Ion–molecule reactions reveal facile radical migration in peptides†

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Ion–molecule reactions between molecular oxygen and peptide radicals in the gas phase demonstrate that radical migration occurs easily within large biomolecules without addition of collisional activation energy.

With the continued development of mass spectrometric methods employing radical chemistry to fragment peptides and proteins in the gas phase, a detailed understanding of the behaviour of radicals in gaseous peptide ions is vitally important. Numerous methods (including electron initiated fragmentation,[1,2] direct bond homolysis by photolysis[3] or collisional activation,[4–10] and photoionisation[11]) can be used to generate peptide radicals. Following radical generation, the subsequent fragmentation chemistry of the peptide is typically dominated by radical-directed processes, making the location of the radical crucial as the initiation point for fragmentation. It is of paramount importance, therefore, to determine whether radicals can migrate within the framework of a peptide or whether barriers exist to prevent such migration.

Recent work, based on the comparison of collisional activation mass spectra of isomeric triglycine radical peptides and accompanying electronic structure calculations, concluded that radical migration is restricted by substantial activation barriers.[12] In contrast, other computational studies have estimated significantly smaller barriers.[13–15] In addition, a body of indirect experimental evidence also suggests that radical migration occurs in peptides prior to fragmentation.[16–18] However, experiments requiring collisional activation or other fragmentation methods are an imperfect probe of the structure of the nascent radical ions given that the possibility of radical migration (over substantial activation barriers), immediately prior to fragmentation, cannot be excluded. Herein, we demonstrate that reactive radicals generated within peptide cations can migrate immediately after their formation and without additional activation energy. This has been achieved through the novel combination of photodissociation for the regioselective generation of peptide radical cations and the use of ion–molecule reactions to probe the final location(s) of the radical.

Gas phase ion–molecule reactions have been used extensively in mass spectrometry as probes of molecular structure,[19] and have proven particularly powerful in differentiating isomeric radical ions.[20,21] Dioxygen has been shown to be a selective reagent, reacting with distonic radical ions (*i.e.*, where the charge and unpaired electron are formally localised on separate atoms within the molecule),[22–24] to form $[M + O_2]$ adduct ions while remaining unreactive toward conventional radical ions.[25–28] Furthermore, dioxygen is often present as a background gas inside commercial mass spectrometers thus obviating the need for instrument modification to observe these ion–molecule reactions. In a recent example, McLuckey and co-workers found that peptide radical cations resulting from electron transfer dissociation of multiply charged polypeptides form $[M + O_2]$ adduct ions upon storage in an ion-trap mass spectrometer. This allows for the facile distinction between z-ions and their complementary even-electron c-ion counterparts.[29]

We have previously demonstrated that peptide radical cations can be generated by the gas phase photodissociation of the C–I bond within iodinated tyrosine residues (see ESI†).[3] Thus formed, these radicals yield site-specific fragmentation upon subsequent collisional activation. This method provides a known starting point for the radical at the 3-position of the tyrosine side-chain. In this study, the archetypal tyrosinyl radical cation, $[Tyr^{\bullet} + H]^+$ (m/z 181), was generated by laser irradiation (266 nm) of protonated 3-iodotyrosine in a specially modified linear ion-trap mass spectrometer. The CID spectrum of $[Tyr^{\bullet} + H]^+$ (Fig. 1a) shows neutral losses of 17 and 46 Da that have previously been assigned for the even-electron $[Tyr + H]^+$ cation to losses of NH_3 and $[CO + H_2O]$, respectively.[30,31] The absence of specific radical-driven dissociation products in Fig. 1a is consistent with localisation of the unpaired electron at the 3-position on the phenyl moiety: with no evidence for radical migration even upon application of activation energy. Fig. 1b shows the mass spectrum obtained from trapping $[Tyr^{\bullet} + H]^+$ in the presence of adventitious O_2 for 3 s. The absence of a significant $[M + O_2]^{\bullet +}$ adduct ion at m/z 213 contrasts with previous studies of distonic radical cations (see earlier), as well as, the analogous reaction of O_2 with the phenylalaninyl radical cation, $[Phe^{\bullet} + H]^+$, formed *via* photolysis of protonated 4-iodophenylalanine (Fig. 1c). The presence of an abundant $[M + 15]$ ion in Fig. 1b suggests distinctive reactivity for $[Tyr^{\bullet} + H]^+$ that can be rationalised in terms of addition of O_2 coupled with the elimination of $HO^{\bullet}$ facilitated by the adjacent hydroxyl group (Scheme 1).

