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Zwitterionic States in Gas-Phase Polypeptide Ions Revealed by 157-nm Ultra-Violet Photodissociation

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Abstract: A new method of detecting the presence of deprotonation and determining its position in gas-phase polypeptide cations is described. The method involves 157-nm ultra-violet photodissociation (UVPD) and is based on monitoring the losses of CO_2 (44 Da) from electronically excited deprotonated carboxylic groups relative to competing COOH losses (45 Da) from neutral carboxylic groups. Loss of CO_2 is a strong indication of the presence of a zwitterionic [(+)···(−)···(+)] salt bridge in the gas-phase polypeptide cation. This method provides a tool for studying, for example, the nature of binding within polypeptide clusters. Collision-activated dissociation (CAD) of decarboxylated cations localizes the position of deprotonation. Fragment abundances can be used for the semi-quantitative assessment of the branching ratio of deprotonation among different acidic sites, however, the mechanism of the fragment formation should be taken into account. Cations of Trp-cage proteins exist preferentially as zwitterions, with the deprotonation position divided between the Asp^9 residue and the C terminus in the ratio 3:2. The majority of dications of the same molecule are not zwitterions. Furthermore, 157-nm UVPD produces abundant radical cations $M^{\bullet+}$ from protonated molecules through the loss of a hydrogen atom. This method of producing $M^{\bullet+}$ ions is general and can be applied to any gas-phase peptide cation. The abundance of the molecular radical cations $M^{\bullet+}$ produced is sufficient for further tandem mass spectrometry (MS/MS), which, in the cases studied, yielded side-chain loss of a basic amino acid as the most abundant fragmentation channel together with some backbone cleavages.

Keywords: radical ions · salt bridges · tandem mass spectrometry · UV photodissociation · zwitterions

Introduction

Electrostatic interactions play an important role in the stability, structure, and function of most proteins and peptides in solutions.[1] These interactions are highly involved in protein–protein and protein–ligand binding and in catalytic reactions.[2] Therefore, most polypeptides are zwitterions in solution. In zwitterions, salt bridges are the strongest electrostatic interactions formed by two proximal opposite charges (cationic and anionic groups). Recent results suggest that salt bridges are important for the stability of solution-phase structures of proteins[3] and larger peptides.[4] The conditions for existence of zwitterions in the gas phase have been extensively debated.[5,6] In the absence of water, the zwitterionic forms of all free amino acids are energetically unfavorable. However, the energy required to stabilize these forms can be reduced by hydration and/or noncovalent binding to metal ions.[7] Likewise, the protonated dimers and trimers of arginine form a gas-phase salt bridge (ion–zwitterion).[8] Larger clusters and polypeptides may overcome the endothermicity of creating ion pairs by self-stabilizing the zwitterionic motif through a [(+)···(−)···(+)] salt bridge. Such an example is bradykinin (BK) $[M+H]^+$ ions, for which both theory[9,10] and experiment[6] suggest a compact zwitterionic structure with a salt bridge between the two protonated terminal arginine residues and the deprotonated C-terminal carboxylic group. Knowledge of the presence or absence of a zwitterionic state is crucial for determining the correct gas-phase structure by computational methods.[11] Techniques with the potential to reveal these motifs, such as ion-mobility mass spectrometry,[9] blackbody infrared radiative dissociation (BIRD),[6] hydrogen–deuterium exchange,[12] the kinetic method,[5] molecular-beam electric-deflection measurements,[13] molecular dynamics simulations,[9] and gas-phase ion–molecule kinetics[14] are often time-con-

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suming or provide indirect information. So far, published studies have been limited to small molecular systems containing only one carboxylic group. Perhaps because of this, only a limited number of zwitterionic and salt-bridge structures have been confirmed for larger gas-phase polypeptide ions.

Potentially, each of the basic sites can be protonated in vacuum and each of the acidic sites can be deprotonated. Structural studies of large systems, such as proteins, requires determination of the charge state of each of the multiple chargeable groups that is present. The position of protonation sites in multiply charged polypeptide cations can be determined by electron-capture dissociation[15] (ECD).[16] Deprotonation of carboxylic acids to multiply charged anions is evidenced as the abundant loss of CO_2 from the oxidized species by electron-detachment dissociation[17] (EDD).[18] Abundant CO_2 losses have also been observed by the 157-nm ultra-violet photodissociation (UVPD) of polyanions.[19] The number of CO_2 losses from the charge-reduced species was equivalent to the number of deprotonations of the anions. The facile decarboxylation can be explained by the electronic excitation of carboxylate groups (electron affinity, $EA < 3.3$ eV)[20] by ~8 eV photons. However, the position of the deprotonated site is not so easy to localize, especially in cations.

Recently, we reported the possibility of detecting zwitterionic states in polypeptide cations by using 157-nm UVPD.[21] We noted that peptide cations known to be zwitterionic in the gas phase tend to lose CO_2 rapidly, whereas this loss in non-zwitterionic cations is not facile. The characteristic CO_2 loss has been attributed to a process similar to that in peptide anions, that is, electronic excitation of the anionic carboxylate followed by electron rearrangement and formation of a biradical species, with subsequent decay of the unstable carboxyl radical through loss of CO_2 upon internal conversion of the energy excess to vibrational excitation [Eq. (1)]:

$$\begin{aligned} &R{-}COO^- + h\nu \rightarrow (R^{-\cdot}{-}COO^{\cdot})^* \\ &\rightarrow (R^{-\cdot}{-}COO^{\cdot})_{hot} \rightarrow (R^-)_{hot} + CO_2 \end{aligned} \quad (1)$$

Here, we present a detailed description and confirmation of this effect, we extend it to molecules with multiple carboxylic acids, and attempt to localize the position of deprotonation by tandem mass spectrometry (MS/MS) of decarboxylated polypeptides.

