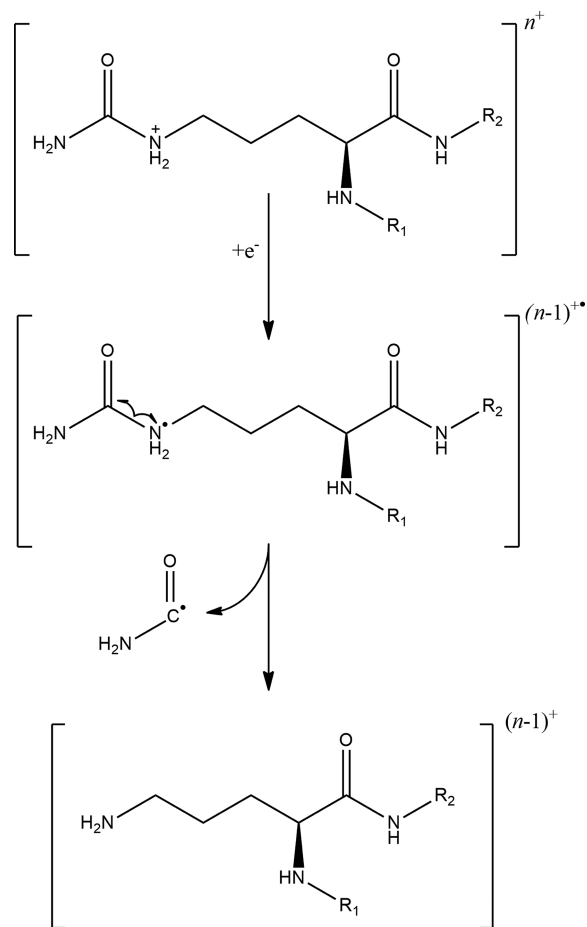


Figure 3. ETD tandem mass spectrum of doubly protonated Ac-SGRGK. Precursor ion is denoted as “2+”, the undetected charge-reduced precursor is only signed as M. Hydrogen atom loss is depicted as '. Neutral losses of ammonia (#) and the arginine-selective carbamimidoyl radical (x) are also shown along with ethanimidic acid (^) loss characteristic to N-terminally acetylated peptides. For ease of interpretation, precursor intensity is reduced to 1/100 of its original value.

products including CH₃-NH₂ (31.042 Da) or HN=C-NH-CH₃ (57.045 Da) (Figure 4),³⁴ which are analogous to

previously reported losses of NH_3 (17.027 Da) and $\text{HN}=\text{C}-\text{NH}_2$ (43.030 Da) characteristic to arginine residues.

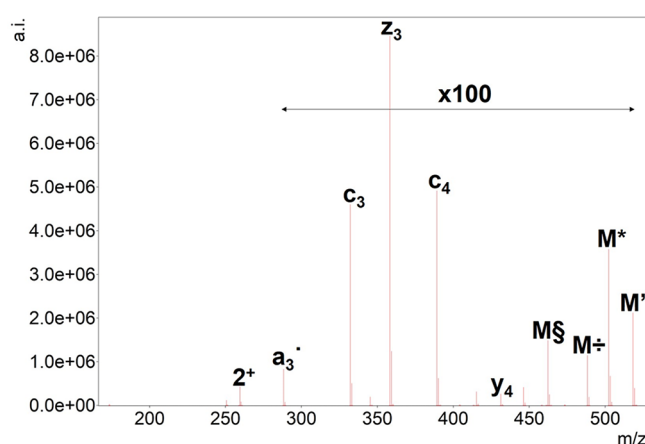


Figure 4. ETD tandem mass spectrum of doubly protonated SGR(Me)GK. Precursor ion is denoted as “2+”, and the undetected charge-reduced precursor is only signed as M. Hydrogen atom loss is depicted as *. Neutral losses of ammonia (*) and the N^G -methylarginine-selective methylamine (±) and *N*-methylcarbodiimide losses (§) are also shown. For ease of interpretation, precursor intensity is reduced to 1/100 of its original value.

