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Radical-driven processes within a peptidic sequence of type I collagen upon single-photon ionisation in the gas phase

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We report on an experimental single-photon absorption study on gas-phase protonated collagen peptides employing a combination of mass spectrometry and synchrotron radiation. Partial ion yields for the main photoabsorption products vary steadily with photon energy over the range from 14 to 545 eV. At low energy, non-dissociative photoionisation competes with neutral molecule loss from the precursor ion, whereas fragmentation of the peptide backbone dominates at soft X-ray energies. Neutral molecule losses from the ionised peptide are found to have low energy barriers and most likely involve amino-acid residue side-chains with radical character, in particular aspartic acid. A particularly interesting finding is photoinduced loss of proline hydroxylation. The loss of this typical collagen post-translational modification might play a destabilizing role in the collagen structure.

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Introduction

The intrinsic properties of biologically-relevant molecular systems in the gas phase have been studied for several decades. To date, the most thoroughly investigated gas-phase molecule of biological relevance has been certainly water, the most abundant molecule in living organisms. The gas phase building blocks of proteins, DNA and lipids have also been studied, as well as their response upon excitation and ionization. From this, the properties of the respective large biomolecular systems have been extrapolated. The driving force for this bottom-up approach on the experimental side was the limitation to molecular beam sources based on thermal evaporation, which excluded most biomolecular species, because of their thermal instability. From the theoretical point of view, available computing power limited reliable calculations to small systems (10–20 atoms).

The development of soft sources of gas-phase molecules such as electrospray ionisation (ESI), Matrix-Assisted Laser Desorption (MALDI), laser ablation of microdroplets and others has triggered a wealth of experimental studies on large biomolecular systems such as peptides, proteins, oligonucleotides, polysaccharides, and even non-covalent complexes of these molecules.

However, to date, most experimental studies focused on molecules originating from the cell nucleus, whereas the extracellular matrix received much less attention. In the case of cartilage, this matrix contains mainly collagen, which is the most abundant protein in the human body. Collagen has a peculiar geometrical structure: three peptidic chains are tightly wound around each other in a triple helix. Assemblies of this helix lead to the unique mechanical properties of cartilage. At the basis of the collagen helical structure lies a regular amino acid sequence of the type (Xxx-Yyy-Gly)_n (where Gly is glycine) and a high proline content of about 20%. The triple helix is also stabilised by proline hydroxylation, a post-translational modification undergone by a number of Yyy residues in collagen.

Despite the importance of collagen, to our knowledge only a single gas-phase study has yet focused on this system¹ and the response of gas-phase collagen upon interaction with photons has never been investigated, yet. Only very recently, we reported on VUV and X single-photon absorption of isolated collagen mimetic peptides.²

Interaction of ionising radiation (mainly photons, electrons, ions and atoms) with biologically-relevant systems in the gas phase is an active area of research. First experimental studies employed electron beams, as affordable and compact sources can deliver the high intensities needed to obtain sufficient collision rates for dilute gas-phase targets. The widespread use of electron impact ionization leads to the construction of widely known mass spectral databases^{3,4} aiming at identifying molecular species by its electron-impact ionisation mass spectrum.

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A fundamental problem of collision studies however remained: in electron impact ionisation studies, there is little control of the energy transferred to the molecule. To tackle this problem, laser induced ionization has been employed. This has led to a deeper understanding of the fundamental physical and chemical processes induced by ionising radiation. For instance, early studies on UV multiphoton ionisation of peptides demonstrated that at low laser flux, it is possible to produce intact radical cations with very low vibrational energy.^{5,6} With increasing photon flux, fragmentation occurs, delivering valuable information on the peptidic sequence. Synchrotron facilities with their high photon flux beamlines have opened up an entirely new area of research, focusing particularly on the properties of biologically-relevant molecules such as neutral building blocks of DNA and proteins.^{7–9} More recently, also photoabsorption in protonated and deprotonated molecular systems of larger size (produced by means of ESI sources) was achieved. For instance, Milosavljevic *et al.*^{10,11} coupled a commercial RF linear ion trap mass spectrometer to VUV and X-ray beamlines at the SOLEIL synchrotron, showing that several electrons can be removed by absorption of a single photon, with almost no fragmentation of the protein. This is rather different from smaller systems such as peptides^{12–14} or DNA mono-¹⁵ and oligonucleotides,¹⁶ which extensively fragment after single photoabsorption. The role of the molecular size has recently been demonstrated for peptides and proteins.¹⁷ In general, fragmentation yields increase with the internal temperature of the system after photoabsorption, with small and hot peptides giving rise to immonium ion formation whereas large and thus cooler proteins are mainly subject to neutral losses and non-dissociative ionisation. Though, even for large proteins such as ubiquitin, immonium ion formation is not fully quenched, indicating the presence of fast local dissociation channels. This has been confirmed in the XUV multiphoton absorption experiment on ubiquitin using the free electron laser FLASH.¹⁸

Until now, however, the detailed mechanism underlying photoinduced backbone cleavage and neutral losses has been far from being understood. In this work, we report single-photon absorption data of a peptidic sequence of type I collagen over a wide energy range (14–545 eV), by coupling a synchrotron beamline with a tandem mass spectrometer.

