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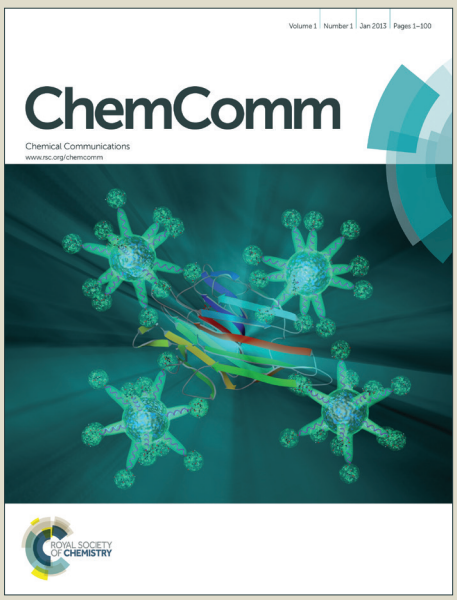

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Kinetic control in the CID-induced elimination of H_3PO_4 from phosphorylated serine probed by IRMPD spectroscopy.

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Francesco Lanucara*[a], Barbara Chiavarino[b], Debora Scuderi[c], Philippe Maitre[c], Simonetta Fornarini[b] and Maria Elisa Crestoni*[b]

Abstract. InfraRed Multiple Photon Dissociation (IRMPD) spectroscopy was used to assay the structural features of the fragment ions resulting from the elimination of H_3PO_4 in the Collision-Induced Dissociation (CID) of protonated serine. Results are interpreted with the aid of density functional theory calculations. Experiment and theory point to an aziridine-ring structure, implying participation of the vicinal amino group in the formation of this species. This finding constitutes a benchmark for investigating the same process in the CID of phosphorylated peptides.

Mass spectrometry (MS) has become an invaluable tool for the qualitative and quantitative analysis of protein post-translational modifications (PTM), including phosphorylation.[1] Identifying the correct phosphorylation site within the sequence of a peptide or a protein by MS entails isolation and activation of the corresponding ions in the gas phase to generate sequence- and position- specific product ions. Due to the pronounced lability of the *O*-phosphoester bond, phosphorylated peptides can easily undergo loss of H_3PO_4 from the modified serine or threonine residue during low energy-CID, often resulting in limited formation of sequence-specific b and y ions, and in difficulties in correctly assigning the phosphorylation site.[1,2] The mechanisms involved in this neutral loss pathway have been extensively investigated both theoretically,[3] and experimentally.[4,5] Charge-remote 1,2-*cis*-β-elimination processes leading to dehydrated serine and threonine residues were originally suggested,[5] however both theoretical calculations and MS experimental evidences point towards charge-directed mechanisms.[3,4] In the proposed pathways, protonation of the phosphoryl oxygen leads to the formation of a more electrophilic β-carbon, which can then be attacked by the nucleophilic amidic nitrogen or oxygen atoms on either side of the C_α of the phosphorylated residue, leading to H_3PO_4 elimination. The competition between different mechanisms is a function of the peptide charge state, proton mobility, and nature of the residues surrounding the modified amino acid.[3,4]

According to previously suggested mechanisms, three main conceivable structures may be envisaged as potentially representing the ions generated from the elimination of H_3PO_4 from protonated phosphorylated serine $(pSerH)^+$,[2-4] (Scheme 1): protonated 2-amino-propenoic acid (route a), resulting from 1,2-*cis*-β-elimination of H_3PO_4; protonated 2-carboxy-aziridine (route b), resulting from a nucleophilic attack of the amino group to the β-carbon of $pSerH^+$; protonated β-lactone (route c), resulting from nucleophilic attack of the carbonylic oxygen on the β-carbon of $pSerH^+$.

O'Hair *et al* provided strong evidences for the formation of a 3-membered aziridine ring upon loss of H_3PO_4 from protonated $pSerH^+$ by a combination of D-labelling, MS^3 and *ab initio* molecular orbital calculations.[6] However, a direct structural proof of these species is yet to be provided.

In this work, we have used InfraRed Multiple Photon Dissociation Spectroscopy (IRMPD)[7] backed by density functional theory calculations to assess the structure of the fragment ions resulting from the neutral loss of H_3PO_4 when $pSerH^+$ is induced to dissociate by CID.

IRMPD spectroscopy has been conveniently used to explore the structural features of a variety of systems in the gas phase, including ionic species of fundamental interests,[8] metal complexes,[9] as well as biomolecules such as (modified) aminoacids,[10] peptides,[11] and proteins.[12] Additionally, this technique has proved of extreme value to probe the structure of product ions often found in the CID of charged peptides,[13] thus furthering our current understanding of the mechanisms involved in the gas phase dissociation of these species.

