Mass Spectral Signatures of Complex Post-Translational Modifications in Proteins: A Proof-of-Principle Based on X-ray Irradiated Vancomycin

Journal Item

Mass spectral signatures of complex post-translational modifications in proteins: a proof-of-principle based on X-ray irradiated vancomycin

Journal:	*Journal of the American Society for Mass Spectrometry*
Manuscript ID	js-2020-00169s.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Abdelmouleh, Marwa; Centre de recherche sur les ions les materiaux et la photonique, Université de Caen Normandie/CEA/CNRS/ENSICAEN Lalande, Mathieu; Centre de recherche sur les ions les materiaux et la photonique, Université de Caen Normandie/CEA/CNRS/ENSICAEN El Feghaly, Johnny; Centre de recherche sur les ions les materiaux et la photonique, Université de Caen Normandie/CEA/CNRS/ENSICAEN Vizcaino, Violaine; Centre de recherche sur les ions les materiaux et la photonique, Université de Caen Normandie/CEA/CNRS/ENSICAEN Rebelo, Andre; Atomic and Molecular Collisions Laboratory, Department of Physics; The Open University Faculty of Science, School of Physical Sciences Eden, Samuel; The Open University Faculty of Science, School of Physical Sciences Schlathölter, Thomas; Rijksuniversiteit Groningen, Zernike Institute for Advanced Materials Poully, Jean-Christophe; Centre de recherche sur les ions les materiaux et la photonique, Université de Caen Normandie/CEA/CNRS/ENSICAEN

Mass spectral signatures of complex post-translational modifications in proteins: a proof-of-principle based on X-ray irradiated vancomycin

Marwa Abdelmouleh,[a] Mathieu Lalande,[a] Johnny El Feghaly,[a] Violaine Vizcaino,[a] André Rebelo,[b,c] Samuel Eden,[b] Thomas Schlathölter[d] and Jean-Christophe Poully[a]*

a. CIMAP, UMR 6252 CEA/CNRS/ENSICAEN/Université de Caen Normandie, Bd Becquerel, 14070 Caen, France

b. School of Physical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

c. Atomic and Molecular Collisions Laboratory, CEFITEC, Department of Physics, FCT - Universidade NOVA de Lisboa, P-2829-516 Caparica, Portugal

d. Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747AG Groningen, Netherlands

* corresponding author: poully@ganil.fr

ABSTRACT: Characterizing post-translational modifications (PTM) of proteins is of key relevance for the understanding of many biological processes, as these covalent modifications strongly influence or even determine protein function. Among the different analytical techniques available, mass spectrometry is attracting growing attention because recent instrumental and computational improvements have led to a massive rise of the number of PTM sites that can be identified and quantified. However, multiple PTM occurring at adjacent amino-acid residues can lead to complex and dense chemical patterns that are a challenge to characterize. By means of X-ray synchrotron radiation coupled to mass spectrometry, and through the test-case of the glycopeptide antibiotic vancomycin, we show that such a pattern has a unique and robust signature in terms of photon energy and molecular environment. This highlights the potential of this technique in proteomics and its value as a tool to understand the biological roles of PTM.

INTRODUCTION

Post-translational modifications (PTM) of proteins are essential in biology, since they tailor protein activity by cleavage and/or creation of covalent bonds. The removed and/or added chemical groups can be quite diverse: the most common PTM are phosphorylation, acetylation and glycosylation. They involve the attachment of a phosphoryl, an acetyl or a carbohydrate group, respectively, to an amino-acid side chain. Other important PTM lead to removal of a peptide from the protein N- or C-terminal side, for instance in collagen. Hydroxylation of prolines and lysines is another PTM, which for instance stabilizes the collagen triple-helix structure responsible for the specific mechanical properties of connective tissues such as skin, cartilage, nails and bones. PTM can also result from oxidative stress, aromatic and sulfur-containing amino-acids being especially prone to cross-linking or oxidation.[1] For instance, these processes are crucial for the synthesis of glycopeptide antibiotics such as vancomycin. The latter is a last-resort drug against Gram-positive bacteria, blocking the renewal of their cell-wall by acting as a ligand for a precursor of the main cell-wall component. Vancomycin is naturally formed by bacteria but has also been synthesized.[2] Once the seven-residue peptidic chain has been created, several PTM have to occur in the five tyrosine side-chains. They involve the addition of OH groups, cross-linking, the addition of chlorine atoms to two of these side-chains, and glycosylation of the central one (see Fig. 1). The numerous and dense cross-linkages in the resultant peptide create a rigid binding pocket allowing for stereospecific recognition of the receptor *via* noncovalent binding. Identifying such a complex modification is extremely challenging, even for state-of-the-art methods such as capillary electrophoresis or mass spectrometry (MS) techniques.[3] The latter have proven to be very powerful for deciphering the site and identity of PTM in proteins such as histones, which undergo a particularly large number of PTM.[4] Generally, two approaches can be followed: top-down or bottom-up. In the first, gas-phase intact proteins are cleaved into fragments whose mass-over-charge (m/z) ratio allows identifying PTM. In the second, proteins undergo enzymatic digestion that produces peptides, which are identified thanks to MS and fragmentation techniques. Protein and peptide fragmentation is often performed by collision-induced dissociation (CID) using a rare gas or N_2, and when coupled to tandem MS, CID has allowed identifying disulfide bridges in conotoxins.[5] However, electron-capture or transfer dissociation are better suited for assigning labile PTM such as O-linked glycosylation or phosphorylation of serines or threonines.[3] A combination of electron-transfer dissociation and CID has been applied for studying cyclotides containing numerous cysteine knots.[6] VUV photoionization has recently been proposed for the identification of multiple PTM in histones.[7,8] In 2019, photodissociation using a UV laser revealed mass spectral signatures of dityrosine cross-linking in peptide dimers.[9]