Experimental

Synthetic peptides PK26-P (average mass = 2274.4 Da) of sequence PGG-PPG-PKG-NSG-EPG-APG-SKG-DTG-AK and PK26-Hyp (average mass = 2322.4 Da) of sequence PGG-PHypG-PKG-NSG-EHypG-AHypG-SKG-DTG-AK (where Hyp is hydroxyproline) were purchased from ProteoGenix (France). Note that we only modified proline residues at Y positions (see the introduction section). Samples of over 95% purity were used with no further purification and were prepared in 50:50 (volume ratio) water/methanol solutions at a 50 μ M peptide concentration with 1% of formic acid.

A home built tandem mass spectrometer, sketched in Fig. 1 and described in detail elsewhere,¹⁹ has been used to record

Fig. 1 Scheme of the home built tandem mass spectrometer.

the mass spectra of the cationic photo-products obtained from the interaction. Briefly, protonated peptides are produced using 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 using a quadrupole mass-filter and subsequently accumulated in a 3D radiofrequency ion trap (low-mass cutoff $m/z = 80$). Trapping is facilitated by collisions with a helium buffer gas injected into the ion trap during the filling process. Photon beam exposure of the trap content, typically during 300 ms 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 peak below 10%. Since the absorption of multiple photons is a sequential process at these fluxes ($10^{12-13} \text{ s}^{-1}$), 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 $p < 0.09$. After the irradiation, fragment ions can carry kinetic energy from the fragmentation process; they are thus cooled down by injection of a second He buffer gas pulse. Precursor ions and cationic fragments are then extracted from the trap and analysed using time-of-flight mass spectrometry. The mass spectra of the non-irradiated trap content and irradiated residual gas are recorded as well in order 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 precursor depletion with a negative intensity. Assuming that absorption of one photon leads to ionisation and/or fragmentation of the precursor ion, this depletion is proportional to the total photo-absorption yield. In the following, all mass spectra are normalised by the precursor depletion.

VUV and soft X-ray photo-absorption experiments were carried out at the BESSY II synchrotron (Helmholtz-Zentrum Berlin) on the U125-2_NIM²⁰ and the U49-2_PGM-1²¹ beamlines, respectively. Protonated peptides were irradiated over a wide range of photon energy, from 14 eV to 545 eV. The photoabsorption signal was too weak for photon energies from 30 to 90 eV, due to too low absorption cross-section and/or photon flux, resulting in a gap in our measurements.

On the soft X-ray beamline, photon energies were selected in order to explore the effect of carbon, nitrogen and oxygen inner shell ionisation or excitation through various molecular transitions. Since it has been shown that the NEXAFS spectra of a

Table 1 X-ray photon energies used in this work and corresponding electronic transitions for C, N and O K-edge photo-excitation and ionisation²⁵

Atom	Transition	Amino acid	Energy (eV)
Carbon	1s → π^* (C=O)	All	288.2
	1s → continuum	All	300
Nitrogen	1s → π^* (C=O)	All	401
	1s → σ^* (C–N)	All	406
	1s → σ^* (C–N)	Pro, Hyp	409.6
	1s → continuum	All	412
Oxygen	1s → π^* (C=O)	All	532
	1s → σ^* (C–OH)	Ser, Thr, Hyp	538
	1s → σ^* (C=O)	All	545

protein in the gas phase are similar to those obtained in thin films,^{22,23} we used the reported photon energies. Moreover, according to the work of Zubavichus *et al.*,²⁴ the NEXAFS spectra of a protein can be interpreted as the sum of their constituent amino acids. Thus, we also used reported photon energies for single amino acids, in particular for the molecular transition in the hydroxyproline residue. Photon energies and the corresponding electronic transitions are listed in Table 1.