Results and Discussion

To distinguish the 44-Da losses from the competitive 45-Da loss, only monoisotopic ions were isolated and fragmented. As demonstrated below, the 44-Da loss corresponded mostly to CO_2 loss from an anionic carboxylate, whereas the 45-Da loss corresponded to $^{\cdot}$COOH loss from a neutral carboxylic acid. To quantify the relative importance of the 44-Da loss to the competitive 45-Da loss, we introduced a CO_2-loss factor $K_{CO_2} = ([-44]-[-45])/([-44]+[-45]))$, in which $[-44]$ is the abundance of the CO_2 loss, and $[-45]$ is the abundance of the $^{\cdot}$COOH loss. The K_{CO_2} factor should give positive values (up to 1.0) for zwitterions and negative values (down to -1.0) for non-zwitterions.

Figure 1a shows a part of the 157-nm UVPD mass spectrum of BK $[M+H]^+$ ions, which are well-established zwitterions. The 44-Da loss from the $[M+H]^+$ parent ions is the most abundant loss in the region just below the molecular mass. This loss stands in sharp contrast to the predominant 45-Da loss ($^{\cdot}$COOH) observed from $[M+2H]^{2+}$ ions (inset in Figure 1a), which are mostly non-zwitterions.[6] For BK $[M+H]^+$, the K_{CO_2} value was 0.80, whereas it was just -0.48 for BK $[M+2H]^{2+}$ ions. This result was consistent with the presence of a salt bridge in $1+$ ions only.

Following Williams and co-workers,[6] who used the dominance of the NH_3 loss to verify by BIRD the presence of a salt bridge in BK $[M+H]^+$ ions, we performed collision-activated dissociation (CAD) for comparison. Due to the removal of isotopic distributions, the H_2O and NH_3 losses also became well-separated and easily quantifiable. CAD of $[M+H]^+$ (Figure 1b) resulted in abundant loss of NH_3 for $1+$ ions, whereas it was not significant for $2+$ ions (data not shown). These results were in full agreement with the BIRD data.[6]

To confirm that the facile 44-Da loss was CO_2 from the C terminus, the methyl ester of BK was synthesized, and esterification of the C terminus was authenticated by CAD MS/MS (data not shown). Subsequently, $[M+H]^+$ ions ($m/z = 1074.56$) of esterified BK were fragmented by applying 157-nm UVPD (Figure 1c). With the C terminus esterified, this peptide could not form a salt-bridge structure. In agreement with this, no loss of CO_2 (theoretical m/z of the corresponding fragment = 1030.6) was observed. Instead, the most abundant fragment in the UVPD was the loss of 59 Da ($^{\cdot}$COOMe), confirming that the 44-Da loss in unmethylated BK $1+$ ions (Figure 1a) occurs from the C terminus.

Because the 45-Da loss was used to calculate K_{CO_2} for detection of the zwitterionic state, its origin required further investigation. One possible pathway for such loss is a concerted breakage of the $^{\cdot}$COOH group, another possibility is H$^{\cdot}$ loss from a neutral carboxylic group followed by CO_2 loss from the RCOO$^{\cdot}$ radicals formed, as in Equation (1). The latter pathway, if significant, could interfere with the assignment of zwitterionic states. On its own, hydrogen-atom loss is an abundant channel in 157-nm UVPD (see H$^{\cdot}$ satellites of the precursor ions in Figure 1a and c). The only question was the origin of this loss. To address this issue, the fragment formed in 157-nm UVPD after the H$^{\cdot}$ loss from BK $1+$ ions was isolated and subjected to CAD (Figure 1d). This fragment corresponded to the radical molecular species $[M+H-H]^{\cdot+}$, or simply $M^{\cdot+}$. The loss of CO_2 from this species was negligible and accounted for $<2\%$ of all fragments with $m/z > 300$. Particularly abundant were chain losses from the Arg residue and peptide backbone fragments of a, x, and y types. Therefore, the H$^{\cdot}$ loss occurred either from