ETHcD Experiments. ETHcD tandem mass spectra obtained for the histone H4 pentapeptides very much resemble HCD tandem mass spectra of singly charged precursors at high energies (Supplementary Figure S3(a–l)). In these cases, however, fragmentation efficiency seems much higher, and the whole m/z region is populated more evenly by fragment ions compared to HCD. Neutral losses are prevalent both for precursors and their fragments. Another feature is that the higher the proton mobility of a given precursor, the higher the similarity of a given ETHcD spectrum to its HCD counterpart. On the other hand, the lower the proton mobility, the higher is the similarity of the given ETHcD spectra to its ETD counterpart. In the case of ETHcD spectra, however, differentiation between two fragments is often ambiguous. For example, the mass of a b_1 ion and a $c_1-\text{NH}_3$ are exactly the same, or the loss of a HNCO molecule from a $[\text{M} + \text{H}]^+$ type precursor is the same as that of a $\text{O}=\text{C}-\text{NH}_2$ from a singly charged, charge-reduced precursor $[\text{M} + 2\text{H}]^{2+}$. In general, several NH_3 and even H_2O losses could be observed for fragments of which the latter is less frequently seen in HCD generated spectra. The mass difference between NH_3 and even H_2O losses is 0.9840 Da, which is identical to the mass difference for deamidation and also citrullination. As examples, ETHcD spectra of the doubly charged precursor of SGXGK and SGRGK peptides at 30% NCE are depicted in Figure 5. As NCE only compensates for mass difference and charge state differences, the lower efficacy of SGRGK fragmentation may be attributed to superior basicity of arginine residues, thus decreasing proton mobility.³⁵ The citrulline effect is very pronounced as the peak corresponding to the y_2 ion is the second highest in the spectrum of the citrullinated SGXGK peptide. In the upper m/z region, the ETD-selective losses could also be identified from the charge-reduced precursor (which is in itself not detectable) including NH_3 , carbamoyl radical, and urea along with a hydroxyl radical loss possibly from the serine side chain. Some of these losses could also be originated from the $[\text{M} + \text{H}]^+$, which is formed by hydrogen

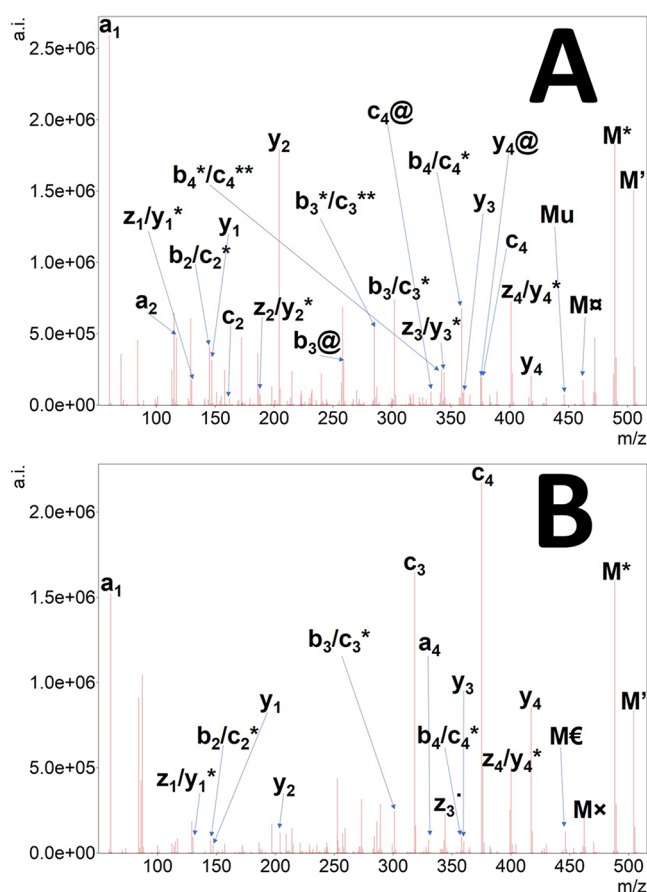


Figure 5. ETHcD tandem mass spectrum of doubly protonated SGXGK (A) and SGRGK (B) histone peptides. Precursor ion is denoted as “2+”, the undetected charge-reduced precursor is only signed as M. Hydrogen atom loss is depicted as *. Neutral losses of ammonia (*), water (deg), the citrulline-selective carbamoyl radical (currency sign) or isocyanic acid (@) and urea (u), and the arginine-selective carbamidoyl radical (x) and carbamide (Euro sign) eliminations are also shown.