Assaying the H_3PO_4 loss process in $pSerH^+$ may constitute a benchmark for probing the same path in phosphorylated peptides. Product ions at *m/z* 88 ($C_3H_6NO_2^+$) were formed by low-energy CID of electrosprayed $pSerH^+$ (*m/z* 186) in the collision cell of a hybrid FT-ICR tandem mass spectrometer (APEX-Qe, Bruker) or in the ion trap of an Esquire 6000 (Bruker) instrument, admitting IR radiation from a free electron laser (FEL) at the Centre Laser Infrarouge d'Orsay (CLIO), and from a tabletop parametric oscillator/amplifier (OPO/OPA) laser source, respectively. Details of the two instrumental platforms are provided in the Electronic Supplementary Information. Formation of ions at *m/z* 88 occurred in both cases, implying elimination of H_3PO_4. Interestingly, in the landmark paper of Ohanessian at al., the same fragmentation pathway was observed upon IRMPD of $pSerH^+$,[10a] confirming that both dissociation techniques promote similar energetic channels. When mass-selected

$C_3H_6NO_2^+$ (*m/z* 88) ions are irradiated with IR light, resonant absorption of IR photons ultimately activates a wavelength-dependent fragmentation process, yielding an ion at *m/z* 70, by elimination of a water molecule. In addition, a minor dissociation channel, leading to product ions at *m/z* 42, was observed only for highly active vibrational resonances (Fig. S1).The fragmentation product pattern was further inspected by energy-resolved CID experiments of selected $C_3H_6NO_2^+$ ions using a hybrid triple quadrupole-linear ion trap mass spectrometer. The appearance of product ions at *m/z* 70 and *m/z* 42 was monitored as a function of increasing values of collision energy, confirming consecutive losses of H_2O and CO, thus indicating dehydration of $C_3H_6NO_2^+$ as the lowest energy fragmentation route (Fig. S2, ESI).

The dependence of the IRMPD yield R (defined as $-\ln [I_P/(I_P + I_F)]$, where I_P and I_F are the abundances of the precursor and product ions, respectively) on the radiation wavenumber, in both the mid-IR (940-1900 cm^{-1}) and the NH/OH stretching ranges (3140-3700 cm^{-1}), is displayed in Figure 1a. The two profiles exhibit intense bands centered at 1010, 1167, 1437, 1530, 1793, 3415, and 3540 cm^{-1}.

In order to gather insight into the vibrational features underlying the IRMPD spectrum, which mainly reflects the absorption of the first resonant IR photon, theoretical calculations at the B3LYP/cc-pVTZ level were carried out, which allowed us to identify various isomers of the sampled $C_3H_6NO_2^+$ ions (Figure S3), and to assign their vibrational transitions (Figures 1, and S4).

According to the relevant thermodynamic data, which include relative enthalpy and free energy values (kJ mol^{-1}) at 298 K (Table S1) the dehydroalanine product ion **1**, where the protonated amino group interacts by hydrogen bonding with the carbonyl oxygen, lies in a global minimum, while its rotamer **2,** differing for the orientation of the carbonyl group, is 19.6 kJ mol^{-1} less stable. The 3-membered aziridine structure **3**, with a protonated amino group and an *anti* conformation of the O=C-Cα-NH_2 moiety, and its rotamers **7**, and **8**, differing for the orientation of the carbonyl and the hydroxyl groups, lie 49.2, 64.8, and 81.9 kJ mol^{-1} higher in energy than **1**, respectively. Elimination of H_3PO_4 via a 1,2-*cis*-β-elimination process is therefore thermodynamically favoured. However, a kinetic preference for the dissociation process (b) over (a) was reported by Reid *et al*,[6] who calculated the activation energy of the neighbouring amino group-assisted pathway (Scheme 1b) to be 40 kJ mol^{-1} lower than that of the β-elimination process (Scheme 1a). Other structures, including the 4-membered β-lactone product ions, protonated at either the amino group in **4**, or the carbonyl in rotamers **5** and **6**, are higher in energy (Table S1) and depicted in Figure S3.

Figure 1 presents the IRMPD spectrum of $C_3H_6NO_2^+$, in the two IR ranges, along with the calculated IR absorption spectra of the most stable structures among those resulting from routes (a), (b), and (c), **1**, **3**, and **4**, respectively. An exhaustive comparative presentation of the linear IR spectra of all calculated species is presented in Figure S4. A good agreement is found between the experimental IRMPD spectrum of $C_3H_6NO_2^+$ and the IR spectrum calculated for the aziridine-ring structure **3.** The experimental and computed vibrational features are summarized in Table 1, and associated to a concise mode description.[14]

In the "fingerprint" region, a peak at 1010 cm^{-1} nicely matches with the NH_2 wagging expected at 1034 cm^{-1}; a band at 1167 cm^{-1}, encompassing three IR bands at 1129, 1150 and 1162 cm^{-1}, is associated to CH and COH bending motions, while the features at 1437, 1530, and 1793 cm^{-1} arise from the CC stretching calculated at 1416 cm^{-1}, the NH_2 bending predicted at 1544 cm^{-1}, and the carbonyl stretch expected at 1774 cm^{-1}, respectively. In the higher energy region, the IRMPD spectrum presents a band at 3415 cm^{-1} assigned to the asymmetric NH stretch calculated at 3412 cm^{-1}, and a feature at 3540 cm^{-1} , due to the carboxylic acid OH stretch predicted at 3545 cm^{-1}.

