Deamidation reactions of protonated asparagine and glutamine investigated by ion spectroscopy

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RATIONALE: Deamidation of Asn and Gln residues is a primary route for spontaneous post-translational protein modification. Several structures have been proposed for the deamidation products of the protonated amino acids. Here we verify these structures by ion spectroscopy, as well as the structures of parallel and sequential fragmentation products.

METHODS: Infrared ion spectroscopy using the free electron laser FELIX has been applied to the reaction products from deamidation of protonated glutamine and asparagine in a tandem mass spectrometer. IR spectra were recorded over the 800–1900 cm⁻¹ spectral range by infrared multiple-photon dissociation (IRMPD) spectroscopy. Molecular structures of the fragment ions are derived from comparison of the experimental spectra with spectra predicted for different candidate structures by density functional theory (DFT) calculations.

RESULTS: [AsnH⁺ – NH₃]⁺ is found to possess a 3-aminosuccinic anhydride structure protonated on the amino group. The dissociation reaction involving loss of H₂O and CO forms a linear immonium ion. For [GlnH⁺–NH₃]⁺, the N-terminal nitrogen acts as the nucleophile leading to an oxo-proline product ion structure. For [GlnH⁺–NH₃]⁺, a sequential loss of [CO + H₂O] is found, leading to a pyrolidone-like structure. We also confirm by IR spectroscopy that dehydration of protonated aspartic acid (AspH⁺) and glutamic acid (GluH⁺) leads to identical structures as to those found for the loss of NH₃ from AsnH⁺ and GlnH⁺.

CONCLUSIONS: The structure determined for AsnH⁺ is in agreement with the suggested structure derived from measured and computed activation energies. IR ion spectra for the NH₃-loss product from GlnH⁺ establish that a different reaction mechanism occurs for this species as compared to AsnH⁺. For both amino acids, loss of NH₃ occurs from the side chain. Copyright © 2016 John Wiley & Sons, Ltd.

The amino acids glutamine (Gln, Q) and asparagine (Asn, N) feature an amide group in their side chain. Deamidation of Asn and Gln residues is a primary route for spontaneous post-translational protein modification.[1] It is a dominant pathway for protein degradation and is a pH-dependent process. Peptides with glutamine as the N-terminal amino acid are known to undergo partial deamidation in solution, which converts the terminal glutamine residue into the cyclic pyroglutamyl residue (2-pyrrolidone-5-carboxylic acid).[2,3] Structural changes in the protein upon such reactions induce biological responses such as molecular clocks for the timing of biological processes or acceleration of amyloid aggregate formation (pathological protein formation).[4,5] Robinson and Robinson[5] strengthen the hypothesis that glutaminyl and asparaginyl residues in peptides serve, through deamidative transformation into glutamyl and aspartyl residues, as molecular timers of biological events such as protein development and aging.

Amides may serve as molecular indicators that the integrity of each protein molecule has been maintained.[5] This fact can be used to investigate for the presence of post-translational modifications.

A variety of studies have addressed the deamidation reactions of protonated Gln- and Asn-containing proteins and peptides in a mass spectrometer, where the reactions are initiated by collisional activation, occur in the gas phase, and can be analyzed in terms of the mass-to-charge ratio of the resulting fragments.[1,3,6,12–19,31,32] In order to gain detailed mechanistic insight into these reactions, the deamidation of protonated Asn and Gln has been studied using a variety of methods including energy-dependent collision-induced dissociation, deuterium exchange, and threshold collision-induced dissociation (TCID) combined with theoretical investigations at various levels of theory.

While there is a general agreement that the thermodynamically most favored site of protonation of Gln and Asn is the α-amino group,[6,7] collisional activation can mobilize the additional proton,[8–10] so that elimination of NH₃ can conceivably occur from either the N-terminal amine or from the side-chain amide group. The latter option has been assumed in the majority of earlier studies[11] as it leads to an acylium ion structure – either as the final or intermediate product – rather than a presumably less stable α-carbocation. Although some studies suggest linear acylium-type product
ions, most studies propose NH₃ loss to occur concomitantly with a nucleophilic attack onto the acylium or carbocation center by one of the nucleophiles in the molecule, leading to cyclic product ion structures. Schemes 1(A) and 1(B) outline the various possibilities for the formation of cyclic structures by deamidation of protonated Asn and Gln, respectively. For each of the conceivable product ion structures, several protonation sites are possible and further increase the diversity of the deamidation chemistry.