Results and discussion

A. Photoionisation and dissociation of the [PK26-P + 3H]³⁺ peptide

The photoabsorption mass spectra of the triply-protonated peptide [PK26-P + 3H]³⁺ ($m/z = 759.2$, noted M^{3+}) for photon energies of 14, 22, 150, 288 and 538 eV are shown in Fig. 2. Below 150 eV, non-dissociative ionisation leading to the formation of the radical peptide ion $M^{4+\bullet}$ ($m/z = 569.4$) dominates the spectra. Above this energy, the spectra contain intense fragment ion peaks, assigned to small ions of $m/z = 80$ –200. It can be noted that we do not observe any peak above the precursor peak, which suggests that no large singly or doubly charged fragments are produced. Overall, we observe a smooth evolution of the spectra with photon energy increasing from 14 to 545 eV. Such a behaviour has been observed for similar energies for the case of the doubly charged oligonucleotide [dGCAT + 2H]²⁺¹⁶ and for neutral vanillin (C₈H₈O₃).²⁶

Mass spectra. At 14 eV, the spectrum is dominated by peaks assigned to the intact radical peptide ion $M^{4+\bullet}$ and loss of neutral molecules from both M^{3+} and $M^{4+\bullet}$. The observation of these losses is consistent with the photo-dissociation spectra of protonated Substance P probed in the 5.2 to 20 eV region,²⁷ *i.e.* around the ionisation threshold (10.3 eV). In the present case, losses of neutral molecules with 18, 30 and 44 Da from M^{3+} and 30, 44 and 72 Da from $M^{4+\bullet}$ are observed, with the 44 Da loss channel being the most intense in both cases. Note that the peak resolution in our spectra does not allow ruling out a minor contribution of other losses, but we focus here on the major ones. The underlying processes will be detailed in the “Valence shell photoabsorption” section. The remaining peaks in the mass spectrum are weaker, and can be assigned to

Fig. 2 Photoabsorption mass spectra of the [PK26-P + 3H]³⁺ peptide for different photon energies. The dashed line shows the position of the precursor peak noted M^{3+} ($m/z = 759.2$). Coloured lines indicate fragments from a given peptide bond cleavage. Background peaks are spotted by an asterisk. Above the spectra, we sketch the peptidic sequence with the conventional nomenclature for amino acids to highlight backbone cleavage sites.

backbone cleavage leading to the formation of the following fragments: z_1^+ ($m/z = 130.2$), y_4^+ ($m/z = 376.4$), z_4^+ ($m/z = 359.4$), z_{13}^{2+} ($m/z = 563.6$), b_{13}^{2+} ($m/z = 567.1$), a_{16}^{2+} ($m/z = 665.7$) and b_{16}^{2+} ($m/z = 679.3$).

In the soft X-ray regime (above the carbon K-edge), $M^{4+\bullet}$ and large fragment peaks vanish while the intensity of the small fragments from peptide backbone cleavage increases and several additional fragmentation channels open up. The respective small peptidic fragments are composed of 1 to 4 amino acids from both N- and C-terminals of the peptide: a_2^+ ($m/z = 127.2$), b_2^+ ($m/z = 155.2$), c_2^+ ($m/z = 172.2$), a_3^+ ($m/z = 184.2$), b_3^+ ($m/z = 212.2$), a_4^+ ($m/z = 281.2$), b_3^+ (309.3), z_1^+ ($m/z = 130.2$), $(x_2 - H_2O)^+$ ($m/z = 226.3$), z_4^+ ($m/z = 359.4$) and y_4^+ ($m/z = 376.4$).

Interestingly, no complementary ions of these fragments are observed. This could be due to further fragmentation of these ions, which would lead to the formation of smaller fragments. For instance, the dipeptides Pro–Gly and Gly–Pro can be found six times in the whole peptide and cleavage of the C–N bond at each side would lead to the formation of an internal ion of $m/z = 155.2$. This fragment could contribute to the peak attributed to b_2^+ (NH–Pro–Gly–CO + H)⁺, which is very intense at a high photon energy.

It worth noting here that no intact multiply ionised peptide is observed, over the entire range of photon energies under study, in contrast to what has been observed for large peptides or proteins.^{19,22,28}

Fig. 3 Relative yield of different ions formed after absorption of one photon on the 14 to 545 eV energy range by the peptide [PK26-P + 3H]³⁺. The gap between 29 and 90 eV is explained in the experimental section. The grey curve shows the sum of the main backbone fragments observed (z_1^+ , a_2^+ , b_2^+ , b_3^+ , $(x_2-H_2O)^+$, a_4^+ , b_4^+ , z_4^+ and y_4^+). Uncertainties have been calculated by Gaussian fitting of the peaks with the Fityk software.