This mechanism is supported by (i) the observation of the expected $DO^{\bullet}$ loss in the spectrum of the $[D_4 - Tyr^{\bullet} + D]^+$ isotopologue and (ii) the retention of additional oxygen with the phenol moiety in the m/z 123 fragment ion formed by subsequent dissociation of the energised m/z 196 product ion (see ESI†). Interestingly, the putative structure of the m/z 196

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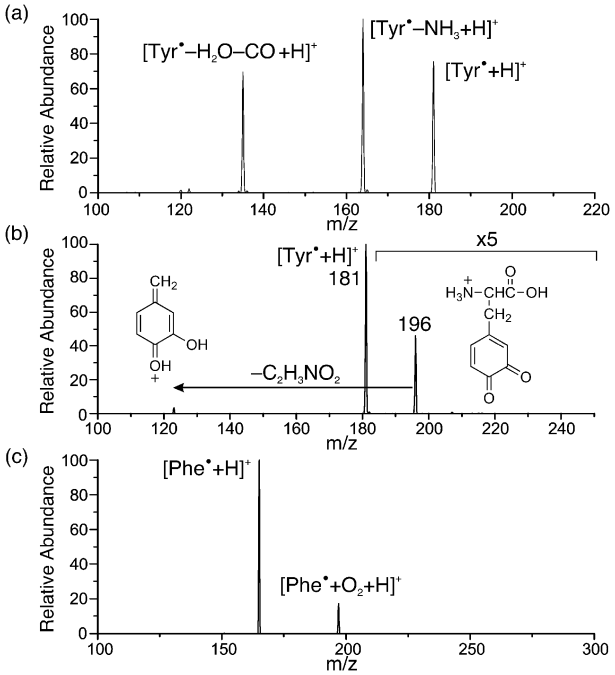
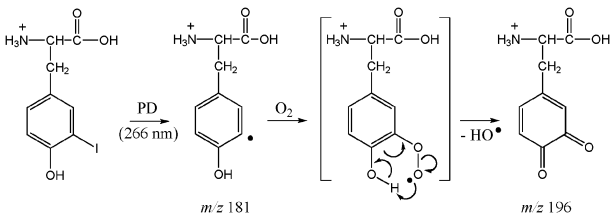


Fig. 1 (a) Collision-induced dissociation mass spectrum of $[Tyr^{\bullet} + H]^+$. (b) The spectrum resulting from the gas phase reaction of $[Tyr^{\bullet} + H]^+$, formed *via* photodissociation (266 nm) of $[Tyr(I) + H]^+$, with O_2. (c) The spectrum resulting from the gas phase reaction of $[Phe^{\bullet} + H]^+$, formed *via* photodissociation (266 nm) of $[Phe(I) + H]^+$, with O_2.



Scheme 1 Proposed mechanism for the reaction of $[Tyr^{\bullet} + H]^+$ with dioxygen.

product ion corresponds to protonated dopaquinone: a reactive intermediate in the tyrosinase catalysed oxidation of tyrosine.[32,33] Taken together these data provide strong evidence for the localisation of the unpaired electron in the 3-position. Furthermore, kinetic analysis of the reaction of *m*/*z* 181 with dioxygen yields strictly linear first-order kinetics with a second order rate constant of 2.06×10^{-11} molecules^{-1} cm^3 s^{-1} and a reaction efficiency of 3.6% (see ESI†). This behaviour is indicative of a single, reactive radical cation at *m*/*z* 181 and we conclude that photodissociation of protonated 3-iodotyrosine at 266 nm yields a single phenyl-type radical structure that undergoes a distinctive concerted reaction in the presence of dioxygen as indicated in Scheme 1. The absence of exothermic rearrangements to stabilised radicals (*e.g.*, phenoxyl, α- or β-radicals) suggests significant activation barriers exist for hydrogen atom transfer within this isolated amino acid.

The hexapeptide radical cation $[RGY^{\bullet}ALG + H]^+$ was synthesised *via* photodissociation of the 3-iodotyrosine analogue. Fig. 2a shows the mass spectrum obtained when this ion is trapped in the presence of O_2 for a period of 3 s.

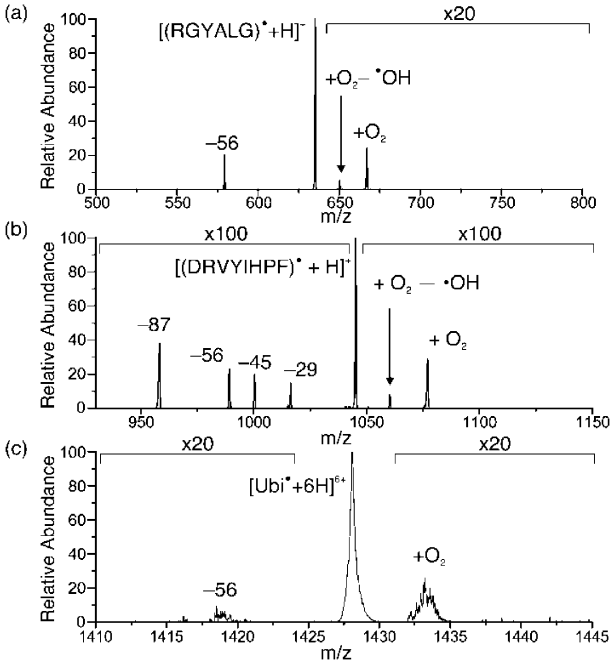
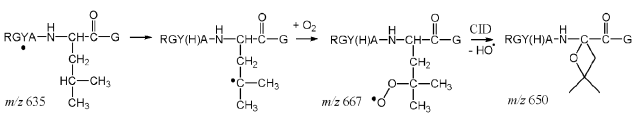