For AsnH⁺, Heaton and Armentrout¹² used TCID to obtain values for the activation energies of the various dissociation pathways, which were compared with detailed high-level computations of the potential energy surface. The study presented here confirms that NH₃ loss occurs from the amide side chain of the molecule as a result of nucleophilic attack by the C-terminal carbonyl oxygen and leads to a product ion having a succinic anhydride structure (see Scheme 1(A)). Other dissociation reactions, in particular the parallel reaction involving H₂O + CO loss from the C-terminus, are also investigated here and will be addressed further below.

For GlnH⁺, the situation is less well understood; there is not an in-depth study of the glutamine potential energy surface including transition states and intermediate reaction species available. A reaction mechanism analogous to that identified for AsnH⁺ would lead to a six-membered glutaric anhydride structure. In contrast to AsnH⁺, however, the alternative mechanisms outlined in Scheme 1(B) lead to energetically more favorable five-membered ring structures. Table 1 outlines the free energy differences between these four-, five- and six-membered ring structures and their different protonation sites. Earlier studies¹⁴,¹³–¹⁵,⁹⁹ have indeed proposed such five-membered structures, in particular the oxo-proline structure (pyroglutamic acid). Further insights into the dissociation mechanisms may also be obtained from studies of the H₂O loss reaction of protonated glutamic acid (GluH⁺), which have often been suggested to lead to the same product ions.

Recently, it has been identified that residue-specific fragmentation of protonated peptides containing glutamine or asparagine occurs and leads to succinimide and glutarimide structures¹⁶,¹⁷ instead of the more standard formation of b₂ ions.¹⁸ It was found that dissociation occurs via a nucleophilic attack of the side-chain amide nitrogen atom of Asn and Gln on the adjacent peptide carbonyl carbon.

Ion spectroscopy is a proven tool for establishing the molecular structures of gas-phase amino acid ions,¹⁹ their (de)protonation sites,²⁰–²¹ and the structures of their gas-phase reaction products in collision-induced dissociation (CID) tandem mass spectrometry (MS/MS).²³ Here, the fragmentation reactions after CID of AsnH⁺ and GlnH⁺ are investigated using ion spectroscopy. Computed spectra for previously established or suggested isomers¹²,¹³ are therefore compared with the measured IRMPD spectra.

Using the product ion structures we are able to propose fragmentation reaction mechanisms for deamidation of AsnH⁺ (confirming previously established pathways¹²) and GlnH⁺. These results confirm that AsnH⁺ and GlnH⁺ indeed follow different fragmentation pathways.

**EXPERIMENTAL**

**IRMPD spectroscopy**

Infrared spectra of the protonated amino acids and their CID product ions were obtained by infrared multiple-photon dissociation (IRMPD)²⁴–²⁶ spectroscopy using Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) as well as a modified 3D quadrupole ion trap mass spectrometer (Bruker, AmaZon Speed ETD), both coupled to the beam line of the infrared free electron laser FELIX.²⁵ All samples were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands) and used without further purification.

| Table 1. Calculated relative free energies at the MP2(full)/6-311+G(2d,2p)//B3LYP/6-31++G(d,p) level at 298 K for the different possible structures after NH₃ loss |
|---|---|---|
| No. | AsnH⁺–NH₃ (kJ/mol) | GlnH⁺–NH₃ (kJ/mol) |
| 1 | 0 | +61 |
| 2 | +59 | +100 |
| 3 | +35 | +92 |
| 4 | +26 | 0 |
| 5 | +32 | +29 |
| 6 | +64 | +11 |
| 7 | +188 | +180 |

The numbers refer to the different structures in Scheme 1.
Deamidation of protonated Asn and Gln investigated by ion spectroscopy

For experiments by FTICR-MS, the protonated amino acids were generated by electrospray ionization (ESI) in a Micromass Z-spray source from approximately 1 mM solutions of the sample in a solution of 50:50 acetonitrile/water containing ~0.1% acetic acid. After the ESI process, the ions were accumulated in a linear hexapole trap before being injected into the ICR cell. Fragment ions are generated by nozzle skimmer dissociation in the high-pressure region at the interface of the ESI source and the hexapole trap/accumulation region. A stored waveform inverse Fourier transform (SWIFT) excitation pulse is used to isolate a single species in the cell. After irradiating the mass-isolated ions for one or a few seconds with tunable IR light (as described below), the mass spectrum of the ions which are left in the cell is recorded using an excite/detect procedure. The instrument and its typical operation have been described in detail elsewhere.\(^{[28]}\)

For experiments in the Amazon ion trap instrument, protonated amino acid ions were generated using ESI from 10\(^{-8}\) to 10\(^{-6}\) M solutions in 50:50 acetonitrile/water with ~0.1% formic acid. The yield at each IR frequency is obtained from three averaged mass spectra. Isolated ions were subjected to CID conditions for 40 ms using an amplitude parameter of approximately 0.3–0.5 V.

