Published in final edited form as:

Proteomics. 2011 April ; 11(7): 1329–1334. doi:10.1002/pmic.201000565.

193 nm Photodissociation of Singly and Multiply Charged Peptide Anions for Acidic Proteome Characterization

James A. Madsen¹, Tamer S. Kaoud², Kevin N. Dalby², and Jennifer S. Brodbelt^{1,*}

¹ Department of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station A5300, Austin, TX, USA 78712

² Division of Medicinal Chemistry, The University of Texas at Austin, 107 West Dean Keaton, Biomedical Engineering Building, Austin, TX, USA 78712

Abstract

193 nm ultraviolet photodissociation (UVPD) was implemented to sequence singly and multiply charged peptide anions. Upon dissociation by this method, *a*-/*x*-type, followed by *d* and *w* side-chain loss ions, were the most prolific and abundant sequence ions, often yielding 100% sequence coverage. The dissociation behavior of singly and multiply charged anions was significantly different with higher charged precursors yielding more sequence ions; however, all charge states investigated (1- through 3-) produced rich diagnostic information. UVPD at 193 nm was also shown to successfully differentiate and pinpoint labile phosphorylation modifications. The sequence ions were produced with high abundances, requiring limited averaging for satisfactory spectral quality. The intact, charge-reduced radical products generated by UV photoexcitation were also subjected to collision induced dissociation (termed, activated – electron photodetachment dissociation (a-EPD)), but UVPD alone yielded more predictable and higher abundance sequence ions. With the use of a basic (pH ~11.5), piperidine-modified mobile phase, LC-MS/UVPD was implemented and resulted in the successful analysis of mitogen-activated pathway kinases (MAPKs) using ultrafast activation times (5 nanoseconds).

Keywords

MS/MS; Photodissociation; Anions; Phosphopeptides; Basic HPLC

Proteome characterization by mass spectrometry (MS) most frequently relies upon the enzymatic digestion of proteins into peptides, followed by tandem mass spectrometric sequencing of peptide cations [1]. This popular protocol has an intrinsic bias towards basic peptides that easily ionize under acidic HPLC conditions and positive polarity MS settings. Being that approximately 50% of peptides/proteins are acidic in nature and that many of the most important PTMs are also acidic (e.g., phosphorylation, sulfonation, etc.) [2, 3], there is a critical need for better analytical methodologies for characterization of the acidic proteome. This deficiency in methods is largely due to the lack of tandem MS techniques available for efficient and predictable dissociation of peptide anions. For example, CID of peptide anions generates spectra that are dominated by neutral losses from both precursor and product ions [4], yielding unforeseen fragmentation behavior and insufficient peptide sequence information (see Supplementary Fig. 1). Due to these shortcomings of CID, several new MS/MS methods have recently been developed to improve peptide anion

jbrodbelt@mail.utexas.edu.

The authors have declared no conflict of interest.

sequencing. Zubarev and coworkers have reported electron detachment dissociation (EDD) that uses high energy electrons to induce informative backbone cleavages of peptide anions [2]. Negative electron transfer dissociation (NETD) in which peptide anions are reacted with electron-deficient radical cations, causing electron transfer from the peptide to the reagent and promoting radical-directed dissociation, has likewise shown promise for peptide sequencing [5, 6]. A photon-based activation technique, activated-electron photodetachment dissociation (a-EPD), uses UV irradiation at 262 nm to produce charge-reduced peptide radical anions that are subsequently dissociated by CID [7]. While these methods have made significant inroads into the characterization of peptide anions, there still remain many hurdles. EDD and a-EPD, for example, are generally low efficiency dissociation methods that require long averaging times to produce reasonable spectra [8]. Also, the activation times required for these methods range from half a second up to multiple seconds for peptide dissociation, impeding the integration of these methods with liquid chromatography timescales [3, 6, 7]. The fragmentation patterns frequently yield many high abundant neutral losses from both parent and product ions which clutter the spectra and are unpredictable [3, 5, 6]; this outcome confounds peptide sequencing and identification. Thus, improvement of these methods or development of alternatives is essential to fully exploit the potential of characterizing the important acidic portion of the proteome. Herein, we report the use of 193 nm photons for peptide anion dissociation of singly and multiply charged species.