atom abstraction from the charge-reduced precursor ($[\text{M} + 2\text{H}]^+$). The presence of both processes could be inferred from the unusual “isotope” distributions (e.g., the peak of NH_3 loss, see Figure S4). Another complicating feature in the spectra worth mentioning is the proper identification of z and \dot{z} ions which only differ by an H^* radical (1.0078). Differentiation of hydrogen abstraction and isotope peaks may be hard. Very high resolution, sophisticated software annotation and high mass accuracy are needed, especially when low-mass posttranslational modifications (citrullination, deamidation) are also present. Usually, all fragments bearing citrulline residues display the loss of HNCO which is rarely seen in simple HCD experiments (Figure S5). This wealth of fragments may also be a disadvantage as it may increase the number of false positive identifications. Therefore, great care must be taken, and manual inspection is highly recommended during evaluation when fragments other than the intact fragment ions (b , y , c) are considered. However, the selective losses in the higher m/z region that can also be found in ETD spectra are reliable—at least in cases where only either citrulline or arginine is in the peptide sequence. For peptides containing one or more arginine and citrulline, the difference between the above-mentioned characteristic peaks is 0.9840

Da, which may also indicate the presence of citrullination beside the HNCO/carbamoyl radical loss.

In the case of Ac-SGXGK peptide, the highest sequential peak was attributed to the citrulline effect, while the second one—comparable to this—was the y_4 ion (Figure 6). Cleavage

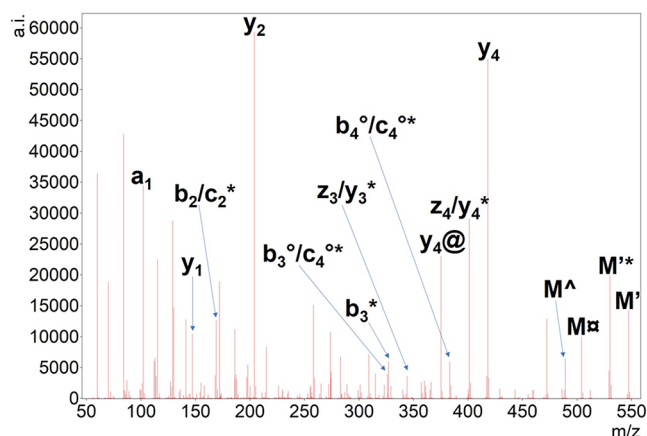


Figure 6. EThcD tandem mass spectrum of doubly protonated Ac-SGXGK histone peptide. The undetected charge-reduced precursor is only signed as M. Hydrogen atom loss is depicted as '. Neutral losses of ammonia (*), water (deg), the citrulline-selective carbamoyl radical (currency sign) or isocyanic acid (@) are also shown. Note that the spectrum is very similar to an HCD one; radical losses could be explained by even-electron fragmentation from M' as well.

preference at the N-terminus of glycine residues in mobile and partially mobile sequences has been reported earlier for CID spectra.³⁶ Characteristic, modification-selective neutral losses in the upper m/z region are present indicating citrullination and acetylation in this histone peptide. The cleavage preference C-terminal to citrulline is also pronounced for the pSGXGK peptide containing a phosphorylated serine residue, while y_2 intensity is much lower for pSGR(Me)GK (Figure 7). “False HNCO losses” from fragments still appeared for peptides that do not comprise citrulline residues (see Figure S6). The peaks corresponding to these fragments were mostly associated with b_3 peaks of methyl-arginine and arginine-containing peptides. We also found a false positive b_2 -HNCO peak for the peptide Ac-SGXGK but none for other Cit-peptides. On the other hand, HNCO loss from precursor ions was only observed for citrulline-containing peptides being faithful reporters of this modification. ETD is often mentioned as a truly complementary technique of HCD. Even in the case of EThcD, it can be seen that a cleavage C-terminal to arginine or glycine—which are normally disfavored upon HCD—is prevalent for Arg-containing peptides (e.g., pSGRGK, Ac-SGRGK). In the case of Cit-containing peptides, EThcD fragmentation was still highly influenced by the citrulline effect. The MS/MS spectra of Ac-pSGXGK contains mainly iminium ions, suggesting much greater proton mobility. Yet, the citrulline effect and characteristic neutral losses are substantial in this case as well.