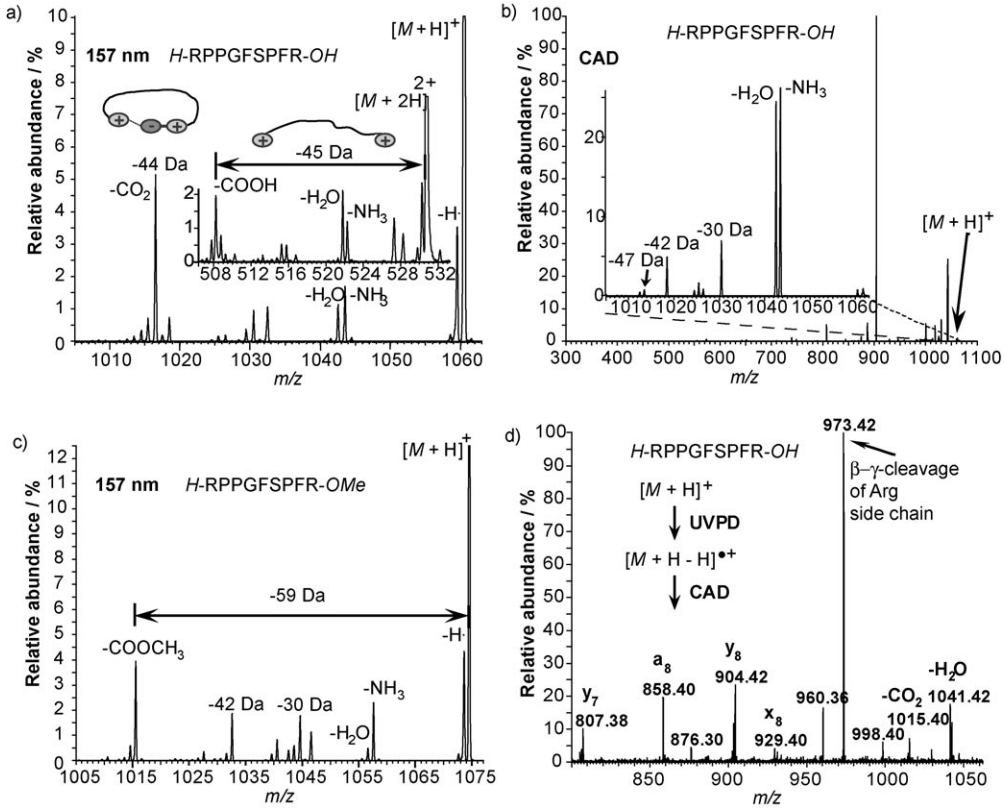


Figure 1. Tandem mass spectra of BK ions: a) 157-nm UVPD of $[M+H]^+$ ions and (inset) $[M+2H]^{2+}$ ions; b) CAD of $[M+H]^+$ ions and (inset) zoom-in of the small-loss region; c) 157-nm UVPD of methyl ester $[M+H]^+$ ions; d) 157-nm UVPD of $[M+H]^+$ ions followed by CAD of $[M+H-H]^{\bullet+}$ species.

the Arg residue or from the backbone, but not from the carboxylic acid. In the latter case, abundant CO_2 losses would occur from molecular radicals, as in CAD of the charge-reduced radical anions $R^-COO^\bullet$ produced in EDD.[18] Thus, we concluded that $^\bullet COOH$ and CO_2 losses in 157-nm UVPD, although both coming from the C terminus, are the results of different reactions.

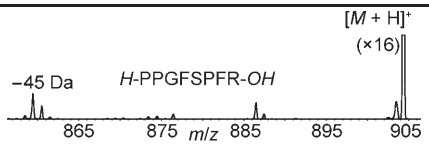
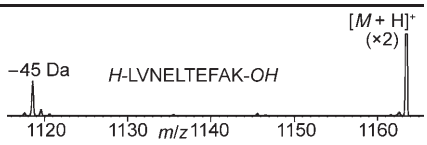
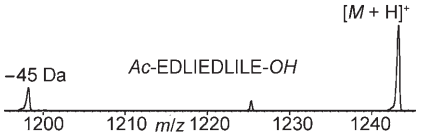
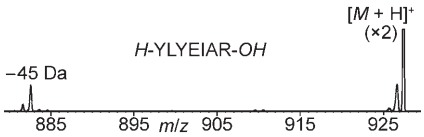
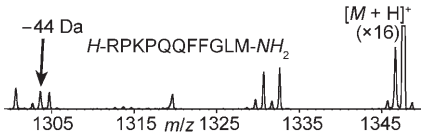
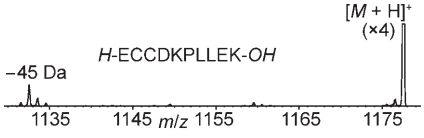
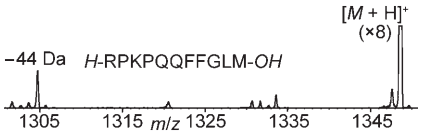
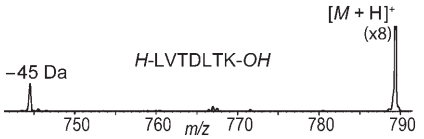
Another concern was associated with small losses (17 and 18 Da) observed in UVPD. Because water and ammonia losses from peptide cations are often proton-mediated processes,[9] the presence of these losses in many UVPD spectra may indicate the possibility of such transfer upon photon absorption. The transferred proton can originate from either a protonated site or a neutral carboxylic group. The exclusion of a significant contribution of the latter process ($-COOH \rightarrow -COO^- + H^+$) is essential to validate UVPD as a method of probing zwitterions. One argument is that such a process is energetically unfavourable: the endothermicity of a carboxylic group deprotonation is ~1300–1500 kJ mol^{-1}, whereas absorption of one 157-nm photon provides only ~800 kJ mol^{-1}. At the same time, to remove a proton from a protonation site requires only ~800–900 kJ mol^{-1}. Hence, the proton donor initiating small losses in UVPD is most likely to be the protonated site and not a neutral carboxylic acid.

Thus, the overall conclusion is that the losses of 44 and 45 Da in BK 1+ ions by UVPD reflect the state of the C terminus: the first loss comes from the anionic $RCOO^-$ group and the second loss originates from a neutral carboxylic group. Thus, the use of the K_{CO_2} factor to determine the relative contribution of zwitterionic structure is justified.

Molecular radicals: Note that the molecular radical cations $M^{\bullet+}$ formed in abundance in 157-nm UVPD are similar to the species that would be produced by electron impact ionization (EI) of neutral gas-phase peptides or by tandem mass spectrometry (MS/MS) of peptide complexes,[22] although the low vapor pressure of peptides makes the formation of $M^{\bullet+}$ ions with EI very difficult. By using 157-nm UVPD, molecular radicals can be produced from any peptide without additional sample treatment or a sample-preparation step. Thus, this method of radical-ion production can be used potentially for high-throughput analysis of tryptic mixtures in proteomics. On the other hand, fragmentation of $M^{\bullet+}$ species requires isolation of a single isotope, which is difficult to automate on many existing mass spectrometers.