Ions of interest were mass isolated and irradiated for 1–4 pulses of tunable IR light. A mass spectrum of the content of the trap is then obtained by scanning out the ion trap.

For both experiments, FELIX produced 6 μs long macro pulses of approximately 20–60 mJ at a 5-Hz repetition rate having a bandwidth of ~0.5% of the center frequency. Absorption of IR radiation increases the internal energy of the system which leads to frequency-dependent unimolecular dissociation. Relating precursor and fragment ion intensities as the fragmentation yield (ΣI(fragment ions)/ΣI(parent + fragment ions)) and plotting the yield of each mass spectrum as a function of laser frequency generates an IR vibrational spectrum. The yield is linearly corrected for the frequency-dependent laser power and the IR frequency is calibrated using a grating spectrometer.

### Computational chemistry

Density functional theory (DFT) calculations were performed at the B3LYP/6-31++G(d,p) level of theory using Gaussian09 revision D01\(^{[29]}\) to optimize the molecular geometries of the precursor and fragment ions and to predict their (linear) IR spectra. Candidate input structures for these calculations were defined based on chemical intuition. The computed harmonic vibrational frequencies were scaled by 0.975 and convoluted with a 15 cm\(^{-1}\) full width at half maximum (FWHM) Gaussian line shape to facilitate comparison with experimental spectra.

A Molecular Mechanics/Molecular Dynamics (MM/MD) approach using AMBER 12\(^{[30]}\) was applied to protonated glutamine and glutamine-NH\(_3\) in order to explore the potential energy surface and to find the lowest energy conformers. After minimization within AMBER, a simulated annealing procedure up to 1000 K was used. Guess geometries were first optimized in the Gaussian09 – revision D01 – package at the B3LYP/6-31++G(d,p) level of theory. A total of 500 structures were obtained in this procedure and grouped based on structural similarity using appropriate rms criteria to give 20–30 candidate structures. Next, these structures were each optimized at the B3LYP/6-31++G(d,p) level as described above. The frequencies were again scaled by 0.975 and convoluted with a 15 cm\(^{-1}\) FWHM Gaussian line shape. All reported 298 K free energies are calculated using MP2(full)/6-311+G(2d,2p) electronic energies with the B3LYP/6-31++G(d,p) optimized structures and thermochemistry. The computational procedure is described in more detail elsewhere.\(^{[31]}\)

### RESULTS AND DISCUSSION

**Protonated asparagine (AsnH\(^+\))**

The IRMPD spectrum of the AsnH\(^+\) precursor ion (m/z 133) has been recorded previously.\(^{[32]}\) Comparison of the experimental spectrum with computed IR spectra for the three most stable conformers suggested that the amino acid is protonated at the N-terminus.

The fragmentation pathways of AsnH\(^+\) and NaAsn\(^+\), including the deamidation pathway, have also been investigated.\(^{[1,12]}\) Two major routes for primary decomposition of AsnH\(^+\) were established: loss of NH\(_3\) leading to an m/z 116 fragment and loss of H\(_2\)O + CO leading to an ion at m/z 87. A fragment at m/z 115 (loss of H\(_2\)O) was also found and suggested to be a result of nucleophilic attack of the side-chain oxygen atom onto the C-terminus. It was observed that the m/z 116 fragment dissociates further by loss of O=C=CH\(_2\) leading to a H\(_2\)N=CH-COOH fragment (m/z 74) and in parallel by loss of CO leading to H\(_2\)N=CH=CH\(_2\)COOH at m/z 88. The m/z 87 fragment can lose an HCN molecule giving H\(_2\)N=C(-O)-CH\(_3\). Dookeran et al.\(^{[13]}\) examined the fragmentation of AsnH\(^+\) and GlnH\(^+\) by energy-dependent CID using a ZAB-2FQ hybrid BEQ mass spectrometer\(^{[33]}\) by varying the collision gas. For AsnH\(^+\), low-energy metastable ion fragmentation was found to lead to dominant loss of H\(_2\)O and CO. At higher collision energies, loss of NH\(_3\) and further fragmentation by elimination of ketene becomes dominant, leading to a linear H\(_2\)N=CH-COOH fragment with m/z 74. Rogalewicz et al.\(^{[15]}\) used a triple quadrupole mass spectrometer to examine the CID MS/MS spectra of protonated α-amino acids with argon at 12 eV lab energy (2.8 eV center of mass energy) under multiple collision conditions. From the estimated activation energy, the loss of NH\(_3\) was predicted to occur from the side-chain protonated amide intermediate, which is favored over NH\(_3\) loss from the N-terminus since that involves formation of a destabilizing carbocation.\(^{[15]}\)

