Published in final edited form as:

Chemistry. 2012 April 23; 18(17): 5374–5383. doi:10.1002/chem.201103534.

Charge-Site Dependent Dissociation of Hydrogen-Rich Radical Peptide Cations upon Vacuum UV Photoexcitation

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Abstract

193 nm vacuum ultraviolet photodissociation (VUVPD) was used to investigate the fragmentation of hydrogen-rich radical peptide cations generated by electron transfer reactions. VUVPD offers new insight into the factors that drive radical- and photon-directed processes. The location of a basic Arg site influences photon-activated C_α-C(O) bond cleavages of singly charged peptide radical cations, an outcome attributed to the initial conformation of the peptide as supported by molecular dynamics simulated annealing and the population of excited states upon UV excitation. This hybrid ETD/VUVPD method was employed to identify phosphorylation sites of the kinase domain of human TRPM7/ChaK1.

Keywords

Mass Spectrometry; Peptides; Radical ions; Proteomics; Photodissociation

Introduction

The gas-phase dissociation of radical (odd electron) molecular ions has increasingly been used as a tool for the structural characterization of proteins. This bloom in the analysis of radical ions has arisen from their unique fragmentation patterns evolving from selective cleavage at specific amino acids and post-translational modifications,^[1, 2] high peptide sequence coverage,^[3, 4] and/or the retention of modifications with preferential cleavage of the peptide backbone.^[3, 4] In general, the radical precursor ions may be classified as either hydrogen-rich or hydrogen-deficient based on whether the number of hydrogen atoms is greater or lower than nominal even-electron species,^[5] and each type results in significantly different fragmentation behavior. For example, activation of hydrogen-deficient radical precursor ions^[1, 2, 5-8] yield in general a few specific cleavages of the peptide backbone as well as highly abundant products evolving from losses of neutral amino acid side-chains,

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Supporting Information Available: Additional UVPD and CID spectra, modeling data, and experimental procedures.

patterns which exhibit site selectivity. Hydrogen-rich radical species, such as those produced upon electron capture dissociation (ECD)^[3] and electron transfer dissociation (ETD)^[4] of multi-protonated peptides, conversely, yield high sequence coverage from backbone cleavages at N-C_α bonds, thus affording *c*- and *z*[•]-type ions. In more mechanistic detail, electron capture/transfer dissociation requires the migration of an H atom to a carbonyl group in the peptide backbone prior to cleavage of an N-C_α bond and production of complementary *c* and *z*[•] ions.^[3, 4] This process is thought to involve rearrangement and transfer of α-carbon radicals to backbone carbonyls, initiating a free radical reaction cascade.^[9] To a lesser extent an H atom can be transferred to an amide nitrogen, thus generating *a/y* fragments.^[4] The performance of ECD and ETD is charge state dependent, with lower charge states often exhibiting little dissociation. To induce radical mobility and/or disrupt secondary structure and thus promote more extensive dissociation, supplemental activation of the intact, hydrogen-rich radical species via collisional activation or infrared photoexcitation has been implemented, both causing heating of the radical species.^[10-13]

Vacuum ultraviolet photodissociation (VUVPD) is another emerging activation method which has been used extensively for characterization of protonated peptides^[8,14-20] and is suitable for energizing and dissociating the stable charge-reduced peptides ions produced upon electron attachment reactions, as illustrated in this report. VUVPD, which typically uses an excimer laser operated at a wavelength of 193 or 157 nm, delivers high-energy photons (6.4 or 7.9 eV) that can excite peptides to higher electronic states and/or promote access to other types of fragmentation pathways such as C_α-C(O) bond cleavages, resulting in *a* + 1 or *x* + 1 radical ions that may undergo secondary dissociation into even electron *a*, *x*, *d*, *w*, *v*, and/or *Y* products through losses of CO and various amino acid side-chains.^[8, 14-20] The interesting fragmentation behavior arising from VUVPD has been exploited recently for proteomic applications.^[14]

Several studies of 157 nm and 193 nm UVPD of peptides have noted a correlation between the types and abundances of product ions relative to the location or nature of the charge site of the peptides, such as those possessing a single proton sequestered on either the N- or C-termini (i.e. conventional even electron precursor ions);^[15-20] These studies have demonstrated that *x*, *v*, and *w* product ions are dominant for VUVPD of singly-protonated peptides with a sequestered charge at the C-terminus (i.e. Arg at the C-terminus), whereas *a* and *d* product ions are preferentially formed upon VUVPD of peptides with a sequestered proton at the N-terminus (i.e. Arg at the N-terminus).^[15-20] Similarly, protonated peptides containing Arg at both termini produced a mixture of product ions. Reilly postulated that photolytic radical cleavage of an alpha carbon-carbonyl-carbon bond was the first step of VUVPD (157 nm) of protonated peptides, leading to radical *a* + 1 or *x* + 1 products depending on the initial location of the protonation site of the peptides.^[20] These *a* + 1 or *x* + 1 radicals underwent subsequent radical elimination to produce the observed series of *a* and *d* ions (for those peptides possessing an arginine at the N-terminus) or *x*, *v*, and *w* ions (for those peptides possessing an arginine at the C-terminus). The rapidity of the VUV photolysis left the charge largely sequestered on the original terminal Arg residue, thus resulting in the more selective preference for production of N-terminal or C-terminal series

in comparison to CID.^[20] Russell et al. noted that Cu^+ -cationized peptides showed less specificity for formation of N-terminal or C-terminal ions upon UVPD, presumably due to the similar affinities of Cu^+ for both Arg and Lys at the termini.^[16]