Conversely, the calculated spectra of dehydroalanine (**1**, **2**), 4-membered β-lactone (**4**-**6**), and 3-membered aziridine (**7**, **8**) structures present very different IR spectra, thus excluding any significant contribution to the sampled ionic population.

As previously reported,[6] a kinetic control must be operative, leading to the preferential formation of a 3-membered aziridine ring **3**, rather than to the more stable dehydroalanine ion **1**. Interestingly, a kinetic preference for an intramolecular nucleophilic substitution reaction over the β-elimination process was also predicted for the model tryptic, phosphorylated peptide GAILpSGAILR.[3] In this case, a combination of quantum mechanical calculations with the Rice–Ramsperger–Kassel–Marcus (RRKM) theory revealed lower activation barriers for the equivalent of pathway (b) in our Scheme 1 than for the β-elimination process (pathway (c) in Scheme 1), suggesting that our results should be confirmed in more complex systems. A very similar scenario was also described for the elimination of H_2O upon CID of protonated serine and threonine, again supporting the formation of an aziridine structure over the dehydroamino-butyric acid/dehydroalanine species.[15]

Table 1. Experimental IR bands and theoretically predicted vibration modes for isomer (**3**).

Wavenumber (cm^{-1})		Vibrational mode[b]
Experimental	Calculated[a]	
1010	1034 (101)	Wag NH_2
1167	1129 (30) 1150 (63) 1162 (138)	β C-H β C-OH, ν C–OH Wag CH_2
1269	1231 (12)	Rock all ring Hs
1363	1297 (14)	β C-OH, C-H
1437	1416 (110)	ν C-COOH, β O-H, β C-H
1530	1544 (99)	Sciss NH_2
1793	1774 (250)	ν C=O
3415[c]	3412 (177)	ν_{asym} NH_2
3540[c]	3545 (186)	ν O-H

[a] In cm^{-1}. Calculated vibrational modes for isomer **3** at B3LYP/cc-PVTZ level of theory. Simulated intensities given in parenthesis are in km mol^{-1}. Bands with intensity lower than 10 km mol^{-1} are not included. All calculated IR frequencies in the 940-1900 cm^{-1} (3150-3700 cm^{-1}) range were scaled by a factor of 0.973 (0.960). [b] Wag = wagging; β = bending; ν = stretching rock= rocking; sciss = scissoring. [c] Frequency region (3150-3700 cm^{-1}) explored with an OPO/OPA laser system.

To conclude, IRMPD spectroscopy was successfully applied to elucidate the structure of the ions resulting from CID-induced elimination of H_3PO_4 from protonated phosphoserine. The search for matching between the experimental IR features and those theoretically predicted for the most stable conformers of candidate product ions point to the formation of *N*-protonated 2-carboxy aziridine. This finding implies that the elimination of H_3PO_4 occurs *via* participation of the vicinal amino group, involved in a nucleophilic attack on the β-carbon of protonated phosphoserine. Despite thermodynamically disfavoured by 49.2 kJ mol^{-1} over the β-elimination product ions (**1** and **2**), preferential formation of the aziridine ring (**3**) is enabled by a

kinetic control of the reaction. Ultimately, we reported the first direct structural characterization of these fundamental ionic species, thus providing a benchmark for investigating the same process in more complex phosphorylated peptides. As it stands, this study provides compelling evidence for the mechanism involved in the loss of neutral H_3PO_4, a process of great relevance for both fundamental studies and applications of MS to the analysis of phosphorylated species.

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Notes and References

[a]*Manchester Institute of Biotechnology, School of Chemistry, University of Manchester 131 Princess Street, M17DN Manchester (UK). Email:francesco.lanucara@manchester.ac.uk; Tel: +44 0161 306 4586*
[b]*Dipartimento di Chimica e Tecnologie del Farmaco, La Sapienza Universita' di Roma, P.le Aldo Moro 5, 00185 Roma (Italy). Email:mariaelisa.crestoni@uniroma1.it; Tel: +39 064991 3596/3878*
[c]*Université Paris Sud, Laboratoire de Chimie Physique, UMR8000 CNRS Faculté des Sciences, Bâtiment 350, 91405 Orsay Cedex (France)*
Electronic Supplementary Information (ESI) available: [Experimental methods and Computational details; Fig. S1-S4; Table S1]. See DOI: 10.1039/c000000x/

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