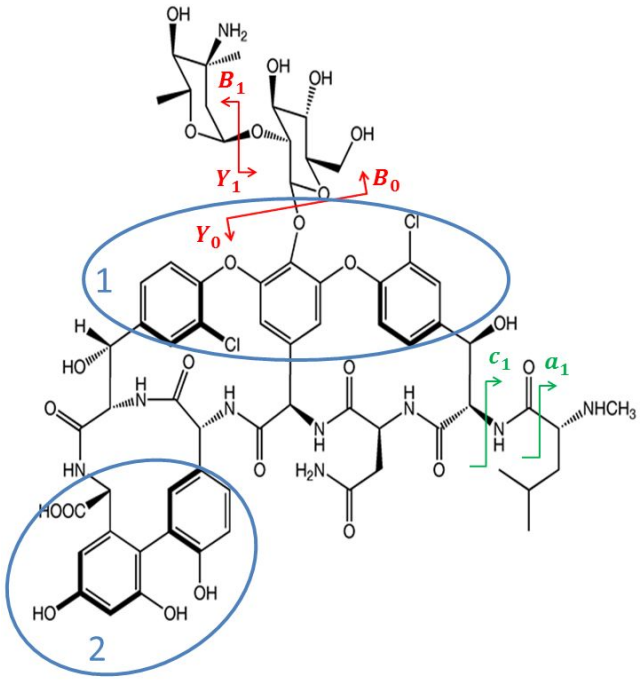


Figure 1. Chemical structure of vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}$, average mass 1449.3 amu). The standard nomenclature is used for the peptide backbone as well as for oligosaccharide fragmentation, and hence for the main fragments observed after photoabsorption. Arrows indicate on which side of the cleaved bond the charge is located. The groups #1 and 2 containing phenyl rings are encircled.

In this article, we report the proof-of-principle that soft X-ray photoionization, as a biomolecular fragmentation technique, gives robust mass spectral signatures of particularly complex and dense PTM. Using vancomycin as a model heavily-modified peptide, we prove that all PTM, not only labile ones, can be identified with mass spectrometry. Our work extends the potential of mass spectrometry techniques in the growing field of PTM identification and quantitation in proteins.

EXPERIMENTAL

Vancomycin hydrochloride and $Ac_2{}^{L}K{}^{D}A{}^{D}A$ have been purchased from Sigma-Aldrich as powders of over 80 % purity, and used without further purification. Solutions have been prepared in 50:50 (volume ratio) water/methanol at 50 µM concentration with 1 % of formic acid to protonate the molecules.

A home built tandem mass spectrometer, described in detail elsewhere,[10] has been used to record mass spectra of the ionic photo-products from the interaction between molecular systems and synchrotron radiation. Briefly, protonated molecular systems are produced with an electrospray ion source and transported into the vacuum chamber through a heated capillary. The molecular ion beam is then focused into an ion funnel and guided into an octopole before being mass-over-charge selected with a quadrupole mass-filter (QMF) and subsequently accumulated in a 3D radiofrequency ion Paul trap. Trapping is facilitated by collisions with a helium buffer gas injected into the ion trap during the filling process. Molecular ions have been irradiated by X-ray photons at the U49-2 PGM-1 beamline of the BESSY II synchrotron (Helmholtz-Zentrum Berlin). Photon beam exposure of the trap content, typically during 300 to 1000 ms, is controlled with a mechanical shutter in order to guarantee that more than 90 % of the product cations result from the absorption of a single photon. To do so, the irradiation time is tuned to induce a depletion of the precursor ion below 10 %. Since the absorption of multiple photons is a sequential process at these fluxes ($10^{12\text{-}13}s^{-1}$), the absorption events are independent, thus a probability p for absorbing one photon gives the probability p^2 for two photons. Neglecting the absorption of more than two photons, we obtain $p^2 + p < 0.1$ and thus $p < 0.09$. Precursor ions and cationic fragments are then extracted from the trap, analyzed by a time-of-flight reflectron mass spectrometer, and detected by microchannel plates. Mass spectra of the non-irradiated trap content (beam-off) and irradiated residual gas are recorded as well, the latter allowing to spot background peaks due to photoionization of residual gas molecules. Then, the beam-on mass spectrum is subtracted from the beam-off one, and the resulting spectrum shows the precursor ion depletion with a negative intensity. Assuming that absorption of one photon leads to ionization and/or fragmentation of the precursor ion, this depletion (area under the peak) is proportional to the total photo-absorption yield. All relative yields have been obtained by calculating the area under each peak, normalizing by the precursor ion depletion, by the total yield of all cationic species formed by photoabsorption, and by detection efficiency.[11]