Photon energy dependence of fragmentation pathways. The relative yields of several product ions are plotted as a function of photon energy in Fig. 3. From 14 to 20 eV, neutral losses from the precursor peptide, (M-18)³⁺ and (M-44)³⁺, decrease whereas the photoionisation yield increases up to a maximum close to 21 eV. These opposite trends are consistent with our assignment for these peaks and show that photoabsorption at these energies can lead to electronic excitation without ionisation, akin to the case of collagen mimetic peptides.² Internal conversion to the ground state can then occur, followed by loss of neutral molecules or backbone fragmentation, mainly on the N-terminal side of Pro residues and C-terminal side of Asp and Glu residues. These preferential cleavage sites are well documented for protonated peptides undergoing low-energy CID,²⁹ which is in line with ground state processes. Losses of 44 and 72 Da from M⁴⁺• closely follow the same trend as M⁴⁺•. Above 22 eV, their yields decrease smoothly and vanish beyond 300 eV. The opposite trend is observed for the backbone fragmentation yield. This behaviour could be due to an increase of vibrational energy deposited into the peptide during the photoionisation process. It would imply that when the photon energy rises, deeper orbitals become accessible and photoabsorption leads to higher excited states, and to more internal energy after internal conversion.

Valence shell photo-absorption. The photoabsorption mass spectra obtained at 14 and 22 eV are in agreement with those reported for smaller protonated peptides, such as leucine-enkephalin^{14,30} or substance P,²⁷ at similar energies, *i.e.* above the ionisation threshold. Moreover, Giuliani *et al.*³¹ have shown that for large proteins, the ionisation energy (IE) increases with the charge state. The presence of the photoionised peptide after absorption of one photon of 14 eV is therefore consistent with previously reported IE of 10.3 eV for singly-protonated substance P²⁷ and lower than 13 eV for small proteins of charge state 4+.³¹

Mass spectra obtained with leucine-enkephalin and substance P also show neutral molecule losses from the precursor peptide M⁺ and the radical peptide cation M²⁺•, which were in

Fig. 4 Zoom in the peptide radical cation and side chain loss region of the mass spectra obtained after absorption of a single 22 eV photon by [PK26-P + 3H]³⁺. Note that our assignment does not rely only on this spectrum (for which the resolution is poor), but on several spectra obtained for the two charge states (3+ and 4+) of the PK26-P and PK26-Hyp peptides.

both cases attributed to amino acid side-chain losses.^{14,32} It can be assumed that the neutral losses observed in our case also originate from amino acid side-chains. From the PK26-P peptide precursor M³⁺, loss of 18 Da is assigned to H₂O from Ser, Thr, Asp or Glu side-chains, present in this peptide. This loss might also come from the C-terminal carboxyl group or from the N-terminal backbone amide group.^{59,60} Loss of 30 Da is assigned to CH₂O from the Ser side-chain and loss of 44 Da to CO₂ from the Asp or Glu side-chain. No loss of 44 Da has been reported for leucine-enkephalin,¹⁴ GAILPTGAILK³³ and gramicidin A,³⁴ which do not contain these two amino acids, suggesting that this loss does not originate from the C-terminal. From the M⁴⁺• radical cation (see Fig. 4), the same assignment can be made for the loss of 30 and 44 Da. The latter has also been observed for photoionization of angiotensin I (DRVYIHPFHL)³⁴ and II (DRVYIHPF),³³ which contain an Asp residue. Loss of 72 Da from the M⁴⁺• radical cation could correspond to C₃H₄O₂ from Glu or C₄H₁₀N from Lys. However, no 72 loss from the lysine side-chain has been reported in the case of substance P,²⁷ which suggests an involvement of the Glu side chain.

In a recent study,³⁵ we observed no loss of neutral molecules from the PK26-P peptide precursor M³⁺ after low-energy collision-induced dissociation (CID) and UV photo-absorption at 220 nm (5.6 eV). This suggests that highly-excited states or ground-state high energy processes are involved in the neutral molecule losses reported here for higher energy photons. This is in agreement with the work of J. P. Reilly's group for the case of peptide photoabsorption at 157 nm (7.9 eV).^{36,37} In contrast, a similar trend followed by both the relative yield of M⁴⁺• and its side chain losses suggests that low energy processes account for these losses (consistent with the appearance energies of a few eV reported in the case of substance P²⁷).