In the present study, we explore the VUVPD and CID patterns of a similar series of peptides in order to evaluate the fragmentation differences between hydrogen-rich radical peptides (generated via initial electron transfer reactions) and their conventional even-electron protonated counterparts (produced directly from ESI). We modified an ETD-enabled linear ion trap mass spectrometer to perform 193 nm UVPD (VUVPD), allowing us to investigate the activation of hydrogen-rich radical peptide ions by the traditional slow-heating collisional activation method (CID) as well as by fast, single photon VUVPD. The fragmentation behavior was evaluated for singly-charged, radical peptide cations formed in the gas phase via electron transfer from fluoranthene anions to doubly protonated peptides and compared to the fragmentation behavior of the corresponding conventional even electron singly protonated peptides. Supplemental Scheme 1 in the supporting information illustrates the main product ions created upon VUVPD and CID of hydrogen-rich radical peptides ($[\text{M}+2\text{H}]^{+\bullet}$) and VUVPD of even-electron peptides ($[\text{M}+\text{H}]^+$) as highlighted in the work herein. The product ions are also structurally defined in the scheme. We focus on the impact of the charge site on the dissociation of peptide radicals possessing a sequestered proton at either an N-terminal or a C-terminal arginine residue.

Results and Discussion

Figure 1a and 1b illustrate the fragmentation patterns obtained upon 193 nm UVPD of $[\text{RVYVHPF} + 2\text{H}]^{+\bullet}$ (generated upon electron transfer reactions of the doubly protonated peptide) and $[\text{RVYVHPF} + \text{H}]^+$ (generated directly by ESI). Interestingly, both the radical and even-electron peptides demonstrated very similar dissociation behavior – yielding several abundant *a*-type ions and a few lower abundance *b* ions. VUVPD of the radical peptide precursors did not result in the *c*-type ions typically associated with ETD and ECD and which are the hallmark of radical-directed cleavages for hydrogen-rich radical peptide cations. Instead, the *a*-type ions upon VUVPD of the peptide radicals are akin to fragment ions expected upon VUVPD of protonated peptides.^[15-20] The production of dominant *a*-type ions for conventional singly protonated peptides upon VUVPD has been noted previously and is rationalized based on sequestration of the proton at the very basic N-terminal Arg.^[15-20] In fact, the only significant difference between the spectra in Figures 1a (radical precursor) and 1b (conventional protonated precursor) was the amino acid side-chain loss specificity and the hydrogen atom content of the a_6 ion. For clarity, these differences are circled in Figure 1a. The hydrogen-rich radical precursor $[\text{RVYVHPF} + 2\text{H}]^{+\bullet}$, for example, favored the radical-directed neutral side-chain loss of histidine (labeled “-H” in Figure 1a), whereas the even-electron peptide favored the loss of the aromatic tyrosine residue (labeled “-Y” in Figure 1b). Thus, UV photoactivation of the singly charged peptide, whether a conventional closed shell species or a radical species, resulted in similar fragmentation behavior. Conversely, CID of $[\text{RVYVHPF} + 2\text{H}]^{+\bullet}$ (a net process termed ETcAD) and $[\text{RVYVHPF} + \text{H}]^+$ yielded very different fragmentation behavior between the radical and even-electron species, as expected based on many past studies of CID versus ETD and the dissociation patterns of odd electron versus even electron ions. For example,

five out of the eight most abundant sequence ions observed upon CID of the hydrogen-rich radical peptide (Figure 1c) arose from cleavages of N-C_α bonds producing *c*- and *z*-type ions, in contrast to the mainly *a* ion series observed upon VUVPD of both the radical and even-electron RVYVHPF precursors (i.e., comparing Figure 1c with Figure 1a and b). CID of the singly-charged, even-electron protonated species, [RVYVHPF + H]⁺, (Figure 1d) yielded mainly the traditional *b*/*y* series with a few *a* ions.

These trends were also observed upon comparison of the CID and VUVPD patterns of other peptides possessing an N-terminal arginine as seen in Figure 2 for RPKPQQFFGLM. Figures 2a and 2b show UVPD spectra of [RPKPQQFFGLM + 2H]⁺⁺ (generated via charge reduction from ETD of the doubly protonated peptide) and [RPKPQQFFGLM + H]⁺ (generated directly upon ESI of the peptide), respectively; and again, these two species yielded similar UVPD fragmentation patterns with a dominant *a*-type ion series. Aside from the two low abundance *a*-type ions observed upon CID of [RPKPQQFFGLM + 2H]⁺⁺ in Figure 2c, the majority of the product ion signals were attributed to N-C_α backbone cleavages that result in *c*- and *z*-type products, species that are typically observed upon fragmentation of radical ions generated upon electron transfer. The even-electron, singly-charged RPKPQQFFGLM was also characterized by CID (Figure 2d), and yielded the expected *b*- and *y*-type products with a few *a* ions that are commonly formed upon collisional activation of protonated peptides. In general, the fragmentation behavior of the hydrogen-rich, radical [RPKPQQFFGLM + 2H]⁺⁺ was significantly different upon 193 nm VUVPD versus CID (comparing Figure 2a and 2c), and the product ion trends were similar to the other N-terminal arginine peptide, RVYVHPF (Figure 1).