Coupling an ArF excimer laser to a linear ion trap mass spectrometer allows implementation of ultraviolet photodissociation (UVPD) at 193 nm as an alternative to CID (details on the experimental setup can be viewed in the supporting information), yielding rich and informative spectra with high sequence coverage from predictable fragmentation patterns (see Fig. 1a). As seen in this UVPD spectrum of the doubly charged aquaporin peptide, the main sequence ions observed are from the complementary a/x series, which are present in high abundance and large numbers. Occasionally, hydrogen migration occurs in conjunction with the formation of these a/x products, leading to hydrogen-rich or hydrogen-deficient ions denoted as $a_n/x_n \pm 1$ in the spectral figures. The conventional a/x ions are more consistently observed. A few c ions and side-chain loss ions (e.g., d , v , ions) are also seen in the spectrum as well as Y -type ions, which originate from backbone cleavages N-terminal to proline; Y ions are produced from radical x -ions and have been observed previously upon vacuum UVPD of peptide cations [9]. The combination of these sequence ions originating from both N- and C-termini resulted in 100% sequence coverage, and also allowed confident assignment of the site of phosphorylation. An interesting characteristic of the spectrum shown in Fig. 1a is the highly abundant charge-reduced, radical species (labeled as $[M - H]^{-}$), which results from photo-induced electron detachment from the selected peptide precursor. The observation of these types of charged-reduced products was reported previously upon UVPD at 262 nm; subsequent application of CID to these charge-reduced products (termed a-EPD) was used to gain additional peptide sequence information [7]. An example a-EPD spectrum (using 193 nm photoexcitation) is shown in Supplementary Fig. 2.

Because ion activation/dissociation is often charge-state dependent, comparisons of the UVPD spectra were undertaken for peptide anions in different charge states. Fig. 1b shows the UVPD spectrum of singly charged RQpSVELHSPQSLPR compared to the UVPD spectra of the doubly charged ion in Fig. 1a. The sequence coverage is slightly lower (85%, corresponding to 11/13 possible backbone cleavages) for the 1- charge state than that obtained for the 2- charge state (100% sequence coverage). Interestingly, a very abundant product ion, $y_{12}^{#*}$, is observed in Fig. 1b and is attributed to the combined loss of phosphoric acid and ammonia from the y_{12} sequence ion. This product arises from the backbone cleavage adjacent (N-terminal side) to the phosphorylated serine residue, suggesting that the highly acidic phosphate group may promote an unusual specific charge dependent cleavage at phosphorylated residues. The same y -type ion with phosphoric acid

and ammonia loss was observed for UVPD of the phosphorylated VPQLEIVNPpSAEER peptide as well (data not shown). In this case, an abundant $y_5^{#*}$ was formed. This preferential pathway could be very useful in pinpointing phosphorylation sites in peptide anions.

Each of the three charge states of the pp60c-src peptide, TSTEPQpYQPGENL, were also subjected to UVPD (Supplementary Fig. 3). Each charge state yielded many abundant a - and x -type ions and high sequence coverage (92%, corresponding to 11/12 possible backbone cleavages) as summarized at the top of Supplementary Fig. 3. The site of phosphorylation could be readily pinpointed due to this high sequence coverage. A particularly salient feature of the UVPD spectrum of the 3- charge state is the decreased low mass cutoff (LMCO), which enables the detection of the abundant PO_3^- and $H_2PO_4^-$ ions. These phosphate reporter anions are important since they permit easy differentiation between phosphorylated and unphosphorylated peptides in complex mixtures [10], and have recently been used for phosphopeptide mapping of melanoma cells [11]. The singly charged peptide again shows preferential cleavage adjacent (N-terminal side) to the phosphorylated residue as noted by the presence of the $y_7^{\#}$ ion. However, this cleavage is significantly less pronounced and is not accompanied by the neutral loss of ammonia as observed for the RQpSVELHSPQSLPR and VPQLEIVNPpSAEER peptides described above, an occurrence that may be attributed to the differences in fragmentation behavior between phosphorylated serine and tyrosine residues upon UVPD. From these TSTEPQpYQPGENL spectra, it is concluded that significant sequence information and predictable dissociation patterns can be achieved using 193 nm photodissociation for a variety of peptide anion charge states, making it a viable option for ESI-LC-MS/MS applications.