RESULTS AND DISCUSSION

The soft X-ray photoabsorption spectra of doubly-protonated vancomycin $[V+2H]^{2+}$ are shown in Fig. 2. In the spectrum at 100 eV, we mainly observe the same species as we have previously observed after photoabsorption in the VUV range.[11] The peak at *m/z* 483.8 corresponds to non-dissociative ionization (NDI) of the precursor ion, a minor process compared to fragmentation following ionization. The yields of the complementary Y_0^{2+} and B_0^{+} fragments are significantly higher (see Fig. 2). Both are due to cleavage of the glycosidic bond (linking the carbohydrate group to the rest of the molecule) after ionization. X-ray photoionization thus allows identifying the mass of the carbohydrate involved in glycosylation. The most intense peaks are assigned to the a_1^{+} and c_1^{+} fragments of the pseudo-peptidic backbone (see Fig. 1). Note that the peak corresponding to the latter (at *m/z* 144) can also be attributed to B_1^{+}, formed by cleavage of the glycosidic bond within the carbohydrate moiety. The relative yield of NDI and large fragments is smaller than in the case of photons in the 14-30 eV range,[11] which can be explained by a rise of the vibrational energy transferred by the photon to the molecular system with photon energy, as it has previously been shown in the soft X-ray range.[12] With increasing photon energy, a more striking difference develops in the mass spectra. Whereas VUV photoabsorption solely leads to fragmentation into peaks at *m/z* 100, 118, 127, 144, 149, a multitude of additional peaks with *m/z* < 300 are induced by soft X-ray photoabsorption (see Fig. 2). In the following, we will show that these peaks can be assigned to internal fragments whose formation requires at least two bond cleavages (see below). The total relative yield of these internal fragments increases with photon energy up to 300 eV, and is compensated by the fall of non-dissociative ionization and all

other fragments, with the exception of a_1^+ (see Fig. 2). From 100 to 300 eV, the relative cross section for inner-valence orbital photoabsorption increases, and thus so does the average electronic excitation. After internal conversion to the electronic ground state and vibrational energy redistribution, this leads to more fragmentation as photon energy rises. This is consistent with the mechanism we proposed in our previous studies on protonated biologically-relevant molecules.[12–14]

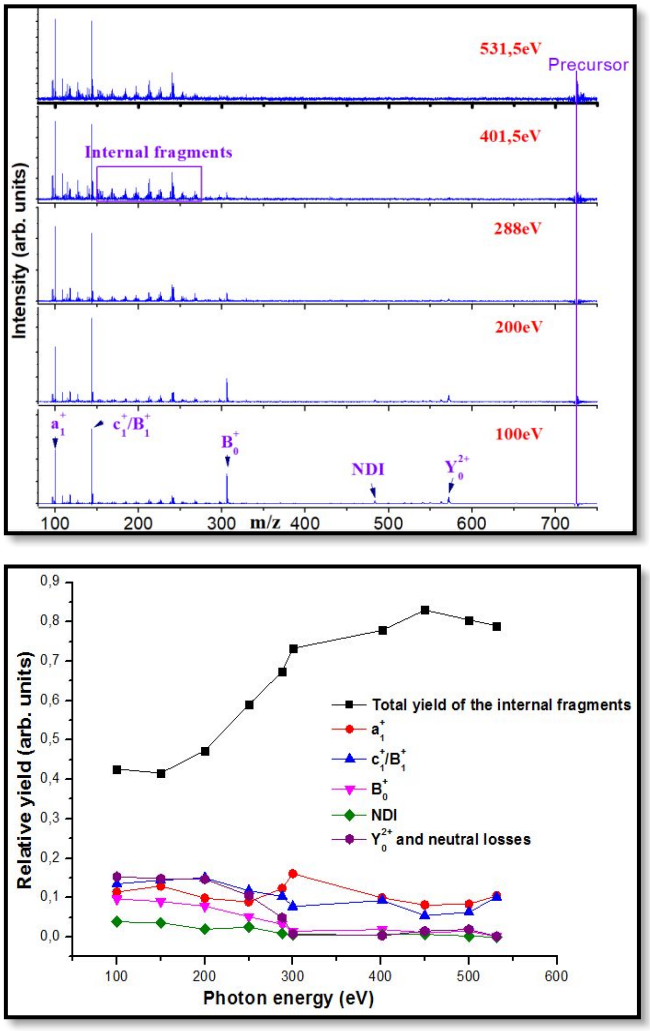


Figure 2. Top: mass spectra of $[V+2H]^{2+}$ after single photoabsorption between 100 and 531.5 eV. The position of the precursor ion (*m/z* 725.6) is represented by a purple line, the peak being negative (see the experimental section for details). The usual nomenclature is used for the peptide backbone as well as for oligosaccharide fragmentation and hence for the main fragments observed after photoabsorption (*cf.* Fig. 1). Internal fragments formed by at least two bond cleavages are indicated. Bottom: relative yield of the species formed after single photoabsorption as a function of photon energy, normalized by the detector efficiency and the total yield of photoinduced cations. NDI stands for non-dissociative ionization.