Role of basic and acidic amino acids: In des-Arg^1-bradykinin $[M+H]^+$ ions (peptide 1, Table 1), the dominating loss was of $^\bullet COOH$ (45 Da). The K_{CO_2} value of −0.32 confirmed that the predominant structure was non-zwitterionic,[6] resulting from removal of the N-terminal arginine residue. On the other hand, the rather small absolute magnitude of K_{CO_2} implies that some fraction of peptide 1 cations may still

Table 1. The 157-nm UVPD mass spectra and relative importance of 44 and 45-Da losses (K_{CO_2} value) from the parent ions of peptide cations.

N	K_{CO_2}	Mass spectrum of $[M+H]^+$, −50-Da region	*N*	K_{CO_2}	Mass spectrum of $[M+H]^+$, −50-Da region
1	−0.32		5	−0.70	
2	−0.98		6	−0.90	
3	0.46		7	−0.46	
4	0.76		8	−0.86	

exist as zwitterions. The rather basic secondary amines of the N-terminal prolines (the gas-phase basicity of des-Arg^1-bradykinin $[M+H]^+$ ions is only ~12 kJ mol^{-1} below that of BK 1+ ions)[23] could, in principle, stabilize a salt-bridge configuration in a fraction of the ion population.

The importance of acidic functional groups for zwitterion formation was investigated by using peptide 2 (Table 1). Because peptide 2 is highly acidic without basic functionalities, formation of a 1+ zwitterion is hindered. In agreement with this, almost no CO_2 losses ($K_{CO_2}=-0.98$) were observed for these cationic species, despite multiple CO_2 losses in UVPD of polyanions (data not shown).

In contrast, peptide 3 (substance P) has two basic residues and an N-terminal amine group, but no acidic groups to deprotonate, thus, it cannot form a zwitterion. However, a 44-Da loss was observed in the 157-nm UVPD mass spectrum. This loss can be confidently attributed to the isobaric $CONH_2$ (44.013 Da) loss,[24] which cannot be distinguished from the CO_2 loss under the limited resolving power of the instrument used. For such a basic peptide, one could expect that addition of an acidic group would promote formation of zwitterions. Indeed, singly charged ions of the acidic form of substance P (peptide 4) are zwitterions, as evidenced by the large value of K_{CO_2} (0.76).

Because zwitterionic 1+ ions require two highly basic and one acidic site for their formation, "normal" tryptic peptides that contain one highly basic site at the C terminus have a small chance of being zwitterions. This hypothesis was tested on seven tryptic peptides of bovine serum albumin (peptides 5–8, other data are similar). In all cases, no zwitterionic states were identified ($K_{CO_2}<0$). Peptide 7 had the highest K_{CO_2} value of −0.46, which is 0.28 greater than the average value for these four peptides. Such a high K_{CO_2} value was explained by the enhanced basicity of this peptide, due to the presence of two lysine residues (missed trypsin cleavage).

Salt-bridges in peptide multimers: As mentioned above, single amino acids are not zwitterions in the gas phase, but their protonated clusters can be zwitterions. The transition between the ionic and zwitterionic states was studied by using protonated multimers of the tetrapeptide MRFA. Figure 2 shows 157-nm UVPD mass spectra of singly-charged MRFA mono-, di-, and trimers. Protonated monomers do not form zwitterions: a predominant loss of 45 Da (•COOH) was observed from the precursor ions (Figure 2a). This loss (*m*/*z* 479) is in stark contrast to the predominant loss of 44 Da (*m*/*z* 480) from the same species arising from decomposition of singly-charged dimers in 157-nm UVPD (Figure 2b). Isolation and subsequent CAD of this peak confirmed it to be that of the molecular ion decarboxylated at the C terminus (data not shown). At the same time, the precursor dimers show in UVPD only the loss of 45 Da (*m*/*z* 1002). Note that this loss does not destabilize the dimer, unlike the 44-Da loss (CO_2) associated with the monomer separation. The conclusion can be drawn that the protonated dimer is a zwitterion, with the C-terminal carboxylic acid of one of the monomers involved in a salt-bridge stabilization of the dimer. Upon 157-nm photon absorption, the photon energy can be converted internally to vibrational excitation, which can result in monomer separation leading to the monomer-ion peak at *m*/z 524. Alternatively, the photon is absorbed by the anionic C terminus. The latter promptly loses CO_2 from the anionic C terminus, as in Equation (1). This gives the C-terminal $-CH_2^-$ anion that has a higher proton affinity (PA) than $-COO^-$ (the gas-phase acidity ΔG_{acid} of CH_3CH_3 is 1723 kJ mol^{-1},[25] compared to 1427 kJ mol^{-1} for CH_3COOH[26]). The higher proton affinity

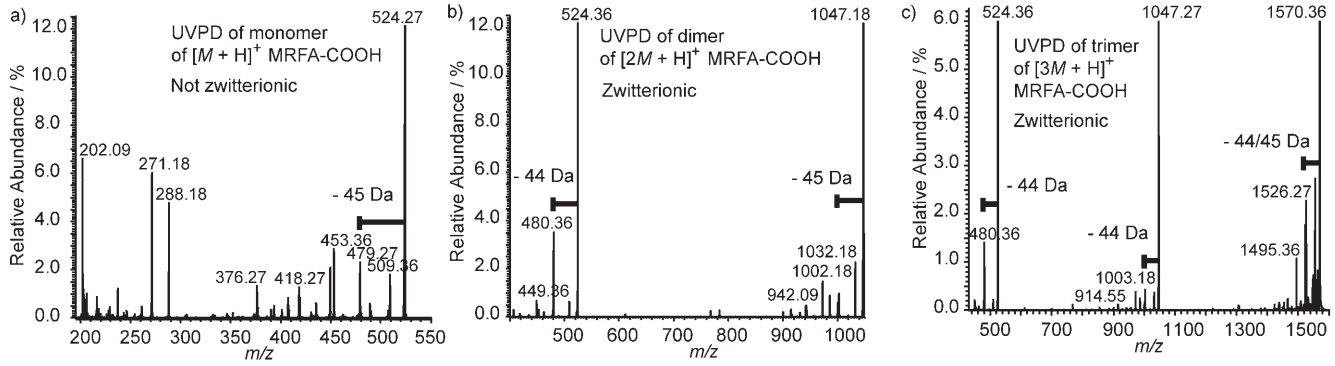


Figure 2. 157-nm UVPD spectrum of singly-charged oligomers of the peptide MRFA: a) of the monomer; b) of the dimer; and c) of the trimer.