The production of high abundance sequence ions across the backbone of a peptide is vital for any MS/MS strategy that will be applied to the analysis of complex mixtures. Sequence coverage distribution is defined as the distribution of backbone cleavages based on the relative abundances of complementary a/x ions, and it was compared for five peptides in the 2- charge state (Fig. 2). Moreover, we also wanted to assess the sequence coverage distribution obtained using a single UV pulse (8 mJ) versus multiple UV pulses (9 pulses, 1 mJ/pulse) for photodissociation, the latter which would be anticipated to cause more extensive dissociation of both precursor and product ions. The sequence coverage distribution is shown in Fig. 2 for multiple pulse UVPD (Fig. 2a) and single pulse UVPD (Fig. 2b). For each peptide (RQpSVELHSPQSLPR, HQGLPQEVNLNENLLR, TSTEPQpYQPGENL, DRVYIHPFHLVIH, and FFVAPFPEVFGK), the sequence coverage distribution was normalized to the most abundance a/x pair obtained by any of the two activation methods. Because peptides containing aromatic groups have higher photoabsorptivities at 193 nm and thus typically yield the highest photodissociation efficiencies [12], the peptides were arranged from the least aromatic (top of figure) to the most aromatic (bottom of figure). Comparison of the distributions in Fig. 2a and 2b illustrate that activation using a singly high energy UV pulse generates similar or often better sequence coverage distribution compared to that obtained upon multiple lower energy laser pulses. An example spectrum using a single 5 ns (8 mJ) pulse for UVPD activation can be seen in Supplementary Fig. 4 for the peptide FFVAPFPEVFGK (2-). The relative abundances of the surviving precursor ions were two to thirty times lower after nine 1 mJ UV pulses than after a single 8 mJ UV pulse. That is, while the most extensive dissociation of the precursor was always achieved using multiple UV pulses, the highest sequence coverage distribution was in fact more often obtained using a single UV pulse, especially for the peptides that contained the greatest number of tyrosine and phenylalanine residues. Interestingly, the sequence distributions in both Fig. 2a and 2b also show an enhanced degree of backbone cleavage next to residues that have UV-absorbing aromatic groups in addition to preferential cleavage at histidine and proline residues. The distributions in Fig. 2

indicate that the highest sequence coverage distribution is achieved using a single UV pulse, a natural fit for high throughput LC-MS/MS applications.