A deeper analysis can be achieved by zooming in the *m/z* 158 - 278 region of the $[V+2H]^{2+}$ spectra, as can be seen in Fig. 3. Interestingly, none of these fragment ions have been observed after collision-induced dissociation (CID) at low energy (on the order of 10 eV),[15] where ionization does not occur. Similar groups of peaks do appear in the mass spectrum of singly-protonated vancomycin reported after CID at 4 keV kinetic energy on argon, but are not mentioned and their exact mass is not possible to obtain from the figure.[16] Since these high-energy collisions induce ionization, like X-rays, this seems to be consistent with these peaks coming from fragments being formed after ionization. In Fig. 3, one can notice that for soft X-ray photoabsorption, groups of peaks of similar intensity appear: they are separated by one mass unit, indicating singly-charged fragments. All precursor ions containing isotopes were present in the trap after *m/z* selection by the QMF (see the experimental section), but even considering the maximum number of carbon and chlorine atoms possible (22 carbons or 16 carbons and 2 chlorines) for the fragment corresponding to the peak of highest mass (270 amu), the natural abundance of ^{13}C and ^{37}Cl cannot explain these patterns. Thus, they are probably due to extensive H scrambling, which has also been found to occur in photoionized 3-aminophenol,[17] an aromatic molecule containing one phenyl ring with one OH group. Indeed, these groups of peaks are typical of mass spectra of neutral H-rich molecules containing aromatic rings, *e.g.* ionized by electron impact,[18] ion impact at keV or MeV energy,[19–22] or by absorption of X-ray photons.[23,24] A good example is ionization of lorazepam, a molecule containing two aromatic rings substituted by one chlorine atom as in vancomycin: groups of peaks separated by 1 mass unit are also observed in the same mass range.[18] Ionization of small carbohydrates (such as that of vancomycin) also yields such groups of peaks, but in a much lower mass range, typically below 100 amu.[25–29] Therefore, we have calculated the mass of potential fragments originating from the groups containing aromatic rings in vancomycin. The results are shown in Scheme 1, where structures of fragments accounting for all groups of peaks seen in the mass spectra are proposed. In Fig. 3, we include vertical bars at the *m/z* of these singly-charged fragments with the maximum number of H atoms (leaving the bond orders unchanged), and without any H atom left. These bars mark off each group of peaks. To confirm that the natural abundance of ^{13}C and ^{37}Cl isotopes does not account for the observed peaks, we also show the simulated isotopic pattern of each fragment in Fig. S1 (see the Supporting Information). It demonstrates that extensive H atom scrambling occurs for these fragments. Interestingly, their formation can be explained by only three pathways, two of them involving the central group containing the three phenyl rings (noted 1 in Fig. 1) and accounting for most of the groups of peaks. The remaining groups are assigned to fragments formed by a third pathway, starting with separation of the biphenol group bound to the C-terminus of the pseudo-peptidic backbone (noted 2 in Fig. 1). In addition, each aromatic ring can be identified separately. Thus, these groups of peaks assigned to internal fragments can be considered as a mass spectral signature of the complex PTM pattern of vancomycin. Internal fragments are not often exploited in MS of biopolymers, because of the intricate data analysis required. Here, the latter is made easier by the following features: the peaks fall at integer *m/z* values, are easily separated even with a modest resolving power, and are structured in distinct groups of peaks separated by 1 amu. Another important point is the influence of photon energy on this mass spectral signature: it can be deduced from Fig. 3. Despite the large energy range (100-531.5 eV) covering C, N and O K-edges, the relative intensity of these peaks varies only very slightly, the overall shape of the groups of peaks

remaining remarkably stable. This is very important if they are used as mass spectral signature for identifying PTM. Consider also that we have shown in the previous paragraph that the total yield of these fragments increases with photon energy from 100 to 300 eV because of the increase in vibrational energy transferred to vancomycin after ionization. Therefore, this behavior might be traced to a high potential energy barrier for creating the largest fragments shown in Scheme 1, due to the need to cleave several bonds, and lower barriers separating the subsequent smaller fragments.

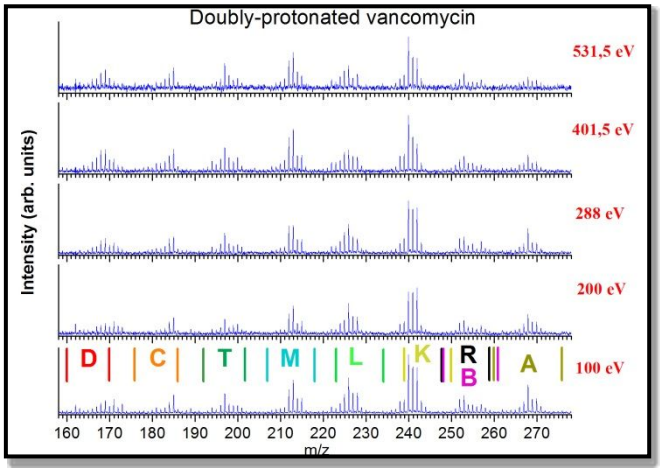
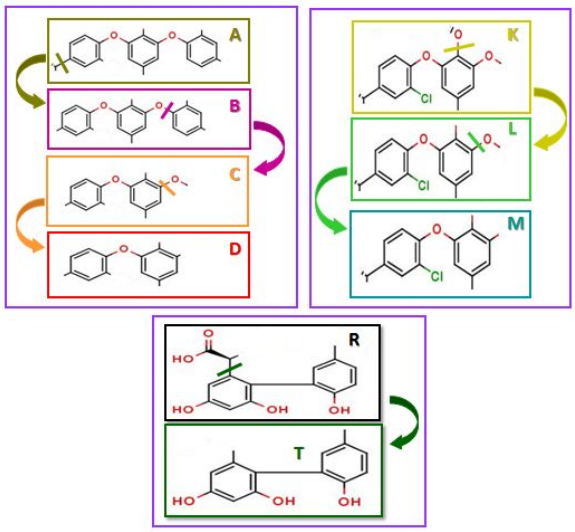