of the $-CH_2^-$ group weakens the anion–proton binding and disrupts the salt bridge, resulting in monomer separation. This is consistent with an expected overall energy deficiency in the salt-bridge stabilization if $-CH_2^-$ is the anion. Generally, the removal of a proton from an acid in the gas phase is considerably more energy demanding than the energy gained by protonating a base ($\Delta H_{acid}(-COOH) - PA_{base}(-NH_2) > 110\ kJ\,mol^{-1}$). Therefore, the change in anion functionality from $-COO^-$ to $-CH_2^-$ will require an additional stabilization energy of $\sim 300\ kJ\,mol^{-1}$ in the $BH^+ \cdot A^- \cdot BH^+$ complex to sustain the salt-bridge structure. This process contributes to the abundance of two ions, depending upon the position of the ionizing proton after the monomer separation: the monomer ion with m/z 524 and its decarboxylated analogue with m/z 480.

Yet another alternative is that the UV photon is absorbed by the neutral C terminus, in which case $^{\bullet}COOH$ is lost. However, this loss does not lead to dimer dissociation, as the neutral C terminus is not involved in monomer binding.

The trimer of MRFA (Figure 2c) is also found to be zwitterionic. Due to larger size and more-extensive hydrogen bonding, it is more stable than the dimer and can remain intact after both $^{\bullet}COOH$ and CO_2 losses (m/z 1525 and 1526, respectively). However, most of the decarboxylated trimers dissociate into dimers and monomers, with both species showing only the loss of 44 Da. This is further evidence that salt bridging is involved in stabilization of the trimers.

Methylation of the C terminus should prevent formation of the salt bridge, as was the case with methylated BK cations (Figure 1c). Thus, oligomer ions can be stabilized only by hydrogen bonding or even weaker interactions, for example, by stacking of the phenyl groups of Phe residues. Figure 3 shows the UVPD mass spectrum of the MRFA–COOMe dimer at m/z 1075.

As for methylated BK cations, the loss of 59 Da was observed (m/z 1016) from protonated dimers of the methylated MRFA peptide. This loss did not destabilize the dimer sufficiently to cause dissociation. No loss of 44 or 45 Da was detected from the parent ions. The absence of any losses from the monomers was in stark contrast to the abundant losses from unmethylated monomers shown in Figure 2. This result is consistent with the absence of a salt-bridge linkage between the monomers and the presence of proton binding. Absorption of the UV photon by the dimer leads either to dissociation into monomers as a result of intramolecular energy conversion, or to the loss of $^{\bullet}COOMe$. If dimer dissociation occurs in the latter case, the proton remains with the intact monomer, due to its higher basicity. The contrast in UVPD behavior of salt-bridged unmethylated oligomers and proton-bound methylated dimer means that 157-nm UVPD can be used to probe the nature of the intracluster binding.

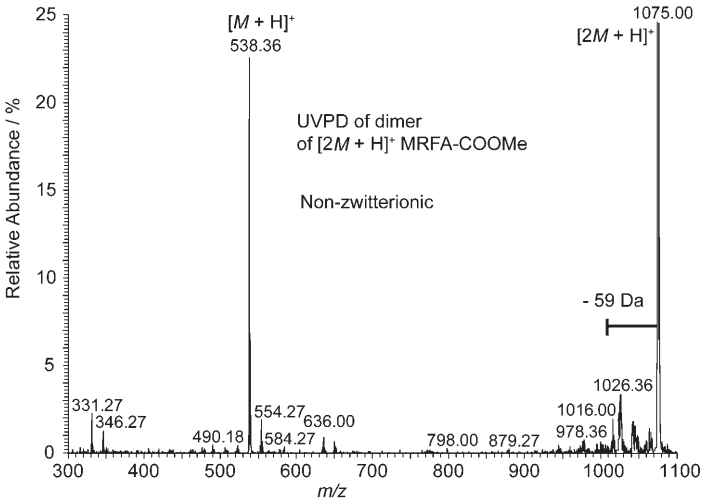


Figure 3. 157-nm UVPD of the protonated dimer of the peptide MRFA with methylated C terminus.

Polypeptides with more than one potential site of deprotonation: Knowledge of the functional groups involved in salt bridging is of considerable interest in protein-structure determination, as this information can restrict the potential conformational space and, hence, reduce the computational cost of molecular modeling. The peptide PLWKQQNLKKEKGLF from the villin head domain[27] is an example of a polypeptide with multiple potential protonation and deprotonation sites.