The striking variations in the VUVPD and CID fragmentation patterns for the N-terminal arginine peptides reverse upon analysis of peptides with a basic amino acid located at the C-terminus instead of the N-terminus. For example, the 193 nm photodissociation spectrum of the C-terminal arginine radical [YLGYLEQLLR + 2H]⁺⁺ ion (generated via charge reduction from ETD of the doubly protonated peptide) is illustrated in Figure 3a. Interestingly, instead of predominantly C_α-C(O) bond cleavages (giving *a* and *x* products) as observed for peptides with sequestered protons at the N-terminus, mostly N-C_α cleavages occurred, yielding mainly *z*-type ions that are the type typically associated with ECD or ETD (Figure 3a). Lower abundance side-chain loss ions at tyrosine and leucine residues and even lower abundance *w*, *v*, and *c* ions were also observed. In contrast, VUVPD of the even-electron [YLGYLEQLLR + H]⁺ ion (generated directly upon ESI) yielded very different fragmentation behavior as compared to the radical precursor with mostly *w*, *v*, *x*, and *y* sequence ions observed as products (along with the loss of the aromatic tyrosine side-chain) (Figure 3b). This latter behavior (i.e. preferential production of *w*, *v*, *x*, and *y* ions upon VUVPD of conventional protonated C-terminal Arg peptides) has been reported previously, and again is attributed to localization of the proton at the basic Arg.^[16-20] This comparison of the VUVPD dissociation patterns of the even-electron and radical YLGYLEQLLR ions illustrates a notable difference from the results of the N-terminal arginine peptides discussed above in which the dissociation behavior was very comparable between the two types of precursors (i.e. both even electron and radical precursors produced similar *a* and *b* ions) upon VUVPD. Low energy CID of [YLGYLEQLLR + 2H]⁺⁺ as shown in Figure 3c

generated mostly z ions and a few b and c sequence ions as well as products arising from the loss of the tyrosine or leucine side-chains. Overall, this CID spectrum was very similar to the VUVPD spectrum of the same peptide species (comparing Figure 3a with 3c), which is again vastly different from the trends observed for the N-terminal arginine peptides described above. CID of protonated YLGYLEQLLR (Figure 3d) yielded the typical b/y sequence ion series. It is worth noting that the z ions generated from VUVPD of $[\text{YLGYLEQLLR} + 2\text{H}]^{+\bullet}$ (Figure 3a) are presumed to occur from radical-directed processes (ETD) as they match the fragmentation ion abundances and hydrogen migration distributions observed for CID of $[\text{YLGYLEQLLR} + 2\text{H}]^{+\bullet}$ (Figure 3c), and are different from the low abundance z -type (or the isobaric $y_n - \text{NH}_3$) ions occasionally observed upon VUVPD for singly-charged, even-electron peptides.^[17,20] The latter z -type ions have been detected infrequently among contiguous series of v,z,y,x ions in several VUVPD mass spectra^[17,20] and have not been observed as the prominent, dominant products like those seen in Figures 3a and Supplemental Figure 1a, thus suggesting a different explanation for their origin in the present study.

The general fragmentation trends and differences described above for YLGYLEQLLR also hold true for other peptides with C-terminal arginine residues as seen in Supplemental Figure 1 for HQGLPQEVLENLLR. That is, VUVPD of the radical and even-electron HQGLPQEVLENLLR ions resulted in different dissociation channels (comparing Supplemental Figure 1a and 1b), whereas CID and VUVPD of $[\text{HQGLPQEVLENLLR} + 2\text{H}]^{+\bullet}$ yielded similar ion series that consisted of mainly c - and z -type ions (comparing Supplemental Figure 1a and 1c). The only notable difference between the two activation methods for the radical $[\text{HQGLPQEVLENLLR} + 2\text{H}]^{+\bullet}$ precursor was that VUVPD generated an abundant Y_{II} (and to a lesser extend y_{II}) ion via cleavage at the N-terminal side of the proline residue. The production of Y ions evolves from photon-induced secondary dissociation of $x + I$ radicals, generating products 2 Da less than traditional y -type ions, and the cleavage selectivity is especially enhanced N-terminal to proline residues.

The work herein showcases the first results of activating singly-charged, hydrogen-rich peptide radicals by 193 nm photodissociation. The VUVPD fragmentation behavior proved to be highly dependent on the location of the basic arginine residue (i.e., the location of the sequestered proton), a factor known to influence the VUVPD behavior of conventional protonated peptides^[15-20] but in an unanticipated way for the peptide radicals reported herein. That is, when the proton was sequestered at the N-terminus for the peptide radicals, $\text{C}_\alpha\text{-C}(\text{O})$ bond cleavage was preferential, yielding a -type ions that are similar to those observed upon VUVPD of protonated peptides,^[15-20] but when the proton was sequestered at the C-terminus, N- C_α cleavages were favored, leading to z - and c -type products that are more typically observed upon dissociation of hydrogen-rich radical ions (as for ETD, ECD, or ETcAD). Upon conventional ECD or ETD, z - and c -type product ions are normally dominant, although a -type ions, less commonly observed for conventional peptides, have been detected for ϵ -peptides, cyclic peptides, and ones that may adopt zwitterionic structures.^[21-23] The formation of a -type ions has been rationalized based on the possibility of amide-nitrogen protonation (instead of solely protonation of basic side chains and the N-terminus) and/or hydrogen loss accompanied by amide bond cleavage and CO elimination,

thus circumventing the primary *c/z* pathways.^[21-23] Upon VUVPD of the peptide radicals, specifically ones with N-terminal basic residues, in the present study the formation of *a*-type ions is far more prevalent than observed upon ETD alone or by ETcaD, suggesting that this pathway is specific to the UV photoactivation process.