Figure 3. Zoom into the region of the mass spectra of $[V+2H]^{2+}$ (see Fig. 2) where the internal fragments appear. Each of them is indicated by a letter above the corresponding group of peaks. Vertical bars correspond to the *m/z* of each singly-charged fragment shown in Scheme 1 with the maximum number of H atoms (leaving the bond orders unchanged), and without any H atom left. The structure of these fragments is given in Scheme 1.

Scheme 1. Proposed structure corresponding to the internal fragments of $[V+2H]^{2+}$ (see Fig. 3). The bond cleavages responsible for their formation are highlighted.



The next question is to know whether the mass spectral signature of the PTM pattern of vancomycin is sensitive to a non-covalently bound molecular environment. To tackle this question, we have studied the vancomycin dimer as well as complexes between vancomycin and $Ac_2{}^{L}K^{D}A^{D}A$ (K is lysine and A alanine), a peptidic model of its receptor, abbreviated R in the following. The chemical structure of the receptor can be found in Fig. S2. In all cases, absorption of one X-ray photon in the 100-531.5 eV range leads to ionization and fragmentation of the precursor molecular system. For instance, Fig. 4 shows that at 100 eV, the $[V+R+2H]^{2+}$ complex dissociates and subsequent intramolecular fragmentation of vancomycin and R occurs. Fragments of the latter have been identified by comparison with the mass spectra of $[R_2+H]^+$ (see Fig. S2). The same fragments as for VUV photons are observed,[11] but large fragments are less abundant, as in the case of isolated vancomycin (*cf.* Fig. 2). Small fragments rise with photon energy from 100 to 300 eV, the total fragmentation yield slightly increasing for both vancomycin and R. Interestingly, the fragmentation yield of vancomycin is compensated by the falling yield of $[R+H]^+$ and *vice versa*, which is unexpected. In our previous study, we have attributed the formation of $[R+H]^+$ to a proton transfer between ionized vancomycin and neutral R.[11] Therefore, the decrease in the $[R+H]^+$ yield while vancomycin fragmentation increases might indicate that proton transfer becomes progressively less likely as photon energy increases. This might be due to more vibrational excitation as the X-ray photon energy rises from 100 to 300 eV, leading to the noncovalent complex dissociating on a faster timescale and quenching proton transfer. This is plausible because MacAleese *et al.*[30] have reported that proton transfer can take up to hundreds of microseconds in an ionized peptide radical cation. These results show that photon energies over 300 eV give the highest yield of vancomycin fragments. Among the latter, and for all noncovalent systems studied here ($[V+R+2H]^{2+}$, $[V+2R+2H]^{2+}$ and $[V_2+3H]^{3+}$), we observe the same internal fragments as for isolated vancomycin, their relative yield increasing with photon energy up to 300 eV (see Fig. 4 and S2-S4). We can also notice that whatever the noncovalent system, the overall relative yield of the internal fragments of vancomycin is lower than in the case of isolated vancomycin. This is consistent with our hypothesis of fragmentation in the ground-state due to vibrational energy transfer, this energy being redistributed into more degrees of freedom when the system gets larger. Furthermore, if we focus on the pattern made by the peaks attributed to these internal fragments, we observe a high similarity whatever the molecular environment (*cf.* Fig. 5). This indicates that the mass spectral signature is robust and can be used to identify the groups responsible for the fragments, and thus the corresponding post-translational modifications in proteins.