DISCUSSION

In this study, the effect of the presence of multiple posttranslational modifications (N-terminal acetylation, serine phosphorylation, and arginine citrullination/methylation) on the tandem mass spectra of human H4 histone peptides was examined by different activation methods including higher-

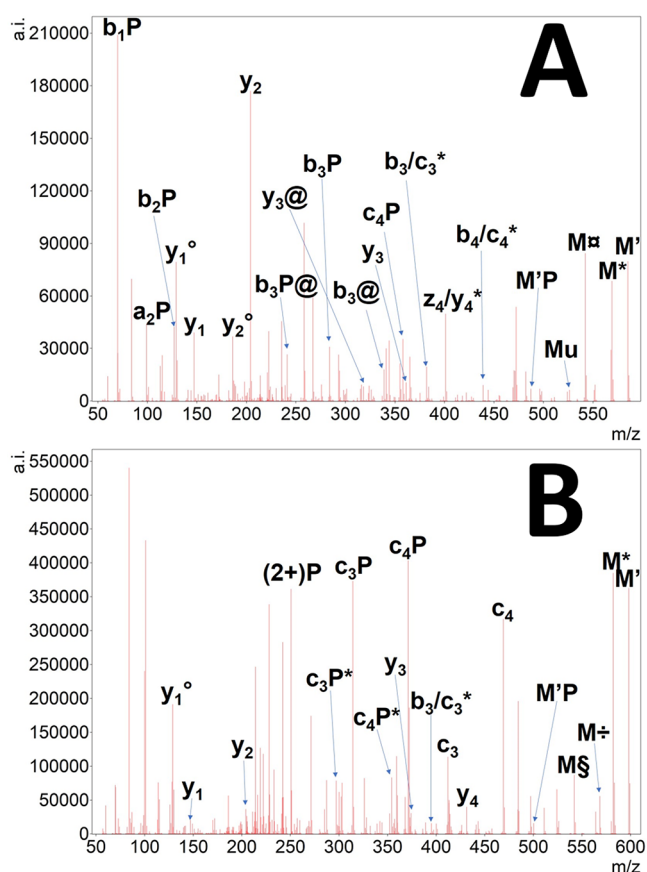


Figure 7. EThcD tandem mass spectrum of doubly protonated pSGXGK (A) and pSGR(Me)GK (B) histone peptides. The undetected charge-reduced precursor is only signed as M. Hydrogen atom loss is depicted as '. Neutral losses of ammonia (*), water (deg), the citrulline-selective carbamoyl radical (currency sign), or isocyanic acid (@) as well as the N^G -methylarginine-selective methylamine (÷) and N-methylcarbodiimide losses (§) are also shown. Note that all b_i related fragments may represent c_i^* ones, and all z_i related fragments may represent y_i^* as well.

energy collisional dissociation (HCD) with collision energy dependence, electron-transfer dissociation (ETD), and electron-transfer higher-energy collisional dissociation (EThcD).

In HCD spectra, the citrulline effect—i.e., the cleavage preference at the C-terminus of citrulline residues—was observed for Cit-peptides in a relatively wide collision energy range. Compared to the arginine-containing reference peptides, peaks corresponding to these fragments were significantly more intense. Isocyanic acid (HNCO) loss was also prominent from precursors (usually 10–20%, but up to 100% depending on proton mobility), but sometimes less prevalent and reliable for fragments (usually 1–30%).

ETD spectra of these doubly charged peptides provided some unexpected and characteristic high m/z peaks corresponding to radical losses from the charge-reduced precursors which could be utilized to confirm citrullination. The loss of a carbamoyl radical ($\cdot\text{CO-NH}_2$) from citrulline side chains—analogue to carbamidoyl radical ($\text{HN}=\cdot\text{C-NH}_2$) elimination of arginine proposed by Hunt et al.—is described here for the first time along with carbamide loss. These products may not be intense when the charge state of the precursor is higher and may be suppressed by arginine side chain losses if arginine is also present in the sequence but are useful for

confirmation of citrullination for lower-charged species. We also found the side chain losses associated with Arg-methylation ($\text{HN}=\text{C}-\text{NH}-\text{CH}_3$ and CH_3-NH_2).³⁶ Acetylation could also be easily identified as the loss of $\text{C}_2\text{H}_5\text{NO}$ (more precisely $\text{H}_3\text{C}-\text{C}(-\text{OH})=\text{NH}$ in this case) can only originate from acetylation in the absence of Asn and Gln residues. Being a backbone cleavage, this peak usually tends to be rather intense compared to other neutral losses.