In the following, we will focus on the origin of the CO₂ loss from M⁴⁺•, which is the most intense loss observed. It has been shown in the case of CID experiments of the peptide radical cations [DGR]^{•38} and z fragment radical cations from [AADAR]^{•39} that CO₂ loss from the Asp side-chain is a major dissociation

pathway. This has been attributed to a very low potential energy barrier, calculated to be 1.25 eV, which is lower than COOH• loss or backbone cleavage.⁴⁰ Interestingly, in the case of [DGR]⁴⁺, CO₂ loss is more abundant from the Asp side-chain than from the C-terminal carboxyl group.³⁸ The mechanism proposed to account for this pathway implies that the radical is left on the peptide after CO₂ loss from Asp, which could therefore induce further fragmentation.⁴¹ This radical-driven dissociation mechanism and the strong yield of CO₂ loss observed are consistent with Asp being the most favourable site of radical migration in the case of the [PK26-P + 3H]⁴⁺ radical cation.

From *z* radical cation fragments containing a Glu residue, formed by electron transfer dissociation of protonated nonapeptides, losses of C₂H₃O₂• (59 Da) and C₃H₄O₂ (72 Da) have been attributed to the Glu side-chain, and no 44 Da loss was detected.³⁹ We also see a loss of 72 Da from the radical PK26-P peptide ion M⁴⁺, and the absence of 44 Da loss is consistent with our attribution of CO₂ loss to the Asp side chain. Moreover, radical driven side-chain losses from lysine were reported to be C₃H₈N• (58 Da) and C₄H₉N (71 Da).⁴² A loss of 72 Da from the lysine side-chain would point to a loss of a protonated side chain, resulting in a charge-reduced peptide, which is not observed experimentally. Therefore, we assign the loss of 72 Da to the Glu side chain.

A number of experiments performed with peptide radical cations formed by electron capture on protonated peptides,^{42–44} electron impact ionisation (20–100 eV)⁴⁵ or femtosecond IR laser ionisation^{33,46} show the same side chain losses from Asp, Glu and Ser as observed here for single-photon ionisation. We summarise the data obtained in this work as well as found in the literature in Table 2. As a conclusion, the similarity of our results to those obtained using other ionisation methods indicates that no matter how a radical site is created on a peptide, it will result in specific side-chain losses associated with low potential energy barriers that outcompete charge-driven or charge-remote processes as often involved in CID experiments of protonated peptides.²⁹

Inner shell photo-absorption. Absorption of a single soft X-ray photon by an atomic K-shell leads to very similar mass spectra for [PK26-P + 3H]³⁺, dominated by small singly-charged fragment ions. Interestingly, for a given atom (*e.g.* C, N or O), the same spectra were obtained for all the transitions included in Table 1. This indicates that the initially localised energy is redistributed within the peptide before fragmentation, and that the “memory” of the absorbing site is lost.

B. Photoionisation and dissociation of the [PK26-P + 4H]⁴⁺ peptide: influence of the initial charge stage

Fig. 5 shows the photoabsorption mass spectra obtained for the [PK26-P + 4H]⁴⁺ (*m/z* = 569.6, noted M⁴⁺) peptide at 14, 22, 150, 288 and 538 eV photon energies. We observe a similar behaviour to that observed for the triply-protonated peptide. At low energy, the intact radical peptide cation M⁵⁺• dominates the spectra and we observe the same side-chain losses from both M⁴⁺ and M⁵⁺•, with CO₂ loss being the most intense. Above 300 eV, M⁵⁺• is no longer observed and small backbone fragments,

Table 2 Mass of the neutral molecules lost from protonated peptides. The amino acids whose side-chains are assumed to be involved in the losses are highlighted in bold and coloured

Peptide	Ref.	Precursor's losses (amu)	Ionised peptide's losses (amu)
PK-26-P PGGPPGPKGN SGEPG APG SKGDTGAK	This work	18, 30, 44	18, 30, 44, 72
leucine-enkephalin YGGFL	14,30	18, 91, 107, 108	Not detected
Substance P RPPKQQFFGLM-NH ₂	32	15, 17, 28, 29, 35, 43, 44, 45, 47, 59, 61, 62, 72, 74, 75, 87, 91, 100	15, 16, 17 29, 43, 45 47, 56, 58 59, 61, 74 87, 100
angiotensin I DRVYIHPFHL	34	44	17, 18 44, 106
angiotensin II DRVYIHPF	33	Not assigned	44, 106
Gramicidin A VGALAVVV WLWLWLW	34	Not detected	Not detected
EGVNDNE EGFFSAR	33	Not detected	44
GAILpTGAILK	33	Not detected	Not assigned
LFpTGHP ESLER	33	18 98	30, 44 56, 72, 85
Melittin GIGAVLKVLTGTPAL ISWIKRRKQ-NH ₂	34	44	44
YG ₁₀ F	12,34	Not detected	17, 18, 107 18+107 17+107
D SHAKRHHG YK RKF HEKHHSHR G Y	47	Not assigned	18, 30, 44 62, 72, 106

similar to those observed for the case of [PK26-P + 3H]³⁺, are the most intense peaks.