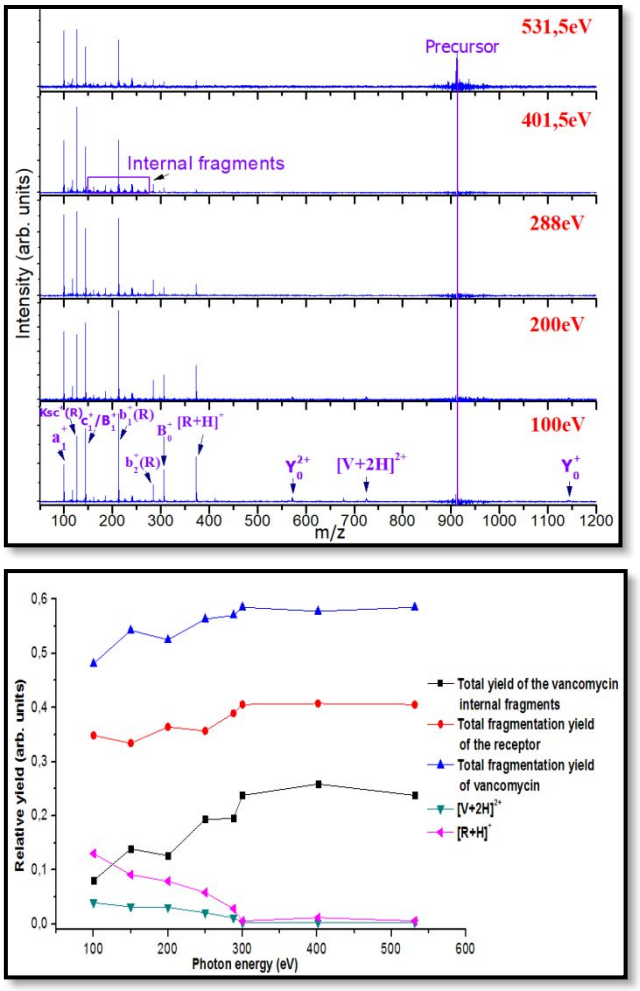


Figure 4. Top: mass spectra of the $[V+R+2H]^{2+}$ complex after absorption of one photon of energy between 100 and 531.5 eV. The position of the precursor ion (*m/z* 911.6) is represented by a purple line. The usual nomenclature is used for the peptide backbone as well as for oligosaccharide fragmentation and hence for the main fragments observed after photoabsorption (*cf.* Fig. 1). Internal fragments of vancomycin formed by at least two bond cleavages are indicated. Bottom: relative yield of the different relaxation channels after single photoabsorption as a function of photon energy, normalized by the detector efficiency and the total yield of photoinduced cations.

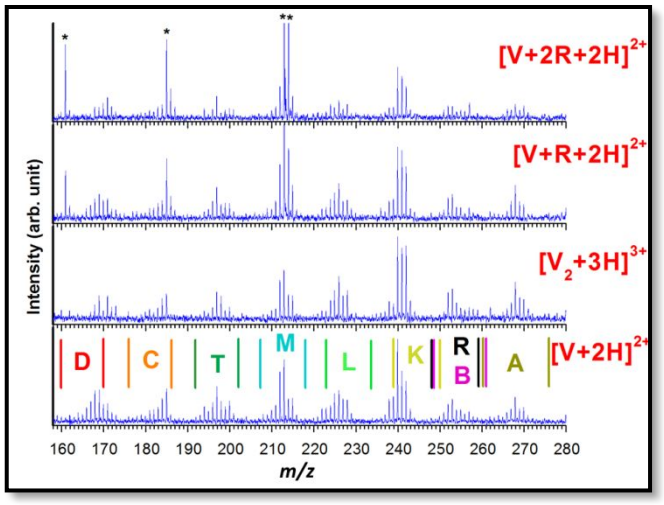


Figure 5. Zoom in the *m/z* 158-280 region of the mass spectra of vancomycin in different environments, after absorption of one 401.5 eV photon. The peaks attributed to fragments of R are spotted by an asterisk. Vertical bars correspond to the *m/z* of each singly-charged fragment shown in Scheme 1 with the maximum number of H atoms (leaving the bond orders unchanged), and without any H atom left.

CONCLUSIONS

In this contribution, we have shown that X-ray photoabsorption of a highly modified tyrosine-rich peptide coupled to mass spectrometric analysis of the resulting fragments gives a signature that can be employed to characterize particularly complex and dense PTM, especially those resulting from oxidative stress. Indeed, this mass spectral signature is robust with respect not only to the X-ray photon energy over a large range (100-531.5 eV), but also to the molecular environment of the peptide. This proof-of-principle expands the potential of mass spectrometry techniques in proteomics. Further work is now required to test the applicability of this method to other peptides, proteins and a wider range of PTM.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Simulated mass spectra of singly-charged internal fragments of vancomycin shown in Scheme 1; Photoabsorption mass spectra $[R_2+H]^+$, $[V_2+3H]^{3+}$ and $[V+R_2+2H]^{2+}$; Total yield of internal fragments of vancomycin in different environments (PDF)

AUTHOR INFORMATION

Corresponding Author

* poully@ganil.fr

Author Contributions

All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

We thank HZB for the allocation of synchrotron radiation beamtime and T. Kachel for his support during experiments. The

French “Conseil Régional de Normandie” is acknowledged for a PhD funding (#15P01339) and CNRS for a PICS grant (07390) supporting the collaboration between CIMAP and the Open University. This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 730872. AR acknowledges Fundação para a Ciência e a Tecnologia (FCT-MCTES) for subsiding him with a PhD scholarship (grant number PD/BD/114449/2016) under the Radiation Biology and Biophysics Doctoral Training Programme (RaBBiT, PD/00193/2012) organized by the Applied Molecular Biosciences Unit - UCIBIO (UIDB/04378/2020) and CEFITEC Unit (UIDB/00068/2020) from Faculdade de Ciências e Tecnologias of Universidade Nova de Lisboa. The Sir John Mason Academic Trust is also acknowledged for the financial support provided to AR for a beamtime at HZB.

REFERENCES

(1) Hawkins, C. L.; Davies, M. J. Detection, Identification, and Quantification of Oxidative Protein Modifications. *Journal of Biological Chemistry* **2019**, *294* (51), 19683–19708. https://doi.org/10.1074/jbc.REV119.006217.

(2) Nicolaou, K. C.; Mitchell, H. J.; Jain, N. F.; Winssinger, N.; Hughes, R.; Bando, T. Total Synthesis of Vancomycin. *Angewandte Chemie International Edition* **1999**, *38* (1-2), 240–244. https://doi.org/10.1002/(SICI)1521-3773(19990115)38:1/2<240::AID-ANIE240>3.0.CO;2-5.