Jarrold and coworkers have evaluated the gas-phase conformations of peptides via ion mobility measurements and have shown that peptides protonated at the C-terminus exhibit helical motifs whereas peptides protonated at the N-terminus adopt compact globular structures.^[24] We hypothesized that these types of conformational differences might also play a role in the dissociation behavior reported herein. We undertook gas-phase molecular dynamics simulated annealing of RAAYAHAF₂A and AAF₂AHAYAAR, two peptides that displayed the same trends observed upon VUVPD and CID of the radical and even-electron species as described for the previous peptides (see spectra in Figure 4 for RAAYAHAF₂A and AAF₂AHAYAAR). The resulting conformations of both the zwitterionic and non-zwitterionic forms are shown in Figure 5; the total potential energy and radius of gyration averaged over ten simulated annealing runs are given in Supplemental Table 1. As seen in Figure 5a and 5c, RAAYAHAF₂A yielded compact globular conformations for both zwitterionic and non-zwitterionic forms; however, AAF₂AHAYAAR yielded a significantly more elongated and helical motif for the non-zwitterionic conformation (Figure 5b). Being that singly-charged peptides containing one highly basic site at the C-terminus (like most tryptic peptides) are rarely zwitterions,^[25] the elongated helical motif is a more likely gas-phase structure for AAF₂AHAYAAR. Thus, the efficient C_α-C(O) bond cleavage that produces *a*-type ions may be favored to occur from more compact globular conformations like the ones observed for N-terminal arginine containing peptides, possibly due to their more exposed UV-absorbing aromatic residues and/or amide bonds stemming from less backbone hydrogen-bonding compared to that found in helical, elongated motifs. Also, upon UV excitation of the odd electron peptide cations, the more compact, globular conformations with extensive intramolecular hydrogen-bonding may dissociate prior to unraveling to a more elongated form, thus allowing access to unique fragmentation pathways such as the formation of *a*-type ions that might not normally be anticipated upon dissociation of radical ions. The impact of conformational differences on the dissociation of the hydrogen rich peptide radicals may be minimized during the lower energy, step-wise excitation process of CID compared to UVPD because of opportunities for peptide unfolding prior to dissociation. The photon-directed C_α-C(O) bond cleavage process likely occurs from excited energy states independent of any radical-directed dissociation behavior. Otherwise, the VUVPD and CID patterns of the hydrogen-rich radical species would be similar. These observations do not require invoking new reaction mechanisms for ETD/ECD or VUVPD or the hybrid ET-VUVPD method. Instead, the results suggest that differences in the observed dissociation pathways of the N-terminal Arg peptide radicals and the C-terminal Arg peptide radicals upon VUVPD versus CID may be related to their favored conformations in the gas phase and how fast they unfold and facilitate the radical cascade reaction cascade^[9,28-31] relative to the rapid, high-energy activation and dissociation promoted by VUV photoexcitation.

Support for the impact of the aromatic residues is derived from an analogous UVPD and CID comparison of the simpler model peptides, RAAAAAAAAA and AAAAAAAAAAR, ones having no aromatic chromophores. Molecular dynamics simulations indicate that these peptides adopt conformations similar to the ones displayed in Figure 5, with a more compact, globular form for RAAAAAAAAA and a more helical conformation for AAAAAAAAAAR (see Supplemental Figure 2). However, the UVPD spectrum of the hydrogen-rich RAAAAAAAAA does not exhibit the dominant *a* ions observed for all the other N-terminal Arg peptides that contain aromatic side-chains. Instead, the UVPD spectrum displays *b* ions as the most abundant products for the even-electron peptide and *c* ions for the radical species (similar to CID, data not shown). Apparently the lack of aromatic chromophores alters the populations of key excited states upon UV excitation, thus excluding the *a*-type fragmentation pathways.

Furthermore, it has been hypothesized that the intact, charge-reduced radical species generated from ETD/ECD are *c*- and *z*-type products held together by non-covalent interactions, and upon supplemental activation, the noncovalent bonds are broken, thus producing mainly *c* and *z* ions.^[10, 11, 13, 26, 27] Moreover, it has been alternatively proposed that supplemental activation can re-ignite the cascade of radical rearrangement reactions, entailing intramolecular hydrogen migrations and transfer of α -carbon radicals that are essential for radical-mediated dissociation, thus propagating additional *c*- and *z*-ion pathways.^[9, 28-31] Our experiments herein yield further evidence that ETD does not occur immediately following the electron transfer process and that the intact radical species is mostly comprised of the intact peptide with a localized radical. Upon supplemental collision activation, this radical could migrate during peptide dissociation and yield mainly *c* and *z* ions.^[9] The evidence for this type of dissociation is clear for peptides with N-terminal arginines in which dissociation via VUVPD likely occurs in the excited state, and *a*-type ions are preferential regardless whether the precursor is a radical or even-electron species (i.e., the spectra are similar). The fragmentation behavior would be considerably different between the two species if the intact radical precursor was comprised of mainly *c*- and *z*-type products held together by noncovalent interactions, and even for peptides with C-terminal arginines, a significant number of internal fragments would be expected.