Two differences between the photoabsorption spectra of [PK26-P + 4H]⁴⁺ and [PK26-P + 3H]³⁺ are, however, observed. The first one is the larger variety of backbone fragments observed for [PK26-P + 4H]⁴⁺ at 14 eV, which are due to dissociative photoexcitation. Over 20 fragments can be identified here whereas only 5 are observed for the triply-protonated case. The second difference is the strong decrease of the CO₂ loss from the Asp side-chain of M⁵⁺•. In the previous section, we attributed this channel to a reaction triggered by the radical formed after ionization, which can occur anywhere in the peptide at these energies. Thus, this radical has to be transferred to the Asp side-chain for this reaction to occur, but with a probability depending on the geometrical structure of the peptide. A possible explanation for these observations for M⁴⁺ is the change of the peptide structure driven by the increased Coulomb repulsion due to the additional proton. Therefore, we have performed molecular dynamics simulations to test this hypothesis.

REMD simulations. In order to study the conformation of the PK26-P peptide as a function of protonation state, we performed

Fig. 5 Photo-absorption mass spectra of the $[\text{PK26-P} + 4\text{H}]^{4+}$ peptide for different photon energies. The dashed line shows the position of the precursor peak noted M^{4+} ($m/z = 569.6$). Coloured lines indicate fragments from a given peptide bond cleavage. Background peaks are spotted by an asterisk. Above the spectra, we sketch the peptidic sequence with the conventional nomenclature for amino acids to highlight backbone cleavage sites.

replica-exchange molecular dynamics (REMD) simulations.⁴⁸ This method allows an efficient sampling of the potential energy surface of a molecular system, in order to find the structure of the lowest-energy conformations. For the $[\text{PK26-P} + 3\text{H}]^{3+}$ peptide, we bound the three protons to the three lysine side chains, which are probably the most favourable protonation sites (gas-phase proton affinity $\sim 988 \text{ kJ mol}^{-1}$ ⁴⁹). Since the N-terminal belongs to a proline, it is the second most favourable protonation site (gas-phase proton affinity $\sim 960 \text{ kJ mol}^{-1}$ ⁵⁰). In the case of $[\text{PK26-P} + 4\text{H}]^{4+}$, we thus attached the fourth proton to the N-terminus. We used the AMBER 99 force field and 30 replicas were sampled from 20 to 1000 K with a geometric progression. Each replica was propagated for 20 ps for thermalisation to occur, and then for an additional 2 ns with a 1 fs time step. For thermal equilibrium, we used a Berendsen thermostat with a 0.1 ps^{-1} coupling constant. Exchange between neighbouring replicas was attempted every 100 fs. For each replica, a snapshot was taken every 40 ps and the 1500 geometries obtained were further optimised with the AMBER 99 force field.

The lowest potential-energy conformers found using this method are shown in Fig. 6. The $[\text{PK26-P} + 3\text{H}]^{3+}$ conformer shows a folded structure while that of $[\text{PK26-P} + 4\text{H}]^{4+}$ is more unfolded. In the case of the triply-protonated peptide, the average distance between the N-terminal and the C-terminal for conformers up to 1.0 eV above the lowest-energy one is about 22.5 Å. This distance increases to 34.2 Å with the additional fourth proton. Moreover, it is worth noting that the first unfolded structure for $[\text{PK26-P} + 3\text{H}]^{3+}$

Fig. 6 Optimised lowest-energy conformers obtained by REMD simulations for peptides $[\text{PK26-P} + 3\text{H}]^{3+}$ (top) and $[\text{PK26-P} + 4\text{H}]^{4+}$ (bottom). H, C, N and O atoms are coloured in white, grey, blue and red, respectively. Proline side-chains are filled in with blue colour in order to spot them easily.

is found 0.6 eV above the lowest energy conformer. Similarly, the first folded structure for $[\text{PK26-P} + 4\text{H}]^{4+}$ appears 1.0 eV above the lowest energy conformer. These values are sufficiently high to ensure that the global shape of the peptides is reliable.