(3) Doll, S.; Burlingame, A. L. Mass Spectrometry-Based Detection and Assignment of Protein Posttranslational Modifications. *ACS Chem. Biol.* **2015**, *10* (1), 63–71. https://doi.org/10.1021/cb500904b.

(4) Huang, H.; Lin, S.; Garcia, B. A.; Zhao, Y. Quantitative Proteomic Analysis of Histone Modifications. *Chem. Rev.* **2015**, *115* (6), 2376–2418. https://doi.org/10.1021/cr500491u.

(5) Gupta, K.; Kumar, M.; Balaram, P. Disulfide Bond Assignments by Mass Spectrometry of Native Natural Peptides: Cysteine Pairing in Disulfide Bonded Conotoxins. *Analytical Chemistry* **2010**, *82* (19), 8313–8319. https://doi.org/10.1021/ac101867e.

(6) Foreman, D. J.; Parsley, N. C.; Lawler, J. T.; Aryal, U. K.; Hicks, L. M.; McLuckey, S. A. Gas-Phase Sequencing of Cyclotides: Introduction of Selective Ring Opening at Dehydroalanine via Ion/Ion Reaction. *Anal. Chem.* **2019**, *91* (24), 15608–15616. https://doi.org/10.1021/acs.analchem.9b03671.

(7) Greer, S. M.; Brodbelt, J. S. Top-Down Characterization of Heavily Modified Histones Using 193 Nm Ultraviolet Photodissociation Mass Spectrometry. *Journal of Proteome Research* **2018**, *17* (3), 1138–1145. https://doi.org/10.1021/acs.jproteome.7b00801.

(8) Greer, S. M.; Sidoli, S.; Coradin, M.; Jesperser, M. S.; Schwammle, V.; Jensen, O. N.; Garcia, B. A.; Brodbelt, J. S. Extensive Characterization of Heavily Modified Histone Tails by 193 Nm Ultraviolet Photodissociation Mass Spectrometry via a Middle-Down Strategy. *Analytical Chemistry* **2018**, *90* (17), 10425–10433. https://doi.org/10.1021/acs.analchem.8b02320.

(9) Mukherjee, S.; Fang, M.; Kok, W. M.; Kapp, E. A.; Thombare, V. J.; Huguet, R.; Hutton, C. A.; Reid, G. E.; Roberts, B. R. Establishing Signature Fragments for Identification and Sequencing of Dityrosine Cross-Linked Peptides Using Ultraviolet Photodissociation Mass Spectrometry. *Analytical Chemistry* **2019**, *91* (19), 12129–12133. https://doi.org/10.1021/acs.analchem.9b02986.

(10) Bari, S.; Gonzalez-Magaña, O.; Reitsma, G.; Werner, J.; Schippers, S.; Hoekstra, R.; Schlathölter, T. Photodissociation of Protonated Leucine-Enkephalin in the VUV Range of 8–40 EV. *The Journal of Chemical Physics* **2011**, *134* (2), 024314.

(11) Abdelmouleh, M.; Lalande, M.; Vizcaino, V.; Schlathölter, T.; Poully, J.-C. Photoinduced Processes within Noncovalent Complexes Involved in Molecular Recognition. *Chemistry – A European Journal* **2020**, *26* (10), 2243–2250. https://doi.org/10.1002/chem.201904786.

(12) Schwob, L.; Lalande, M.; Rangama, J.; Egorov, D.; Hoekstra, R.; Pandey, R.; Eden, S.; Schlatholter, T.; Vizcaino, V.; Poully, J.-C. Single-Photon Absorption of Isolated Collagen Mimetic Peptides and Triple-Helix Models in the VUV-X Energy Range. *Physical Chemistry Chemical Physics* **2017**, *19* (28), 18321–18329. https://doi.org/10.1039/c7cp02527k.

(13) Egorov, D.; Schwob, L.; Lalande, M.; Hoekstra, R.; Schlathölter, T. Near Edge X-Ray Absorption Mass Spectrometry of Gas Phase Proteins: The Influence of Protein Size. *Phys. Chem. Chem. Phys.* **2016**, *18* (37), 26213–26223. https://doi.org/10.1039/C6CP05254A.

(14) Egorov, D.; Hoekstra, R.; Schlathölter, T. A Comparative VUV Absorption Mass-Spectroscopy Study on Protonated Peptides of Different Size. *Phys. Chem. Chem. Phys.* **2017**, *19*, 20608–20618. https://doi.org/10.1039/C7CP03203J.

(15) Jørgensen, T. J. D.; Delforge, D.; Remacle, J.; Bojesen, G.; Roepstorff, P. Collision-Induced Dissociation of Noncovalent Complexes between Vancomycin Antibiotics and Peptide Ligand Stereoisomers: Evidence for Molecular Recognition in the Gas Phase. *International Journal of Mass Spectrometry* **1999**, *188*, 63–85.

(16) Florencio, M.; Despeyroux, D.; Jennings, K. R. COLLISION GAS EFFECTS IN THE COLLISION-INDUCED DECOMPOSITION OF PROTONATED AND CATIONIZED MOLECULES OF CARBOHYDRATE ANTIBIOTICS. *Organic Mass Spectrometry* **1994**, *29* (9), 483–490. https://doi.org/10.1002/oms.1210290907.