The promising results summarized above motivated us to adapt UVPD for LC-MS/MS applications, as demonstrated for a mixture of mitogen-activated pathway kinases (MAPKs). Typical LC mobile phases used for peptide separations are acidic and limit the ionization efficiencies of acidic species analyzed under negative polarity ionization. Flora and Muddiman showed increased ionization of acidic peptides by adjusting directly infused, working solutions to a higher pH using piperidine [13]. We have replicated these experiments and confirmed increased ion signals as well as a shift to higher charge states upon addition of piperidine to the solutions (data not shown). We were motivated to use piperidine as an organic modifier in the LC mobile phase to promote improved negative ionization after peptide separation to complement UVPD. An equimolar mixture of digested MAPKs, including c-Jun *N*-terminal kinase 2 (JNK2), mitogen-activated protein kinase kinase 7 (MKK7), mitogen-activated protein kinase 1 (ERK2), and p38 mitogen-activated kinase 14 (p38MAPK α), was analyzed. Mitogen-activated protein kinase pathways are well-defined cell signaling modules that regulate many diverse cellular events. These proteins essentially control all cellular processes and thus are thought to play key roles in cancer progression [14]; they also yield many acidic peptides after trypsin digestion making them amenable to the proposed LC-MS/UVPD method. Fig. 3a shows the base peak chromatogram obtained for the MAPK tryptic peptide mixture using a highly basic mobile phase (pH 11.5) consisting of 10 mM piperidine in each eluent. Good separation and satisfactory peak shapes are obtained using this protocol. The analogous data-dependent UVPD acquisitions yielded high quality photodissociation spectra (i.e., high signal-to-noise) by averaging only 10 microscans using a single 5 ns UV pulse per scan as seen in Fig. 3b and 3c for the doubly charged p38MAPK α peptide, TLFPGTDHIDQLK, and the triply charged ERK2 peptide, LKELIFEETAR, respectively. Both spectra display mainly *a/x* sequence ions along with a few abundant *d* and *w* side-chain loss ions and other diagnostic ions (occasional *Y*, *c*, *z*, *y*, and *b* ions); high backbone sequence coverage (100% for both peptides) was derived from this rich fragmentation behavior. These examples illustrate the high sensitivity and sequence information achievable by using UVPD (5 ns activation) on a fast chromatographic timescale.

In this report, we have illustrated the benefits and applicability of 193 photodissociation for “bottom-up”, acidic proteome characterization. The relative ease and affordability of coupling a laser to a commercial mass spectrometer has accelerated our efforts to implement this MS/MS technique on other mass spectrometers (e.g., orbitraps, hybrid quadrupole time-of-flights, etc.) for various large scale and targeted proteomic applications. We are also currently writing script to manipulate the UVPD spectra to make them more applicable to commercial database searching algorithms, which were mainly developed for positive mode analysis using CID. Coupled with our previous work [12], the present findings establish a compelling benchmark for obtaining high sequence information of both acidic and basic peptides via alternating positive/negative UVPD scans in a single chromatographic separation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

J.S.B. acknowledges funding from the NSF (CHE-1012622), and the Welch Foundation (F-1155). J.A.M. acknowledges the ACS division of Analytical Chemistry and The Society for Analytical Chemists of Pittsburgh for

support. K.N.D. acknowledges funding from the Welch Foundation (F-1390) and the National Institutes of Health (GM59802). T.S.K. acknowledges a scholarship from the Egyptian Ministry of Higher Education.