It has been shown that Coulomb-driven unfolding of ubiquitin induces a quenching of the radical migration.⁵¹ In the case of the PK26 peptides, several initial ionisation sites are reachable after photoabsorption, because at low energy, delocalised valence orbitals are probed and at high energy, when 1s electrons are involved, a high number of C, N and O atoms are present. If we assume that CO_2 loss is driven by the radical created by ionisation, this radical has thus to be transferred to the Asp side-chain. Therefore, this unfolding-induced quenching is consistent with our observation of a reduced CO_2 loss from $[\text{PK26-P} + 3\text{H}]^{3+}$ to $[\text{PK26-P} + 4\text{H}]^{4+}$. It is also possible that this effect favours other competing fragmentation pathways triggered by the radical at other sites. This might be the reason for the larger variety of backbone fragments observed in the case of the $[\text{PK26-P} + 4\text{H}]^{4+}$ peptide. A similar effect has been observed by Xu *et al.*³⁸ on $[\text{DGR}]^{+*}$, by adding a CH_3 group substituting for the H atom of the backbone C_α bound to the Asp side chain, in order to quench the transfer of this H and thus migration of the radical on this residue. They observed that upon CID, this modified peptide radical cation does not undergo CO_2 loss, but instead backbone cleavage leading to an intense y_2^+ fragment, originally absent in the mass spectrum of the unmodified $[\text{DGR}]^{+*}$. To our knowledge, this effect observed in the case of photoionisation and triggered by the initial protonation state has never been reported in the literature for such large peptides. However, we cannot rule out a possible role of the fourth proton in the enhanced backbone fragmentation for $[\text{PK26-P} + 4\text{H}]^{4+}$, if some of it is due to charge-driven processes.

C. Photoionisation and dissociation of the PK26-Hyp peptide: influence of hydroxyprolines

Proline hydroxylation is only a small modification of the proline side-chain, but has a crucial role in collagen stability *in vivo*.

To investigate the putative effect of this modification on the behaviour of PK26 peptides under ionising radiation, we performed photoabsorption experiments on the $[\text{PK26-Hyp} + 3\text{H}]^{3+}$ and $[\text{PK26-Hyp} + 4\text{H}]^{4+}$ peptides under the same experimental conditions as those for the PK26-P peptides. Over the 14 to 545 eV photon energy range, the mass spectra obtained for the PK26-Hyp peptides are very similar to those obtained for PK26-P peptides. However, a striking difference can be observed for neutral molecule losses from the peptide radical cation: the increase of H_2O loss is particularly strong in the case of $[\text{PK26-Hyp} + 4\text{H}]^{4+}$. This effect is highlighted in Fig. 7. As described previously, $[\text{PK26-P} + 3\text{H}]^{3+}$ and $[\text{PK26-Hyp} + 3\text{H}]^{3+}$ show similar side-chain losses from the peptide radical cations: CH_2O (30 Da), CO_2 (44 Da) and $\text{C}_3\text{H}_4\text{O}_2$ (72 Da), from Ser, Asp and Glu side-chains, respectively. An additional weak peak shows up for the hydroxylated peptide and corresponds to a loss of 18 Da. We thus attributed it to H_2O loss from the photoionised peptide radical cation. This H_2O loss is enhanced in the case of $[\text{PK26-P} + 4\text{H}]^{4+}$ and $[\text{PK26-Hyp} + 4\text{H}]^{4+}$, even becoming the dominant loss for the latter. We also observe a decrease of the intact peptide radical cation, consistent with the appearance of this strong dissociation pathway. Since H_2O loss and $[\text{PK26-Hyp} + 4\text{H}]^{5+}$ follow the same trend as photon energy (data not shown), as the peaks assigned to loss of neutral

molecules from side chains, it strongly suggests a similar dissociation process induced by the radical.

For protonated peptides, the H_2O molecule is generally lost from serine and threonine side chains. In the case of CID, it is induced by charge-directed processes, which requires a mobile proton.^{52–54} It worth noting that in the case of CID of the $[\text{GSR}]^{+\bullet}$ and $[\text{GTR}]^{+\bullet}$ radical cations, abundant H_2O loss has been observed, in contrast to a range of other $[\text{GXR}]^{+\bullet}$ peptides.⁵⁵ However in our case, if these two amino acids were involved in H_2O loss, we should not observe any difference between PK26-P and PK26-Hyp. This hypothesis can therefore be excluded. On the other hand, Canon *et al.* have not observed H_2O loss from substance P²⁷ after VUV photoabsorption above the ionisation energy, which probably excludes a number of side chains as well as the C-terminal carboxyl group.

Nevertheless, Gupta *et al.*⁵⁶ have observed H_2O loss induced by collisional activation of the radical fragment $[\text{z}_{10} + \text{K}]^{+\bullet}$ (where K is potassium) from antiamoebin II. This fragment contains two hydroxyproline residues, from which they have attributed H_2O loss. As this fragment is positively charged by the potassium ion and not by a proton, it is consistent with this loss being induced by the radical. We can therefore assign the observed H_2O loss from the PK26-Hyp peptide radical cations to the hydroxyproline side-chain, which notably contains an OH group.