(17) Bockova, J.; Rebelo, A.; Ryszka, M.; Pandey, R.; da Fonseca Cunha, T.; Limao-Viena, P.; Mason, N. J.; Poully, J. C.; Eden, S. Mapping the Complex Metastable Fragmentation Pathways of Excited 3-Aminophenol(+). *International Journal of Mass Spectrometry* **2019**, *442*, 95–101. https://doi.org/10.1016/j.ijms.2019.05.006.

(18) *NIST Chemistry WebBook, NIST Standard Reference Database Number 69*, National Institute of Standards and Technology.; P.J. Linstrom and W.G. Mallard.

(19) J. de Vries; R. Hoekstra; R. Morgenstern; T. Schlathölter. Multiple Ionization and Fragmentation of the DNA Base Thymine by Interaction with C^{q+} Ions. *Eur. Phys. J. D* **2003**, *24*, 161–164.

(20) Alvarado, F.; Bari, S.; Hoekstra, R.; Schlathölter, T. Quantification of Ion-Induced Molecular Fragmentation of Isolated 2-Deoxy-D-Ribose Molecules. *Phys.Chem.Chem.Phys.* **2006**, *8*, 1922–1928.

(21) Poully, J.-C.; Vizcaino, V.; Schwob, L.; Delaunay, R.; Kocisek, J.; Eden, S.; Chesnel, J.-Y.; Mery, A.; Rangama, J.; Adoui, L.; Huber, B. Formation and Fragmentation of Protonated Molecules after Ionization of Amino Acid and Lactic Acid Clusters by Collision with Ions in the Gas Phase. *Chemphyschem* **2015**, *16* (11), 2389–2396. https://doi.org/10.1002/cphc.201500275.

(22) Agnihotri, A. N.; Kasthurirangan, S.; Nandi, S.; Kumar, A.; Galassi, M. E.; Rivarola, R. D.; Fojon, O.; Champion, C.; Hanssen, J.; Lekadir, H.; Weck, P. F.; Tribedi, L. C. Ionization of Uracil in Collisions with Highly Charged Carbon and Oxygen Ions of Energy 100 KeV to 78 MeV. *Physical Review A* **2012**, *85*.

(23) Itälä, E.; Huels, M. A.; Rachlew, E.; Kooser, K.; Hagerth, T.; Kukk, E. A Comparative Study of Dissociation of Thymidine Molecules Following Valence or Core Photoionization. *J. Phys. B-At. Mol. Opt. Phys.* **2013**, *46*.

(24) Itälä, E.; Ha, D. T.; Kooser, K.; Rachlew, E.; Huels, M. A.; Kukk, E. Fragmentation Patterns of Core-Ionized Thymine and 5-Bromouracil. *Journal of Chemical Physics* **2010**, *133*.

(25) Ptasinska, S.; Denifl, S.; Scheier, P.; Märk, T. D. Inelastic Electron Interaction (Attachment/Ionization) with Deoxyribose. *Journal of Chemical Physics* **2004**, *120*, 8505–8511.

(26) Vall-Ilosera, G.; Huels, M. A.; Coreno, M.; Kivimaki, A.; Jakubowska, K.; Stankiewicz, M.; Rachlew, E. Photofragmentation of 2-Deoxy-D-Ribose Molecules in the Gas Phase. *Chemphyschem* **2008**, *9*, 1020–1029.

(27) Shin, J.-W.; Dong, F.; Grisham, M. E.; Rocca, J. J.; Bernstein, E. R. Extreme Ultraviolet Photoionization of Aldoses and Ketoses. *Chemical Physics Letters* **2011**, *506*, 161–166.

(28) Ghosh, D.; Golan, A.; Takahashi, L. K.; Krylov, A. I.; Ahmed, M. A VUV Photoionization and Ab Initio Determination of the Ionization Energy of a Gas-Phase Sugar (Deoxyribose). *J. Phys. Chem. Lett.* **2012**, *3*, 97–101.

(29) Shin, J.-W.; Bernstein, E. R. Vacuum Ultraviolet Photoionization of Carbohydrates and Nucleotides. *Journal of Chemical Physics* **2014**, *140*, 044330.

(30) MacAleese, L.; Hermelin, S.; Hage, K. E.; Chouzenoux, P.; Kulesza, A.; Antoine, R.; Bonacina, L.; Meuwly, M.; Wolf, J.-P.; Dugourd, P. Sequential Proton Coupled Electron Transfer (PCET): Dynamics Observed over 8 Orders of Magnitude in Time. *J. Am. Chem. Soc.* **2016**, *138* (13), 4401–4407. https://doi.org/10.1021/jacs.5b12587.

For Table of Contents Use Only

Mass spectral signatures of complex post-translational modifications in proteins: a proof-of-principle based on X-ray irradiated vancomycin

Marwa Abdelmouleh, Mathieu Lalande, Johnny El Feghaly, Violaine Vizcaino, André Rebelo, Samuel Eden, Thomas Schlathölter and Jean-Christophe Poully

Gas-phase X-ray photoabsorption coupled to mass spectrometry of a heavily-modified peptide provides a robust signature of its post-translational modifications.