The results above demonstrate the utility of combining both ETD and VUVPD into a hybrid method to gain insight into the peptide activation mechanisms for each technique. Moreover, the unique blend of competitive radical- and photon-directed dissociation pathways, which are dependent upon charge location, opens up new avenues for generating richer and more informative spectra that may increase confidence in peptide scoring for *in silico* proteomics applications. ETD has shown great promise in the determination of post-translational modifications (especially phosphorylation),^[4, 32] but has proven to be less effective for characterization of singly charged peptides (due to neutralization) or doubly charged peptides (in which charge reduction without dissociation is dominant).^[10] Since VUVPD produces particularly rich diagnostic fragmentation patterns for low charge states,^[8] the ET-VUVPD hybrid method seemed like a natural fit for the confident identification of peptide phosphorylation sites. Therefore, this method was applied to characterize the kinase domain of human TRPM7 channel-kinase, containing the last 462 amino acids (1403-1864) of

TRPM7/ChaK1 (GenBankTM accession number AF346629). This channel-kinase plays critical functional roles in neurodegenerative disorders,^[33] anoxic cell death,^[34] and hypomagnesemia;^[35] furthermore, after autophosphorylation, TRPM7 has been shown to be massively phosphorylated.^[36] LC-MS/MS utilizing ETD (MS²) followed by either VUVPD (ET-VUVPD) or CID (ETcaD)^[10] was employed for analysis of the charge-reduced radical products (MS³) from two picomoles of trypsinized TRPM7/ChaK1. A total of six replicate LC-MS/MS runs were performed (three for ET-VUVPD and three for ETcaD). Figure 6a illustrates a representative ETD spectrum from the phosphopeptide ion [AALIPVWLQDRPpSNR + 2H]²⁺, which exemplifies the lack of diagnostic information generated from ETD of lower charged peptide precursors. Neither the peptide sequence nor the site of modification could be identified from this spectrum. However, upon activation of the [M + 2H]⁺ ion by subsequent VUVPD, a very rich diagnostic set of product ions are generated that can be used to identify both the peptide sequence and the site of phosphorylation modification (Figure 6b). The spectrum in Figure 6b is an example of the type of high quality data that can be obtained via ET-VUVPD on a chromatographic timescale, even for low concentration proteins. As seen in Table 1 (and discussed in more detail in the next section), the peptide AALIPVWLQDRPpSNR was not identified by automated spectral analysis when ETcaD was used as the activation technique.

Table 1 compares the TRPM7/ChaK1 phosphopeptide identifications from ET-VUVPD, ETcaD, and ETD after automated database searching using the MassMatrix^[37, 38] (MM) algorithm (for which more experimental details are included in supplemental information). In total, ET-VUVPD identified eight phosphopeptides (five unique identifications) from three replicate runs, ETcaD identified six phosphopeptides (five unique identifications) from three replicate runs, and ETD identified seventeen phosphopeptides (five unique identifications) from all six runs. These peptides were filtered by strict MM scoring thresholds (see supplemental information), and each spectrum was manually validated. Interestingly, ET-VUVPD excelled for singly-charged, hydrogen-rich radical precursors while ETcaD excelled for doubly-charged, radical species (see Table 1 and Supplemental Figure 3). This observation supports the ion activation dogma that VUVPD yields the richest spectra for singly-charged peptides whereas CAD frequently offers greater success for activation of multiply-charged ions. A total of eight, nine, and eleven unique phosphopeptides were identified when combining ET-VUVPD with ETD, ETcaD with ETD, or integrating the results from all three methods, respectively. Clark et al. previously expressed residues 1158-1864 of TRPM7 in HEK 293 cells and used mass spectrometry to identify its phosphorylation sites.^[36] In the present study a smaller protein TrpM7-KD (aa 1403-1864) was purified following over-expression in *E. coli*. Three new phosphorylation sites were identified in this protein in the present study, none of which had been reported. These sites include Thr-1430, Thr-1631 and Ser-1787. Interestingly, two out of the three novel phosphorylation sites (Thr-1430 and Ser-1787) were identified by ET-VUVPD from the peptides ApTEGDNTEFGAFVGHHR and pSCDMVFGPANLGEDAIAK, and ETD alone identified two of the three sites (Thr-1631 and Ser – 1787) from the peptides VQCpTWSEHDILK and RpSCDMVFGPANLGEDAIAK with Ser-1787 identified by both ET-VUVPD and ETD. While ETcaD identified no novel phosphorylation sites, it did yield useful complementary information that strengthened the confidence of other

phosphorylation site determinations. Thus, the most structurally meaningful information was achieved by utilizing all three radical-directed dissociation methods (ETD, ET-VUVPD and ETcaD). We anticipate that further improvements in phosphopeptide characterization will be gained by “training” automated searching algorithms to recognize the specific fragmentation behavior produced by ET-VUVPD (i.e., spectra that include *w*, *v*, and other side-chain loss product ions). However, designing a new scoring model for ET-VUVPD spectra is beyond the scope of this study.

Conclusions

Vacuum ultraviolet photodissociation (VUVPD) at 193 nm was implemented on an ETD-enabled ion trap mass spectrometer to investigate the fragmentation behavior of hydrogen-rich radical peptide cations and aid in the understanding of the processes that drive radical- and photon-directed dissociation. The VUVPD fragmentation behavior of the peptide radicals proved to be highly dependent on the location of the sequestered proton. That is, when the proton was sequestered at the N-terminus, C α -C(O) bond cleavage was preferential, yielding mainly *a*-type ions similar to the dominant ions observed upon VUVPD of conventional protonated peptides, and when the proton was sequestered at the C-terminus, N-C α cleavages were favored, leading to *z*- and *c*-type products that are commonly generated by ECD or ETD. This charge-site dependent outcome was attributed to the initial conformation of the peptide (compact, globular versus elongated as supported by molecular dynamics simulated annealing) and the resulting differences in dissociation pathways upon fast, high energy UV activation. This hybrid ETD/VUVPD method (ET-VUVPD) was employed to confidently identify novel phosphorylation sites of the kinase domain of human TRPM7/ChaK1 when coupled with LC-MS/MS and automated database searching.