The 2+ charge state of this villin peptide was shown by 157-nm UVPD to contain a salt bridge (K_{CO_2} value of 0.4)

(Figure 4, left). The salt bridge can involve a carboxylic group from either the C terminus or Glu^{11} residue. To localize the sites involved in decarboxylation, CAD mass spectra of the parent ions $[M+2H]^{2+}$ and the decarboxylated species $[M-CO_2+2H]^{2+}$ were compared (Figure 4, right). Both spectra showed almost complete sequence coverage by b- and y-type fragment ions. The top panel in Figure 4 shows regions of the mass spectrum around the b_{10}, b_{11} and y_4 ions from the $[M+2H]^{2+}$ species. For comparison, the lower panel shows the same regions from the CAD mass spectrum of the $[M-CO_2+2H]^{2+}$ ions. The comparison suggests that CO_2 losses in 157-nm UVPD occur from both potential deprotonation sites. Based on the relative abundances of the fragment ions and their −44-Da satellites in the CAD spectrum of decarboxylated species, it was estimated that approximately 15% of the zwitterionic ions have a salt bridge formed from deprotonation of Glu^{11}, whereas the remaining 85% of the zwitterionic ions arise from deprotonation of the C terminus. This interpretation suggests at least two different ion conformations. Given the possibility of ion isomerization due to sideband excitation in the process of ion isolation, the existence of two ion populations during 157-nm photon absorption is not improbable.

Zwitterionic states in a protein: By projecting the results of the amino-acid cluster studies[8] onto proteins, one can conclude that because of their size, proteins ions can easily stabilize gas-phase zwitterionic structures. To reveal the presence of a salt bridge in a protein is not sufficient; one also needs to locate it. Here, we applied 157-nm UVPD to study the zwitterionic state of the smallest known protein, the "Trp-cage" (NLYIQWLKDGGPSSGRPPPS), which has a well-defined structure in the gas phase.[28] This molecule has two highly acidic groups, D^9 and the C terminus, and three basic sites, the N terminus, K^8, and R^{16}. The pI value of the Trp-cage is 9.4, and at physiological pH ≈ 7 in solution it has a 1+ net charge, with R^{16} and K^8 protonated and D^9 deprotonated. In ESI mass spectra, the average charge state is between 2 and 3, depending upon the experimental conditions, with the individual charge states ranging from 1+ to 3+. Recently, Iavarone et al. suggested that, in the gas phase, 2+ ions of the Trp-cage can retain their solution-phase zwitterionic structure with a salt bridge between K^8, D^9, and R^{16}.[29] However, ECD results[28] supported by molecular dynamics simulations (MDS)[30] favour the protonation of Q^5 and R^{16} residues in Trp-cage dications.

In 157-nm UVPD (Figure 5), only 1+ ions of Trp-cage appeared to be predominantly zwitterions ($K_{CO_2} \approx 0.4$), whereas the dications showed preferential loss of 45 Da ($K_{CO_2} < 0$; exact values are difficult to obtain because of imperfect isotopic isolation for these large species). Localization by CAD MS/MS of the residue involved in the salt bridge was not as straightforward as with the villin peptide that gave abundant b,y fragmentation. In CAD analysis, Trp-cage 1+ ions produced only two abundant fragments, y_{11} and b_{16} (Figure 6a). In the absence of mobile protons (e.g., the charge state does not exceed the number of arginines in the sequence), protons to initiate backbone cleavage are recruited either from the acidic aspartic acid[31] or from the protonated arginine residues, leading to cleavages C-terminal to D^9 and R^{16} residues. Therefore, if Asp is deprotonated prior to CAD fragmentation, its carboxylic group cannot initiate such cleavage, and, thus, the D^9 residue involved in a salt bridge cannot generate the $y_{11}'^{+}$ fragment. To confirm this suggestion, D^9 in Trp-cage was replaced by asparagine residue (N), and very little $y_{11}'^{+}$ was formed in CAD (Figure 6b). At the same time, an abundant b_{16} fragment was still present. The D→N mutant gave an abundant CO_2 loss in UVPD, indicating that Trp-cage cations can be zwitterions, even if the only acidic site present is the C terminus (data not shown).

Disregarding the possibility of isomerization during collisional excitation, one could conclude from the ratio of the abundances of $y_{11}'^{+}$ and b_{16} ions in Figure 6a (4:1) that the Asp residue in 1+ Trp cage is preferentially neutral. This conclusion could, however, be wrong, as the relative proba-

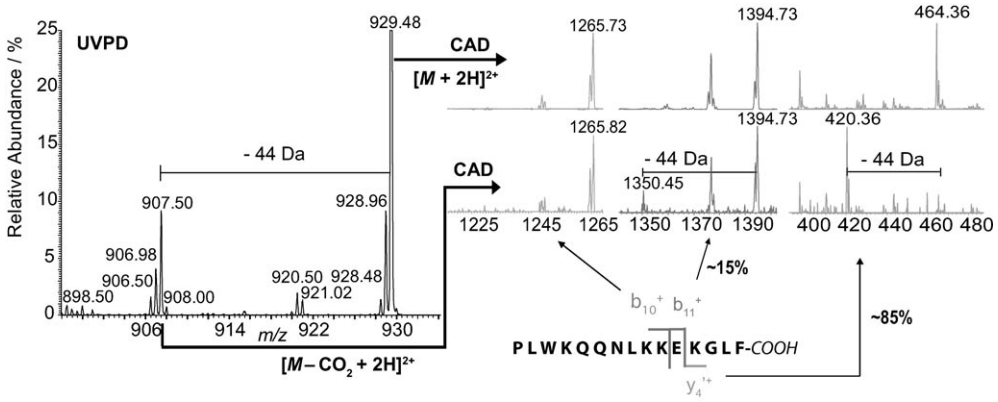


Figure 4. Left: Part of the 157-nm UVPD mass spectrum of the 2+ ion of a villin peptide showing the dominant loss of CO_2 ($K_{CO_2} = 0.4$) from two potential deprotonation sites; C terminus and E^{11}. Right: Parts of the CAD mass spectra of the precursor ions (upper panel) and decarboxylated ions (lower panel). The comparison reveals the positions and probabilities of decarboxylation in the precursor species (sequence diagram, bottom): approximately 15% of decarboxylation occurs at E^{11} and approximately 85% at the C terminus.