Here, the structure of the fragments generated from CID of AsnH\(^+\) having m/z 116 and m/z 87 are examined using IRMPD ion spectroscopy. The intensity of the m/z 115 ion generated was too small for investigation. CID on the m/z 116 fragment resulted in fragments at m/z 88, 74 and 46 fragments.

**Fragment: AsnH\(^+\)−NH\(_3\)**

Figure 1(A) shows the experimental IR spectrum of the mass-selected m/z 116 fragment ion along with the calculated spectrum for the lowest energy structure found (see Table 1), the N-protonated 3-aminosuccinic anhydride
Experimental IRMPD spectrum of the m/z 116 fragment ion from protonated Asn (in black) and DFT-calculated spectrum (green, as structure 1 in Scheme 1(A)) of the 3-aminosuccinic anhydride structure established by Heaton and Armentrout.

Figure 1. (A) Experimental IRMPD spectrum of the m/z 116 fragment ion from protonated Asn (in black) and DFT-calculated spectrum (green, as structure 1 in Scheme 1(A)) of the 3-aminosuccinic anhydride structure established by Heaton and Armentrout.

Loss of water is a well-known fragmentation channel for protonated amino acids with a carboxylic acid group in their side chain (Asp and Glu). Elimination of H2O from protonated aspartic acid (AspH+) (in black) and calculated spectrum (purple, as structure 2 in Supplementary Fig. S2, Supporting Information) of the iminium ion structure previously proposed in various reports. The arrow in the figure indicates the protonation site.

The four-membered ring protonated β-lactam carboxylic acid structure (structure 5 in Scheme 1(A)) was suggested to be the result of a nucleophilic attack by the terminal amino nitrogen on the side-chain amide carbon atom.

The relative free energy of this isomer is +32 kJ/mol compared to the succinic anhydride isomer, although protonation at the lactam carbonyl oxygen (4 in Scheme 1(A)) reduces the relative free energy difference to +26 kJ/mol. Nonetheless, the experimental IR bands around 1820 cm\(^{-1}\) and 1479 cm\(^{-1}\) are not well reproduced by the computed spectra for either of these lactam structures, as shown in Supplementary Fig. S1 (Supporting Information). The acylium ion structure reported by Dookeran et al.[13] is still higher in energy and can also be discarded based on the IR data. The calculated spectra of all structures from Scheme 1(A) are shown together with the experimental spectrum in Supplementary Fig. S1 (Supporting Information).

Related: H2O loss from protonated aspartic acid (AspH+)

Loss of water is a well-known fragmentation channel for protonated amino acids with a carboxylic acid group in their side chain (Asp and Glu). Elimination of H2O from protonated aspartic acid (m/z 134) leads to an ion at m/z 116 and the reaction mechanism has been suggested to be analogous to NH3 loss from AsnH+, discussed above. Note that analogously to the suggested reaction pathways for protonated Asn, other authors[13,34] have proposed a linear acylium structure (not shown in Fig. 1(A), see Supplementary Fig. S1, Supporting Information) for the ion produced by loss of H2O from protonated Asp.