Experimental Section

Materials

Competent cells used for amplification and expression were obtained from Novagen (Gibbstown, NJ). Ni-NTA Agarose, QIAprep Spin Miniprep Kit, QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit were provided by Qiagen (Valencia, CA). Native Pfu DNA Polymerase was purchased from Stratagene (La Jolla, CA). Enzymes for restriction digestion and ligation were obtained from New England BioLabs (Ipswich, MA) and Invitrogen Corporation (Carlsbad, CA), respectively. Yeast extract, tryptone and agar were purchased from US Biological (Swampscott, MA). The entire buffer components used for protein purification and experimental procedures were obtained from Sigma (St. Louis, MO) and Fisher scientific. RVYVHPF, α -casein, trypsin, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Amersham Biosciences (Pittsburgh, PA) provided the FPLC system and the columns for purification. YLGYLEQLLR and HQGLPQEVLENLLR were generated by trypsin digestion of α -casein using a 1:20 enzyme:substrate ratio and an incubation time and temperature of 16 hours and 37° C. All solvents were purchased from Fisher Scientific (Fairlawn, NJ). RAAYAHAFAA, AAFAHAYAAR, RAAAAAAAAA, and AAAAAAAAAAR were synthesized by GenScript (Piscataway, NJ). Human TRPM7 channel-kinase, containing the last 462 amino acids

(1403-1864) of TRPM7/ChaK1 (GenBankTM accession number AF346629), was expressed in *E. coli*, as described in Supplemental Information.

Mass Spectrometry, Liquid Chromatography, and Automated Spectral Analysis

An ETD-enabled, Thermo Fisher Scientific (San Jose, CA) LTQ XL linear ion trap (LIT) mass spectrometer was modified to perform 193 nm UVPD (VUVPD). Fluoranthene was used as the reagent anion with ETD reaction times of 100 ms. Photons entered the LIT through a 1.8 cm hole that was drilled into the center of the ion volume (located inside the ETD ion optics). VUVPD was performed with a Coherent ExciStar XS excimer laser (Santa Clara, CA) operated at 193 nm. The laser setup was similar to that previously described,^[39] except a CaF₂ lens was used to transmit 193 nm photons into the LIT. For all VUVPD experiments, the laser was pulsed once per scan with an energy of 8 mJ/pulse and a pulse duration of 5 ns (the actual activation period allowed by the LTQ software and hardware was 30 μ s). A q-value of 0.1 was also used. ET-VUVPD (MS³) experiments were carried out using ET reaction times of 100 ms followed by isolation and subsequent VUVPD of the charge-reduced, hydrogen-rich radical. Standard parameters (35% NCE, q-value equal to 0.25 and activation times of 30 ms) were used for all MS² CID experiments. ETcaD (MS³) experiments were carried out using ET reaction times of 100 ms followed by isolation and subsequent “soft” CID (20% NCE and a q-value of 0.16) of the charge-reduced, hydrogen-rich radical. Model peptides and protein digests (10 μ M) were directly infused at 3 μ L/min.

TRPM7/ChaK1 was digested with trypsin at a 1:20 enzyme:substrate ratio (pH of ~8) overnight. Liquid chromatography was performed using a Dionex UltiMate 3000 RSLCnano system (Sunnyvale, CA), and a Dionex Acclaim PepMap column (75 μ m $\times$ 15 cm, 3 μ m particle size). Eluent A consisted of 0.1% formic acid in water and eluent B 0.1% formic acid in acetonitrile. A linear gradient from 5% eluent B to 40% eluent B over 60 min at 300 nL/min was used. Samples were injected at approximately two picomoles of digested protein. Data-dependent nanoLC-MS/MS was performed as follows: the first event was the full mass scan (m/z range of 400 - 2000) in the positive mode followed by ETD on the most abundant ion and then either VUVPD or CID on the most abundant ion from the ETD scan (this cycle was repeated twice before a new full mass scan was performed). The maximum injection time for full mass scans and MS/MS events was set to 100 ms, the dynamic exclusion duration was 50 s, and the exclusion list size allowed for 500 specified m/z values. A single repeat count was used. ETD (MS²), ET-VUVPD (MS³), and ETcaD (MS³) parameters were the same as described above. Full mass and MS² spectra were the culmination of three averages, and MS³ spectra were the culmination of ten averages.

MassMatrix^[37, 40, 41] was used for automated LC-MS/MS analysis. A precursor mass tolerance of 3.0 Da, and a fragment mass tolerance of 1.0 Da were used for processing. Phosphorylation of serine, threonine, and tyrosine were set as variable side-chain modifications. Experimental MS² and MS³ spectra were searched against a database consisting of the TRPM7/ChaK1 sequence. Peptide hits were filtered based on a minimum pp score of 5.0, a minimum length of six amino acids, a maximum peptide ranking of one, and manual validation of spectra. The pp score is a statistical measure of the number of matched product ions in an experimental spectrum. Whereas, the pp2 score is a statistical

measure of the total abundance of matched product ions in an experimental spectrum. Prior to MassMatrix searching, MATLAB (Natick, MA) script was written to subtract undissociated precursor ions from both ET-VUVPD (MS³) and ETcaD (MS³) spectra. The *m/z* range less than 300 was also eliminated for ET-VUVPD (MS³) spectra to reduce searching interference from photoionization background.