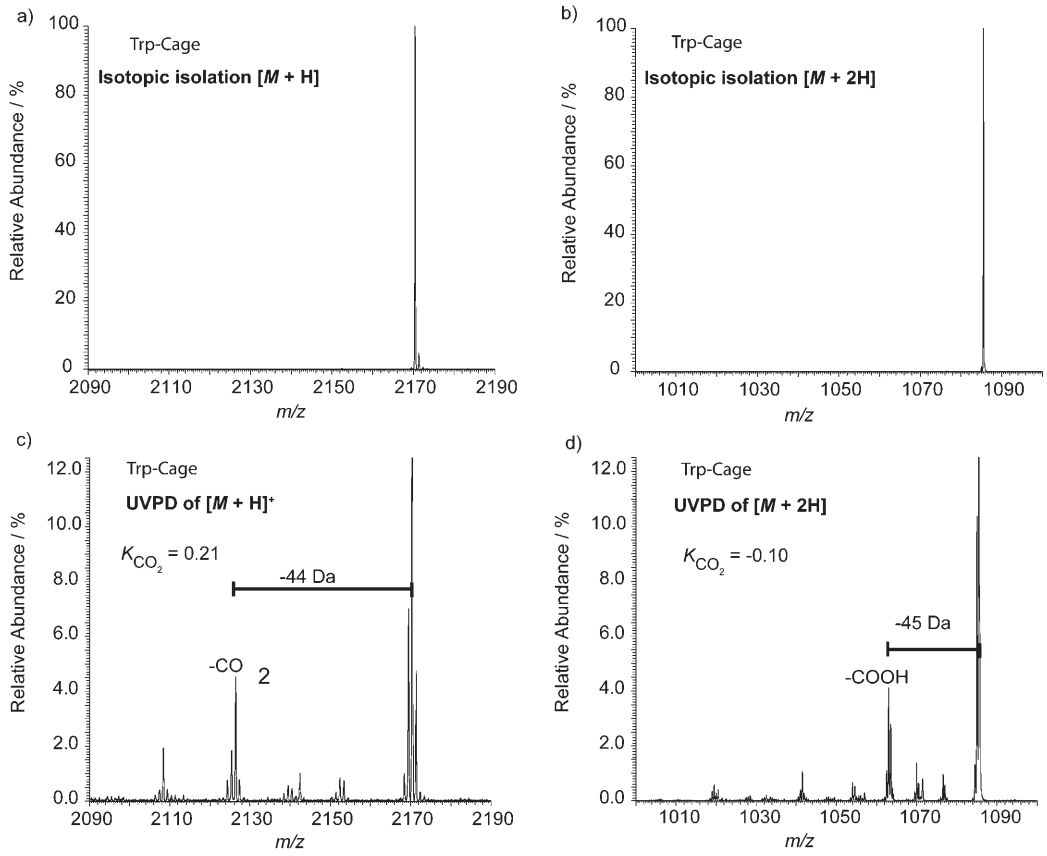


Figure 5. Isotopic isolation of Trp-cage molecular ions: a) monocations, b) dications. The 157-nm UVPD of isotopic peak of Trp-cage molecular ions: c) monocations, d) dications.

bilities of the C–N bond cleavages after D^9 and R^{16} can be different for the cases of neutral and deprotonated Asp. The CAD MS/MS spectra of molecular species and D→N mutant decarboxylated in 157-nm UVPD are shown in Figure 6c and d. Decarboxylated native Trp-cage ions gave a predominant −44-Da satellite peak to y_{11} ions, with an abundance ratio of 9:1. Taken at face value, this result indicates that the CO_2 loss occurs preferentially from the C terminus, which is, thus, implicated in a dominant salt-bridge structure. However, y_{11} is a poor indicator of the branching ratio of the deprotonation sites, because, as discussed above, its formation is greatly facilitated at the neutral state of D^9. From the point of view of branching ratios, b_{16} ions are more reliable, as the only condition of their formation is the protonation of R^{16}, which is almost guaranteed, due to the high basicity of Arg. The ratio of the abundances of the b_{16} ion and its −44-Da satellite in Figure 6c indicates that in ≈60% of Trp-cage 1+ ions, the residue D^9 is deprotonated, whereas in ≈40% of the ion population, deprotonation occurs at the C terminus. The absence of the −44-Da satellite to the b_{16} ion if Asp is replaced by Asn (Figure 6d) is consistent with all deprotonation occurring at the C terminus.

This last comment relates to the presence of "intact" y_{11} ion (*m*/*z* 994.6) in the CAD spectrum of decarboxylated Trp-cage 1+ ions (Figure 6c). But how can this ion be formed? Either decarboxylated D^9 can still induce preferential cleavage C-terminal to its location, or scrambling of the radical site can occur upon vibrational excitation. The latter effect can, in principle, compromise the localization of the decarboxylation position by CAD, however, the impact of this on the measurement of the branching ratio is probably low, given the low abundance of the "intact" y_{11} ion in Figure 6c.