Using IRMPD spectroscopy, it is now straightforward to verify whether the m/z 116 product ions from AspH+ and AsnH+ possess the same molecular structure. The IR spectrum (dashed grey trace) of AspH+–H2O is overlaid onto that of AsnH+–NH3 in Fig. 1(A). In terms of band frequencies, it is clear that all bands are reproduced within the experimental accuracy. We hence conclude that the loss of H2O from protonated aspartic acid leads to an ion of the same structure as that of AsnH+–NH3, i.e. a 3-aminosuccinic anhydride structure. This does not correspond to the structure proposed in the literature.[13,34]

Fragment: AsnH+–(CO + H2O)

In addition to deamidation, loss of CO+H2O forms a major low-energy dissociation channel of AsnH+, leading to a product ion at m/z 87. This dissociation pathway dominates over NH3 loss at the lowest collision energies.[13] Previous studies have explained this reaction pathway as being due to a proton transfer to the carboxyl group leading to sequential loss of H2O and CO from the C-terminus, leading to a product ion with an immonium ion structure.[12] The full potential energy surface for the reaction was reported by Heaton and Armentrout.

Figure 1(B) shows the experimental spectrum of the m/z 87 fragment ion from AsnH+ (black trace) along with the calculated spectrum for the immonium ion structure (purple trace); the protonation site is indicated with an arrow. In the calculated spectrum, the band at 1700 cm\(^{-1}\) is assigned to an amine bond stretching mode, the peak at 1597 cm\(^{-1}\) is attributed to a amide NH2 bending mode, and the bands at 1371 cm\(^{-1}\) and 1123 cm\(^{-1}\) are CH3 bending modes. The match with the computed IR spectrum for the immonium ion structure is rather good. Alternative structures are conceivable, e.g. different protonation sites or structures resulting from cyclization through nucleophilic attack by the side-chain nitrogen or oxygen atoms onto the β-carbon leading to four-membered ring structures. However, as shown in Supplementary Fig. S2 (Supporting Information), these alternatives are substantially higher in energy and feature computed IR spectra that do not match the experimental data.

Suggested reaction mechanism

The spectroscopically identified product ion structures suggest the reaction mechanisms shown in Scheme 2 for the loss of NH3 and H2O+CO. Loss of NH3 occurs from the side-chain amide by a nucleophilic attack of the C-terminal carbonyl oxygen, leading to a succinic anhydride structure. Loss of CO and H2O occurs by detachment of the protonated C-terminal carboxylic acid group leaving behind an immonium ion, in accordance with what has been established previously.[12]
the spectrum was measured in the FT-ICR MS. The arrows in the highest contribution to the experimental spectrum. This higher energy conformer (blue shaded) appears to give orientation and H-bonding of the protonated amino group.

The higher energy conformer (red) and an alternative conformer at +17 kJ/mol (black) compared with computed spectra for the lowest energy structure (blue shaded).

The two conformers have very similar structures and we expect that interconversion between them does not involve a significant barrier. It is therefore likely that a mixture of the two conformers exists and the spectrum appears to indicate this, particularly by the enhanced intensity in the 1400–1600 cm$^{-1}$ range. Spectral broadening of IRMPD bands is not uncommon in cases where structures can dynamically interconvert$^{35–37}$ and such a scenario is possibly at play here as well.

The other features in the spectrum are assigned as the C=O stretch of the carboxylic acid group at 1781 cm$^{-1}$, the amide carbonyl stretch at 1677 cm$^{-1}$, modes having mainly N-H bending character at 1651, 1609, and 1587 cm$^{-1}$, and the O-H bending mode of the carboxylic acid group at 1164 cm$^{-1}$.

**Fragment: GlnH$^+$–NH$_3$**

Various studies have reported that, in contrast to AsnH$^+$, elimination of NH$_3$ is the only dominant low-energy dissociation pathway for GlnH$^+$, which is confirmed in our experiments. Elimination of NH$_3$ can conceivably occur from either the N-terminus or the side-chain amide group (see Scheme 1(B)). Detachment of the N-terminal amide would lead either to 2-aminooxycarbonytetracyanide (blue structure in Scheme 1(B)) if cyclization occurs through the side-chain oxygen or to 2-pyrrolidone-5-carboxylic acid (also referred to as pyroglutamic acid or oxo-proline) if cyclization occurs through the side-chain nitrogen (red structure in Scheme 1(B)). Elimination of NH$_3$ from the side-chain amide has been considered to be more likely, and will lead to the same oxo-proline structure (blue in Scheme 1(B)) if cyclization occurs through the N-terminal nitrogen. If cyclization occurs through the C-terminal carboxyl oxygen, a 3-aminoglutamic anhydride (green structure in Scheme 1(B)) is formed. The latter reaction is analogous to that identified for NH$_3$ loss from AsnH$^+$, leading instead to the five-membered succinic anhydride structure. The energies of these suggested structures are listed in Table 1.