Fig. 2 (a) The spectrum resulting from the gas phase reaction of $[RGYALG + H]^{\bullet +}$, formed *via* photodissociation (266 nm) of $[RGY(I)ALG + H]^+$, with O_2. The analogous spectra for the radical cations (b) $[DRVYIHPF + H]^{\bullet +}$ and (c) $[ubiquitin + 6H]^{\bullet 6+}$.

Significantly, in this spectrum the distinctive $[M + O_2 - HO^{\bullet}]^+$ is of low abundance, suggesting few, if any, of these ions retain the radical in the 3-position on tyrosine. Furthermore, the observation of the $[M + O_2]^{\bullet +}$ adduct ion at *m*/*z* 667 indicates the presence of an alternative reactive isomer in the radical ion population. Collision-induced dissociation of the $[RGYALG + H + O_2]^{\bullet +}$ at *m*/*z* 667 yields *m*/*z* 650, indicative of $HO^{\bullet}$ loss, while deuterium labelling of this peptide at the α-carbon on leucine results in $DO^{\bullet}$ loss under the same experimental conditions (see ESI†). This suggests that the *m*/*z* 667 ion includes a peroxyl radical at leucine, resulting from migration of the radical to this residue *prior* to the reaction with O_2 (*cf.* Scheme 2). Deuterium labelling on the γ-carbon of leucine confirms direct transfer of the radical from tyrosine to leucine (ESI†). While these data indicate radical migration to leucine can occur, the presence of other isomers resulting from rearrangement of the nascent radical cation cannot be excluded. Indeed, pseudo-first order kinetic analysis of the reaction of $[RGYALG + H]^{\bullet +}$ with dioxygen reveals significant curvature suggesting the ion population consists of at least two isomeric radical cations (see ESI†); (i) a fast reacting radical with the unpaired electron likely localised on the leucine side-chain and (ii) a slow or unreactive isomer. Radical migration from the 3-positon on tyrosine to any of the α-position(s) along the peptide backbone is predicted to be exothermic by as much as 30 kcal mol^{-1}.[34] Such radicals may



Scheme 2 Proposed mechanism for the reaction of $[RGYAL^{\bullet}G + H]^+$ with dioxygen.

account for the less reactive fraction of the $[RGYALG + H]^{\bullet +}$ ion population. Indeed, the authentic α-radicals (at least initially), formed by collision-induced side-chain cleavage, are substantially less reactive or unreactive with O_2 (see ESI†).

We therefore conclude that following photodissociation of the C–I bond on the iodinated tyrosine residue, the radical does not remain localised at the 3-position but rather migrates to one or more locations throughout the peptide. Similar behaviour is observed for the reaction of the angiotensin radical cation $[DRVYIHPF + H]^{\bullet +}$ (Fig. 2b) and even for multiply charged ubiquitin (Fig. 2c). The presence of $[M + O_2]^+$ adduct ions in these spectra is indicative of the presence of radicals at sites remote from the initial location on tyrosine.

Interestingly, the spectra shown in Fig. 2a–c also reveal significant amounts of side-chain fragmentation similar to pathways previously described for the CID of peptide radicals,[34] *e.g.*, the loss of the isobutene (−56 Da) from the leucine side-chain in Fig. 2a. Furthermore, these fragmentation pathways reveal a time-dependence (see ESI†) and appear to be independent of the concentration of dioxygen (the latter was established by comparing the $[RGYALG + O_2]^{\bullet +}$ and $[RGYALG - 56]^{\bullet +}$ ion abundances while varying the availability of air in the electrospray source region). These observations suggest that fast migration of the radical away from the tyrosine residue may be followed by subsequent slow unimolecular rearrangements, some of which give rise to side-chain fragmentation.

The results presented herein unambiguously demonstrate that radical migration can occur in large peptides without the addition of significant activation energy. The possibility for facile radical migration must therefore be considered in fragmentation experiments where radical peptides are generated. If needed, ion–molecule reactions such as those demonstrated above can provide a facile route to monitor radical migration in peptides.

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