Conclusion

The CO_2 loss in 157-nm UVPD monitored by measuring K_{CO_2} reveals the presence of deprotonation in gas-phase polypeptides, a strong indication of a salt-bridge structure in polypeptide cations. This UVPD/CAD approach is complementary to ECD for charge localization in polypeptide polycations. The significant differences in the K_{CO_2} values for the peptides studied demonstrate the discriminatory power of this method. The CAD of decarboxylated cations can localize the position of deprotonation in zwitterions. For the quantitative assessment of the branching ratio of deprotonation among different acidic sites, fragment abundances can be used, provided the mechanism of their formation is taken into account. As a secondary result of this current study, a

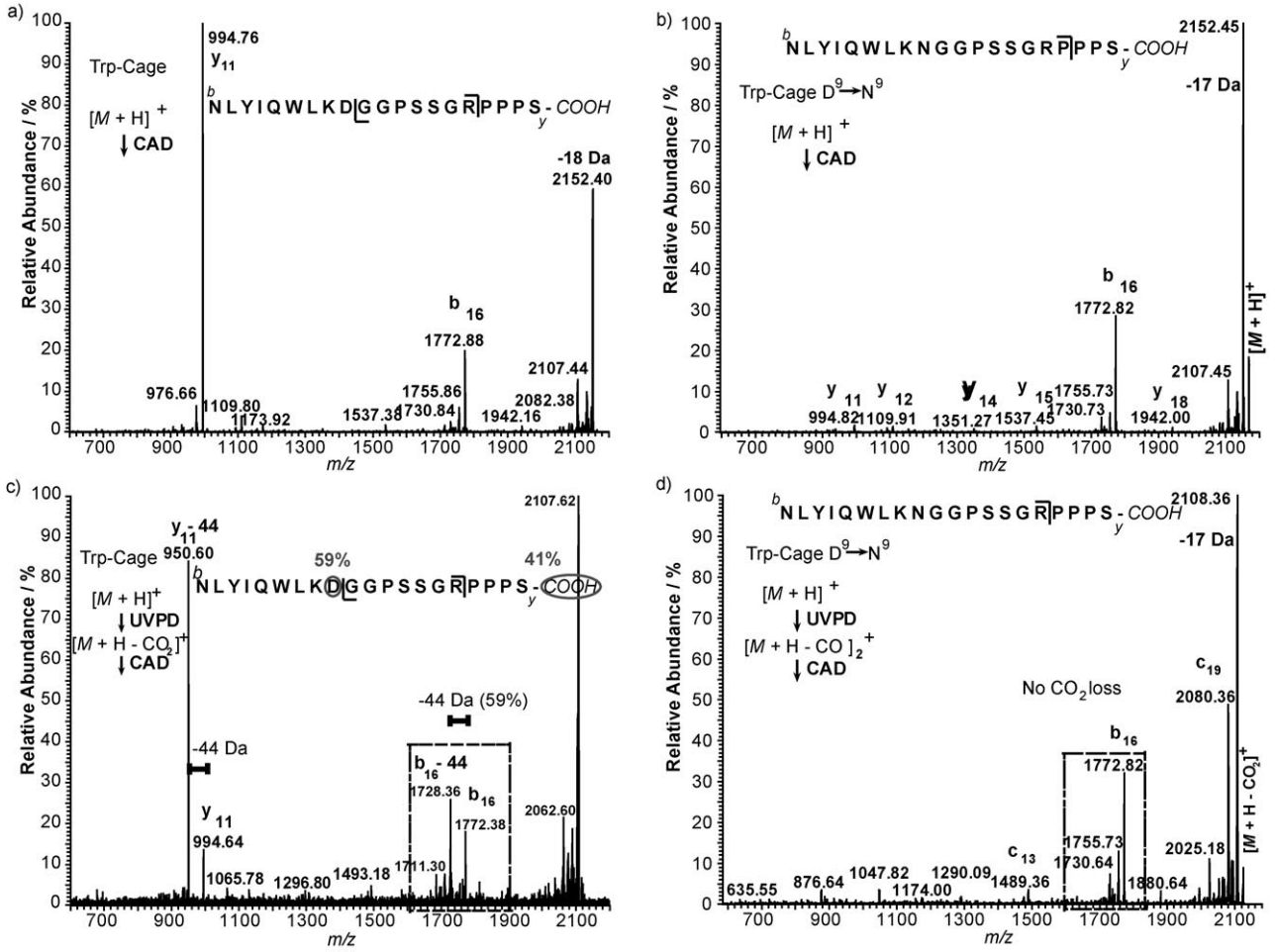


Figure 6. CAD of Trp-cage 1+ ions of: a) native molecule, b) $D^9 \rightarrow N^9$ mutant, c) CAD MS/MS of native molecular species decarboxylated by 157-nm UVPD, d) the same as (c), but for $D^9 \rightarrow N^9$ mutant.

new method of production of radical cations $M^{\bullet+}$ was identified: loss of a hydrogen atom from the protonated species upon 157-nm UVPD irradiation. The method is general and can be applied to any gas-phase peptide cation, and the molecular radical cations $M^{\bullet+}$ produced are sufficiently abundant to facilitate further MS/MS.

Experimental Section

Peptides were synthesized by employing automated solid-phase synthesis and the 9-fluorenylmethoxycarbonyl (Fmoc) protection strategy by using a research-scale ResPep peptide synthesizer (Intavis AG, Gladbach, Germany). For amidated peptides, the universal modified Rink amide MBHA (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl) resin was used as solid support. The methyl ester of BK was prepared by acid-catalyzed esterification, by dissolving the peptide (1 mg) in a solution (300 μL) of 2 M sulfuric acid in methanol. After 3 h of stirring at RT, the solution was lyophilized and redissolved in electrospray solvent (2:49:49 acetic acid/methanol/water (v/v/v)). The C-terminal position of the methylation site was confirmed by performing MS/MS.

All peptides were diluted in electrospray solvent to a concentration of approximately 10^{-6} M. Mass-spectrometric experiments were performed by using a linear ion-trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray interface operated in the positive-ion mode. An excimer F_2 (157 nm) UV laser Excistar S500 (Tuilaser, Germering, Germany) was connected to the mass spectrometer by means of a CaF_2 window on-axis with the ion trap. Unfocused laser pulses of 1 mJ and 15 ns fired with a repetition rate of 500 Hz passed through a 1.5-mm collimator installed in front of the window in a compartment flashed during laser operation with dry nitrogen. Isolated precursor ions were UV-irradiated for 7 ms.

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