ETHcD spectra of these histone peptides yielded the most numerous fragments for citrullinated peptides including the ($^*\text{CO}-\text{NH}_2$)/HNCO losses from (charge-reduced) precursors, HNCO losses from almost all fragments that bears a citrulline residue and citrulline effect in the case of Cit-peptides. Methylamine and *N*-methylcarbodiimide losses were also prevalent from the precursors of Arg(Me) containing peptides. Phosphorylated and acetylated peptides displayed their characteristic elimination reactions, too. These neutral loss intensities were very variable being usually ~1–20% but orthophosphoric acid loss in some cases reached ~70–80%. ETHcD not only improved neutral losses' yield for all types of PTMs but sequential fragment yields as well. Some fragments could not be differentiated from each other in ETHcD, but most of these products refer to the same sequential information, potentially improving identification. However, the overlapping of isotope peaks due to the immense amount of neutral losses may pose a concern for a software-based annotation. The technique has its own pitfalls as well due to the high probability of generating combined losses having the same formulas but inferring to structurally different ions. If this is the case for such simple systems, fragmenting larger sized peptides would increase spectral complexity and may lead to erroneous assignments especially for quantitative studies. Thus, while ETHcD was found to be beneficial for the localization of larger and labile modifications⁴ (e.g., glycosylation and phosphorylation), care must be taken when applied to small modifications (e.g., citrullination) and large precursor charge states.

Nevertheless, we suggest using ETHcD for analyzing these PTMs for low mass peptides by restricting the automatic annotation to b, y, and c sequential ions and the above-mentioned characteristic neutral losses from precursors only, followed by manual inspection of the remaining product ion neutral losses, if necessary. In this case, a complete sequence coverage as well as exact PTM site determination becomes possible without significantly increasing false identifications. Another option is to use HCD for confirmation of the modifications and subsequent ETD fragmentation for unambiguous sequence information as was used previously for citrullinated peptides.³⁷

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.9b00036>.

Figure S1: Fragment distribution of the peptides (a) SGXGK, (b) SGR(Me)GK, (c) SGRGK, (d) Ac-SGXGK, (e) Ac-SGR(Me)GK, (f) Ac-SGRGK, (g) pSGXGK, (h) pSGR(Me)GK, (i) pSGRGK, (j) Ac-pSGXGK, (k) Ac-pSGR(Me)GK and (l) Ac-pSGRGK upon higher-energy collision-induced dissociation (HCD) as a function of collision energy. Figure S2: ETD tandem mass spectra of peptides (a) SGRGK, (b)

Ac-SGRGK, (c) pSGRGK, (d) Ac-pSGRGK, (e) SGR(Me)GK, (f) Ac-SGR(Me)GK, (g) pSGR(Me)GK, (h) Ac-pSGR(Me)GK, (i) SGXGK, (j) Ac-SGXGK, (k) pSGXGK, and (l) Ac-pSGXGK. Figure S3: ETHcD tandem mass spectra of peptides (a) SGRGK, (b) Ac-SGRGK, (c) pSGRGK, (d) Ac-pSGRGK, (e) SGR(Me)GK, (f) Ac-SGR(Me)GK, (g) pSGR(Me)GK, (h) Ac-pSGR(Me)GK, (i) SGXGK, (j) Ac-SGXGK, (k) pSGXGK, and (l) Ac-pSGXGK. Figure S4: Representation of "unusual isotope distributions" in the ETHcD spectrum of the peptide pSGXGK. Figure S5: Representation of isocyanic acid loss (@) from all fragments that contain the citrulline residue in an ETHcD tandem mass spectrum. Figure S6: Representation of a false neutral loss of isocyanic acid (@) for the peptide pSGR(Me)GK which lacks a citrulline residue (PDF)

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Notes

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■ ABBREVIATIONS

B, one-letter code for an amino acid if not stated otherwise

CID, collision-induced dissociation
Cit, three-letter abbreviation for citrulline
ETD, electron-transfer dissociation
ETHcD, electron-transfer higher energy collisional dissociation
HCD, higher energy collisional dissociation
MS/MS, tandem mass spectrometry
NCE, normalized collision energy
pS, phosphorylated serine residue
PTM, posttranslational modification
X, one-letter abbreviation for citrulline (in the absence of IUPAC recommendation)

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