Figure 3 shows the experimental IRMPD spectrum for the $m/z$ 130 fragment corresponding to GlnH$^+$–NH$_3$ (black trace). The blue trace represents the vibrational spectrum predicted for the oxo-proline structure proposed in earlier studies$^{33,38}$ and identified as the deamidation product of sodiated glutamine.$^{39}$ Computed spectra for protonation on the lactam nitrogen (structure 4 in Scheme 1(B)) atom as well as on the oxo O-atom (structure 5 in Scheme 1(B)) are shown. In the calculated spectrum of Fig. 3(A), the band at 1786 cm$^{-1}$ is assigned to C=O stretching of the carboxylic acid group, the absorption at 1687 cm$^{-1}$ can be attributed to lactam C-N stretching. The bands at 1480, 1419, 1276, 1198 and 1140 cm$^{-1}$ have predominantly C-H bending character. Changing the protonation site to the nitrogen atom (Fig. 3(B)) yields a predicted spectrum that does not match the experiment, as is most obvious from the bands at 1786 and 1687 cm$^{-1}$. The relative free energy is +29 kJ/mol (see Table 1).
Related: H$_2$O loss from protonated glutamic acid (GluH$^+$)

Protonated glutamic acid (GluH$^+$, m/z 148) is similar to GlnH$^+$ except for a carboxylic acid group in the side chain replacing the amide group. Elimination of H$_2$O from GluH$^+$ has been suggested$^{[13,38]}$ to lead to the same product ion as that formed by NH$_3$ loss from GlnH$^+$. In order to verify this, the IRMPD spectrum of GluH$^+$–H$_2$O (grey trace) is overlaid onto the IRMPD spectrum of GlnH$^+$–NH$_3$ (blue trace) in Fig. 3. Although the relative intensities of the bands show some deviations, attributed to differences in the experimental conditions, the band frequencies (1786, 1681, 1480 and 1140 cm$^{-1}$) are reproduced closely, leading us to conclude that the product ions from GluH$^+$–H$_2$O and GlnH$^+$–NH$_3$ are the same.

Fragment: GlnH$^+$–NH$_3$ – (H$_2$O + CO)

In contrast to AsnH$^+$, loss of H$_2$O + CO from GlnH$^+$ is not observed in parallel with deamidation. Instead, the GlnH$^+$ – NH$_3$ fragment ion undergoes subsequent loss of H$_2$O + CO to arrive at a product ion at m/z 84. Dookeran et al.$^{[13]}$ proposed that CO and H$_2$O are formed from elimination of the carboxylic acid group from protonated oxo-proline leading to a pyrrolidone-like structure (shown in Fig. 4). Based on deuterium-labeling experiments, an alternative acyclic structure for the m/z 84 fragment ion from GlnH$^+$ and GluH$^+$ has also been suggested.$^{[38]}$ Computed spectra for both isomers are shown in Fig. 4 along with the experimental IR spectrum for the m/z 84 fragment from GlnH$^+$–NH$_3$. We suggest that the small size of the molecule in combination with its rigidity causes the multiple-photon absorption and dissociation process to be relatively inefficient, so that weaker bands (especially at 1250 and 1050 cm$^{-1}$) are not observed in the experimental IRMPD spectrum.

The m/z 84 fragment is a result of the detachment of CO and H$_2$O after loss of an NH$_3$ from GlnH$^+$. This is in contrast to the fragmentation mechanism of protonated asparagine, where the m/z 87 ion is formed directly from asparagine.

Attributing the experimental structure to the cyclic isomer in Fig. 4(A), the peak at 1627 cm$^{-1}$ is assigned to the C-N stretching in the lactam. The peak around 1450 cm$^{-1}$ is assigned to C-H bending, the peaks at 1050 and 973 cm$^{-1}$ to C-C stretching.

Figure 3. Experimental IRMPD spectrum of GlnH$^+$–NH$_3$ (black in all panels) compared with calculated spectra (colored according to the structures in Scheme 1(B)) for different isomeric structures for the m/z 130 ion. The best match is identified for the oxo-O protonated oxo-proline structure (top left, is the assigned calculation and therefore shaded). The dotted grey line represents the experimental spectrum for the m/z 130 fragment from GluH$^+$ (H$_2$O loss). The arrows indicate the protonation site of the structure.