In addition, the strong increase of H_2O loss while CO_2 loss is lowered from peptide $[\text{PK26-Hyp} + 3\text{H}]^{3+}$ to $[\text{PK26-Hyp} + 4\text{H}]^{4+}$ might come from the quenching of radical migration. Indeed, we performed the same REMD simulations as for the PK26-P peptide, and also found an unfolding induced by the fourth proton. Three Hyps distributed along the peptide would then become more accessible for the radical than Asp, leading to a higher probability for this dissociation pathway.

The role of hydroxyproline in this water loss has also been investigated using experiments performed on two other peptides: $[(\text{PPG})_{10} + 2\text{H}]^{2+}$ and $[(\text{PHypG})_{10} + 2\text{H}]^{2+}$. Both peptides were irradiated under the same conditions with 150 eV photons. A zoom in the peptide radical cation $\text{M}^{3+\bullet}$ region of the mass spectra is shown in Fig. 7. For $[(\text{PPG})_{10} + 2\text{H}]^{2+}$, we observe two very weak peaks assigned to H_2O and CO_2 loss from the C-terminal group. Indeed, it is the only site on the peptide where an OH and a CO_2 group can be found and therefore lost. For the hydroxylated peptide, we observe a striking increase of the H_2O loss, which is likely to come from the hydroxyproline side chain and confirms our previous hypothesis for the PK26-Hyp peptide. Interestingly, even if the peptide $[(\text{PHypG})_{10} + 2\text{H}]^{2+}$ contains ten hydroxyproline residues, we do not observe loss of several H_2O molecules. It implies that the radical cannot induce a second side-chain H_2O loss. This is also in line with the case of the PK26 peptides, where no multiple side-chain losses have been identified. It is possible that part of the internal energy deposited by the absorbed photon is transferred into the neutral molecule side chain fragment as kinetic energy, leaving no sufficient energy into the peptide for further dissociation. Another possibility is that most of the photon energy is transferred into the photo-electron leaving a low amount of

Fig. 7 Mass spectra in the peptide radical cation region for peptides $[\text{PK26-P} + 3\text{H}]^{3+}$ and $[\text{PK26-Hyp} + 3\text{H}]^{3+}$ (in blue), $[\text{PK26-P} + 4\text{H}]^{4+}$ and $[\text{PK26-Hyp} + 4\text{H}]^{4+}$ (in green) and $[(\text{PPG})_{10} + 2\text{H}]^{2+}$ and $[(\text{PHypG})_{10} + 2\text{H}]^{2+}$ (in red) after absorption of one 150 eV photon.

internal energy in the peptide. None of these hypotheses can be confirmed without coincident measurement of the photoelectron kinetic energy, which cannot be performed with the present set-up.

From a more general point of view, it is interesting to interpret this water loss as a dehydration of collagen that could destabilise its helical structure. Indeed, as mentioned previously, proline hydroxylation helps stabilizing the collagen triple helix structure in the liquid phase.^{57,58} Therefore, if loss of hydroxyl groups also occurs *in vivo* as a result of the interaction with ionising radiation, it might lead to a destabilization of the collagen structure and a change in the cartilage mechanical properties.

Conclusion

We have probed the intrinsic physical and chemical processes induced by ionising radiation in a type I collagen peptidic sequence. VUV and soft-X-ray photoabsorption lead to ionisation, loss of neutral molecules as well as backbone fragmentation. The latter process increases in probability with photon energy, while non-dissociative ionisation falls between 20 and 545 eV, which is consistent with the amount of internal energy deposition rising with photon energy in this range. At low photon energy above the ionisation energy (14–20 eV), neutral molecules are lost from amino-acid residue side-chains of the precursor peptide, but also of the photoionised peptide radical cation. The low barrier for the latter losses, as well as comparison with earlier studies reported in the literature, are in line with radical-induced processes that mainly target the Asp side-chain in the studied peptides, leading to abundant CO₂ loss. Interestingly, we observe a quenching of this channel due to the decrease of radical migration upon unfolding of the peptide. Moreover, the unfolded peptide with hydroxyproline, a typical collagen post-translationally modified residue, shows extensive H₂O loss from the hydroxyproline side-chain. This process might destabilise collagen *in vivo* upon ionising radiation. However, studies on the irradiation of collagen in the condensed phase are needed to check this hypothesis.

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