Peptide Modeling

Molecular dynamics simulated annealing was used to obtain representative low energy conformations of the peptides in gas phase. These calculations were performed using the TINKER molecular modeling package^[42] with the force field AMBER ff99.^[43] By performing annealing (i.e., slow cooling) from a high temperature, the peptide is able to surmount energetic barriers towards lower energy conformations. This method has been used in a number of gas phase peptide studies.^[44–47] Ten copies of each of these peptides were annealed from a starting temperature of 1000 Kelvin and slowly cooled towards 0 Kelvin. The temperature was maintained using the stochastic thermostat developed by Bussi et al.^[48] These calculations were performed over 5×10^7 steps with a time step of one femtosecond using the modified Beeman integrator.^[49] The low energy conformations were visualized and plotted with Visual Molecular Dynamics (VMD).^[50] Zwitterions were modeled with protonation at arginines and N-termini, and deprotonation at C-termini. Peptide variants with neutral capping groups were modeled to mimic non-zwitterions (protonation only at arginine) where an acetyl cap was used on the N-terminus while an amide cap was used on the C-terminus. The resulting low energy conformations were further characterized by the radius of gyration, R_g (see Supplemental Table 1). The quantity R_g represents the root mean squared distance of each atom of the peptide from the centroid of the peptide—i.e., the mean position of all atoms of the peptide. In general, this quantity encapsulates the length scale of the volume that the peptide occupies in space. For example, a small radius of gyration suggests that the peptide occupies a small volume in space whereas a large radius of gyration suggests that the peptide occupies a large volume in space.

Supplementary Material

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Acknowledgments

J.S.B. acknowledges funding from the NSF (CHE-1012622), and the Welch Foundation (F-1155). D.E.M and R.R.C acknowledge Welch Foundation (F-1514) and the NSF (CHE 0848571). KND acknowledges funding from the NIH (GM 59802) and the Welch Foundation (F-1390). We would also like to thank Dr. Alexey Ryazanov for providing the TRPM7/Chak1 plasmid DNA, and Andrew P. Horton for spectral processing MS³ data from LC-MS/MS runs.

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Figure 1.

193 nm VUVPD of (a) [RVYVHPF + 2H]⁺• (m/z 918, generated via charge reduction from ETD of the doubly protonated peptide) and (b) [RVYVHPF + H]⁺ (m/z 917, generated directly upon ESI of the peptide). CID of (c) [RVYVHPF + 2H]⁺• (m/z 918, generated via charge reduction from ETD of the doubly protonated peptide) and (d) [RVYVHPF + H]⁺ (m/z 917, generated directly upon ESI of the peptide). Products with a superscript letter (e.g., a^Y) represent a loss of the corresponding amino acid side-chain from the sequence ion. Water and ammonia loss are denoted by a degree sign (°) and an asterisk (*), respectively.

Figure 2.

193 nm UVPD of (a) $[RPKPQQFFGLM + 2H]^+ \bullet$ (m/z 1350, generated via charge reduction from ETD of the doubly protonated peptide) and (b) $[RPKPQQFFGLM + H]^+$ (m/z 1349, generated directly upon ESI of the peptide) and CID of (c) $[RPKPQQFFGLM + 2H]^+ \bullet$ (m/z 1350, generated via charge reduction from ETD of the doubly protonated peptide) and (d) $[RPKPQQFFGLM + H]^+$ (m/z 1349, generated directly upon ESI of the peptide). Radical product ions are denoted with $\bullet$. The mixture of a_n+1 and a_n+2 species are symbolized by $a_n+1(2)$. Water and ammonia loss are denoted by a degree sign ($^\circ$) and an asterisk (*), respectively.

Figure 3.

193 nm VUVPD of (a) [YLGYLEQLLR + 2H]⁺• (m/z 1269, generated via charge reduction from ETD of the doubly protonated peptide) and (b) [YLGYLEQLLR + H]⁺ (m/z 1268, generated directly upon ESI of the peptide) and CID of (c) [YLGYLEQLLR + 2H]⁺• (m/z 1269, generated via charge reduction from ETD of the doubly protonated peptide) and (d) [YLGYLEQLLR + H]⁺ (m/z 1268, generated directly upon ESI of the peptide). Products with a superscript letter represent a loss of the corresponding amino acid side-chain from the sequence ion. Water and carbon dioxide loss are denoted by a degree sign (°) and an asterisk (*), respectively.

Figure 4.

193 nm UVPD of (a) $[\text{RAAYAHAF AA} + 2\text{H}]^{+\bullet}$ (m/z 1050, generated via charge reduction from ETD of the doubly deprotonated peptide) and (b) $[\text{RAAYAHAF AA} + \text{H}]^+$ (m/z 1049, generated directly upon ESI of the peptide), and CID of (c) $[\text{RAAYAHAF AA} + 2\text{H}]^{+\bullet}$ (m/z 1050, generated via charge reduction from ETD of the doubly deprotonated peptide) and (d) $[\text{RAAYAHAF AA} + \text{H}]^+$ (m/z 1049, generated directly upon ESI of the peptide). Ammonia loss are denoted by an asterisk (*). Radical product ions are denoted with $\bullet$. The mixture of a_n+1 and a_n+2 species are symbolized by $a_n+1(2)$. 193 nm UVPD of (e) $[\text{AAFAHAYAAR} + 2\text{H}]^{+\bullet}$ (m/z 1050, generated via charge reduction from ETD of the doubly deprotonated peptide) and (f) $[\text{AAFAHAYAAR} + \text{H}]^+$ (m/z 1049, generated directly upon ESI of the peptide), and CID of (g) $[\text{AAFAHAYAAR} + 2\text{H}]^{+\bullet}$ (m/z 1050, generated via charge reduction from ETD of the doubly deprotonated peptide) and (h) $[\text{AAFAHAYAAR} + \text{H}]^+$ (m/z 1049, generated directly upon ESI of the peptide).