To firmly establish the oxo-proline structure for GlnH$^+$–NH$_3$ (4 in Scheme 1(B)) alternative structures were considered by comparing their predicted spectra with the experimental one. If the NH$_3$-loss mechanisms for AsnH$^+$ and GlnH$^+$ were analogous, a six-membered aminoglutaric anhydride structure (structure 1 in Scheme 1(B)) would have resulted from deamidation of GlnH$^+$. The relative free energy of this structure is 60–100 kJ/mol higher in energy (dependent on the protonation site, see Table 1) than the oxo-proline structure. Calculated spectra for this structure do not match the experimentally observed IR spectrum, for instance, the experimental bands at 1786 and 1687 cm$^{-1}$ are not reproduced in the calculated spectra (see Fig. 3(D)). A final alternative is the substituted tetrahydrofuran structure resulting from elimination of the N-terminal amine and concomitant cyclization through the side-chain oxygen atom (Fig. 3(C), structure 6 in Scheme 1(B)). The relative free energy of this structure is only +11 kJ/mol higher than the oxo-proline structure. The calculated IR spectrum for this species, however, does not match as well as that for the O-protonated oxo-proline structure, especially considering the band at 1786 cm$^{-1}$ and around 1100–1200 cm$^{-1}$. From this we assign the oxo-O-protonated oxo-proline structure as the product ion from deamidation of GlnH$^+$.

Related: H$_2$O loss from protonated glutamic acid (GluH$^+$)

Fragment: GlnH$^+$–NH$_3$ – (H$_2$O + CO)
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Scheme 3. The reaction mechanism (solid arrow) for the fragmentation of GlnH⁺ as suggested from the product ion structures identified from their IRMPD spectra. The dashed arrow indicates an alternative mechanism leading to the same product ion structure.

The only other plausible alternative is the same pyrolidone-like structure with protonation at the oxygen instead of at the nitrogen, which gives a structure that is 57 kJ/mol higher in energy. The computed spectrum for this protoner clearly disagrees with the experimental spectrum (as visible in Supplementary Fig. S3, Supporting Information), so that we assign the structure in Fig. 4(A) to the m/z 84 fragment ion.

Suggested reaction mechanism

From the identified structures in Figs. 3 and 4, a suggested reaction mechanism can be derived, as presented in Scheme 3. Proton transfer from the N-terminus to the side-chain amide nitrogen induces elimination of a neutral ammonia molecule with a concomitant nucleophilic attack of the N-terminal nitrogen onto the carbonyl carbon of the side chain. This leads to an oxo-proline structure protonated on the oxo-oxygen atom (shown in its resonant form in Scheme 3). Further activation transfers the proton to the hydroxyl group of the carboxylic acid eliminating H₂O + CO. This leaves behind a fragment ion at m/z 84 with a pyrolidone-like structure.

Admittedly, the identification of the structure of the m/z 130 ion does not distinguish between a reaction mechanism in which the ammonia molecule is eliminated from the N-terminus or from the side-chain amide. Both reaction pathways lead to the same oxo-proline structure. Various reports have suggested NH₃ to be eliminated from the side chain as it leads to an intermediate carbonium ion (at the side-chain carbonyl) rather than an intermediate carbocation (at the α-carbon), which would occur upon NH₃ loss from the N-terminus and which is estimated to be less stable.

CONCLUSIONS

We have presented IR spectra for CID products of AsnH⁺ and GlnH⁺, including a comprehensive characterization of the product ions from the deamidation reactions. With the assistance of quantum-chemical computations, these spectra identify the product ion structures. AsnH⁺→NH₃ is thus shown to possess a 3-aminosuccinic anhydride structure protonated on the amino group, as had been identified on the basis of measured and computed activation energies. Likewise, the parallel dissociation reaction involving simultaneous loss of H₂O and CO is confirmed to form a linear immonium ion.

The IR ion spectrum for the NH₃-loss product from GlnH⁺ establishes that a different reaction mechanism occurs for this species. While neutral ammonia is again eliminated from the side-chain amide, the N-terminal nitrogen now acts as the nucleophile leading to an oxo-proline product ion structure.

Both AsnH⁺ and GlnH⁺ thus form five-membered ring structures upon deamidation. The fragmentation reactions for H₂O loss from protonated aspartic and glutamic acid have also been spectroscopically investigated. These fragmentation reactions lead to structures that are identical to those found for the loss of NH₃ from AsnH⁺ and GlnH⁺.

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REFERENCES