References

1. de Godoy LMF, Olsen JV, Cox J, Nielsen ML, et al. Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature*. 2008; 455:1251–1254. [PubMed: 18820680]
2. Kjeldsen F, Silivra OA, Ivonin IA, Haselmann KF, et al. C α -C backbone fragmentation dominates in electron detachment dissociation of gas-phase polypeptide polyanions. *Chem--Eur J*. 2005; 11:1803–1812.
3. Kjeldsen F, Horning OB, Jensen SS, Giessing AMB, Jensen ON. Towards Liquid Chromatography Time-Scale Peptide Sequencing and Characterization of Post-Translational Modifications in the Negative-Ion Mode Using Electron Detachment Dissociation Tandem Mass Spectrometry. *J Am Soc Mass Spectrom*. 2008; 19:1156–1162. [PubMed: 18555696]
4. Ewing NP, Cassady CJ. Dissociation of multiply charged negative ions for hirudin (54-65), fibrinopeptide B, and insulin A (oxidized). *J Am Soc Mass Spectrom*. 2001; 12:105–116. [PubMed: 11142354]
5. Coon JJ, Shabanowitz J, Hunt DF, Syka JEP. Electron Transfer Dissociation of Peptide Anions. *J Am Soc Mass Spectrom*. 2005; 16:880–882. [PubMed: 15907703]
6. Huzarska M, Ugalde I, Kaplan DA, Hartmer R, et al. Negative Electron Transfer Dissociation of Deprotonated Phosphopeptide Anions: Choice of Radical Cation Reagent and Competition between Electron and Proton Transfer. *Anal Chem*. 2010; 82:2873–2878. [PubMed: 20210298]
7. Antoine R, Joly L, Tabarin T, Broyer M, et al. Photo-induced formation of radical anion peptides. Electron photodetachment dissociation experiments. *Rapid Commun Mass Spectrom*. 2007; 21:265–268. [PubMed: 17167813]
8. Larraillet V, Vorobyev A, Brunet C, Lemoine J, et al. Comparative Dissociation of Peptide Polyanions by Electron Impact and Photo-Induced Electron Detachment. *J Am Soc Mass Spectrom*. 2010; 21:670–680. [PubMed: 20171119]
9. Zhang L, Reilly JP. Peptide photodissociation with 157 nm light in a commercial tandem time-of-flight mass spectrometer. *Anal Chem*. 2009; 81:7829–7838. [PubMed: 19702244]
10. Carr SA, Huddleston MJ, Annan RS. Selective detection and sequencing of phosphopeptides at the femtomole level by mass spectrometry. *Anal Biochem*. 1996; 239:180–192. [PubMed: 8811904]
11. Old WM, Shabb JB, Houel S, Wang H, et al. Functional proteomics identifies targets of phosphorylation by B-Raf signaling in melanoma. *Mol Cell*. 2009; 34:115–131. [PubMed: 19362540]
12. Madsen JA, Boutz DR, Brodbelt JS. Ultrafast Ultraviolet Photodissociation at 193 nm and its Applicability to Proteomic Workflows. *J Proteome Res*. 2010; 9:4205–4214. [PubMed: 20578723]
13. Flora JW, Muddiman DC. Selective, Sensitive, and Rapid Phosphopeptide Identification in Enzymatic Digests Using ESI-FTICR-MS with Infrared Multiphoton Dissociation. *Anal Chem*. 2001; 73:3305–3311. [PubMed: 11476230]
14. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer*. 2004; 4:937–947. [PubMed: 15573115]

Figure 1.

UVPD (193 nm) spectra of human aquaporin-2 peptide **(a)** RQpSVELHSPQSLPR, 2- and **(b)** RQpSVELHSPQSLPR, 1-. A q -value of 0.1 and an activation of 9 pulses (in a 18 ms activation period), 1 mJ/pulse, at 500 Hz were used for each spectrum. Neutral losses of phosphoric acid, H₂O, and NH₃ are denoted by #, °, and *, respectively.

Figure 2.

Sequence coverage distribution comparison between UVPD using 9 pulses (in a 18 ms period, 1 mJ/pulse) and UVPD using one 5 ns, 8 mJ pulse. The product ion signals were plotted for summed a/x pairs for each backbone cleavage. For each peptide (RQpSVELHSPQSLPR, HQGLPQEVLENENLLR, TSTEPQpYQPGENL, DRVYIHPFHLVIH, and FFVAPFPEVFGK), ion signals were normalized to the most abundant a/x pair between the three activation methods. Peptides are oriented from least aromatic (top of figure) to most aromatic (bottom of figure).

Figure 3.

LC-MS/UVPD analysis of a mitogen-activated protein kinase (MAPK) mixture; (a) base peak ion chromatogram from a separation using 10 mM piperidine spiked into LC eluents, (b) UVPD (193 nm) spectra of the p38MAPK α peptide TLFPGTDHIDQLK, 2-, and (c) UVPD (193 nm) spectra of the ERK2 peptide LKELIFEETAR, 3-. A q -value of 0.1 and an activation of one 5 ns (8 mJ) pulse were used for each 193 nm photodissociation spectrum. Neutral losses of H₂O, NH₃, and CO₂ are denoted by °, *, and “, respectively.