Figure 5.

Molecular dynamics simulated annealing of Ac-RAAYAHAF-FAA-NH₂, (b) Ac-AAFAHAYAA-AR-NH₂, (c) RAAYAHAF-FAA, and (d) AAFAHAYAA-AR in which acetylation (Ac) of the N-terminus and amidation of the C-terminus is incorporated to prevent protonation or deprotonation, respectively, and zwitterion formation. For each peptide, the four lowest conformations are plotted together (the lowest is illustrated as dark gray). Arginine is shown using a stick representation.

Figure 6.

(a) ETD of the TRPM7/ChaK1 phosphorylated peptide (a) $[AALIPVWLQDRPpSNR + 2H]^{2+}$ and (b) 193 nm VUVPD of $[AALIPVWLQDRPpSNR + 2H]^+•$ (after 100 ms of ETD) collected during back-to-back LC-MS/MS scans. The precursor m/z values for the doubly-charged, even-electron and singly-charged, radical species were 908 and 1816, respectively. Phosphoric acid loss is denoted by “-”.

Table 1

Phosphopeptide identification from TRPM7/ChaK1 using ET-VUVPD, ETcaD, and ETD. ET-VUVPD and ETcaD MS³ spectra were collected from three replicate LC-MS/MS runs each, while ETD MS² spectra were the culmination of all six runs (from both ET-VUVPD and ETcaD runs).

Precursor	Method	pp	pp ₂	m/z	Phosphorylated Peptide	Phosphosite
[M+2H] ⁺⁺	ET-VUVPD	5.7	5.6	1789.1	ApTEGDNTEFGAFVGHR	Thr-1430
[M+2H] ⁺⁺	ET-VUVPD	8.5	11.0	1528.3	ILpSNNNTSENTLK	Ser-1463
[M+2H] ⁺⁺	ET-VUVPD	14.7	11.4	1816.5	AALIPVWLQDRPpSNR	Ser-1526
[M+3H] ⁺⁺⁺	ET-VUVPD	16.3	11.4	1818.3	AALIPVWLQDRPpSNR	Ser-1526
[M+2H] ⁺⁺	ET-VUVPD	8.7	5.8	1519.3	LSQpSIPFTPVPPr	Ser-1568
[M+2H] ⁺⁺	ET-VUVPD	10.7	7.6	1519.6	LSQpSIPFTPVPPr	Ser-1568
[M+2H] ⁺⁺	ET-VUVPD	7.9	4.8	1843.8	pSCDMVFGPANLGEDAik	Ser-1787
[M+2H] ⁺⁺	ET-VUVPD	7.9	6.4	1845.1	pSCDMVFGPANLGEDAik	Ser-1787
[M+2H] ⁺⁺	ETcaD	9.4	3.2	1175.0	FKETpSNKIK	Ser-1456
[M+2H] ⁺⁺	ETcaD	12.8	10.0	1528.1	ILpSNNNTSENTLK	Ser-1463
[M+2H] ⁺⁺	ETcaD	17.0	15.0	1529.0	ILpSNNNTSENTLK	Ser-1463
[M+4H] ²⁺⁺	ETcaD	14.7	2.4	1690.8	RPSTEDTHEVDpSKAALIPVWLQDRPpSNR	Ser-1512 Ser-1526
[M+3H] ²⁺⁺	ETcaD	12.5	2.4	1211.6	LSQpSIPFTPVPPrGEPVTVYR	Ser-1568
[M+3H] ²⁺⁺	ETcaD	9.0	3.1	1103.3	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	17.5	11.6	606.2	AALIPVWLQDRPpSNR	Ser-1526
[M+3H] ³⁺	ETD	17.1	11.6	606.5	AALIPVWLQDRPpSNR	Ser-1526
[M+3H] ³⁺	ETD	20.8	12.9	606.6	AALIPVWLQDRPpSNR	Ser-1526
[M+3H] ³⁺	ETD	15.9	10.4	606.6	AALIPVWLQDRPpSNR	Ser-1526
[M+3H] ³⁺	ETD	18.2	9.9	507.3	LSQpSIPFTPVPPr	Ser-1568
[M+3H] ³⁺	ETD	21.4	10.1	507.3	LSQpSIPFTPVPPr	Ser-1568
[M+3H] ³⁺	ETD	21.4	11.7	507.3	LSQpSIPFTPVPPr	Ser-1568
[M+3H] ³⁺	ETD	7.0	2.9	512.9	VQCpTWSEHDILK	Thr-1631
[M+3H] ³⁺	ETD	13.4	2.2	736.3	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	15.2	2.4	736.3	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	15.9	5.9	736.4	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	11.2	4.3	736.4	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	12.5	4.0	736.4	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	15.0	2.5	736.5	LTFAFNQMKPKpSIPYSPR	Ser-1694
[M+3H] ³⁺	ETD	5.5	2.6	667.5	RpSCDMVFGPANLGEDAik	Ser-1787
[M+3H] ³⁺	ETD	5.6	1.9	667.5	RpSCDMVFGPANLGEDAik	Ser-1787
[M+3H] ³⁺	ETD	7.0	1.9	667.9	RpSCDMVFGPANLGEDAik	Ser-1787