Water Loss from Protonated XxxSer and XxxThr Dipeptides Gives Oxazoline—Not Oxazolone—Product Ions

Jos Oomens,* Lisanne J. M. Kempkes, Thijs P. J. Geurts, Luuk van Dijk, Jonathan Martens, Giel Berden, and P. B. Armentrout

ABSTRACT: Neutral loss of water and ammonia are often significant fragmentation channels upon collisional activation of protonated peptides. Here, we deploy infrared ion spectroscopy to investigate the dehydration reactions of protonated AlaSer, AlaThr, GlySer, GlyThr, PheSer, PheThr, ProSer, ProThr, AsnSer, and AsnThr, focusing on the question of the structure of the resulting \([\text{M} + \text{H} - \text{H}_2\text{O}]^+\) fragment ion and the site from which \(\text{H}_2\text{O}\) is expelled. In all cases, the second residue of the selected peptides contains a hydroxyl moiety, so that \(\text{H}_2\text{O}\) loss can potentially occur from this side-chain, as an alternative to loss from the C-terminal free acid of the dipeptide. Infrared action spectra of the product ions along with quantum-chemical calculations unambiguously show that dehydration consistently produces fragment ions containing an oxazoline moiety. This contrasts with the common oxazolone structure that would result from dehydration at the C-terminus analogous to the common b/y dissociation forming regular b2-type sequence ions. The oxazoline product structure suggests a reaction mechanism involving water loss from the Ser/Thr side-chain with concomitant nucleophilic attack of the amide carbonyl oxygen at its \(\beta\)-carbon, forming an oxazoline ring. However, an extensive quantum-chemical investigation comparing the potential energy surfaces for three entirely different dehydration reaction pathways indicates that it is actually the backbone amide oxygen atom that leaves as the water molecule.

INTRODUCTION

Peptide sequencing utilizing tandem mass spectrometry is a key technology in proteomics, and collision-induced dissociation (CID) is the most commonly used method to induce fragmentation. CID results predominantly in backbone cleavages of the peptide at the amide bond, giving b-type N-terminal and y-type C-terminal product ions. Dissociation is in most cases promoted by proton migration upon collisional activation, where the new location of the proton on one of the backbone amide linkages becomes the target of a nucleophilic attack. The nucleophile is often either the adjacent backbone amide oxygen or the N-terminal amine nitrogen. In the former—and most common—case, the N-terminal b-type product ion contains an oxazolone ring moiety, while in the latter case, head-to-tail cyclization forms a macrocyclic b-type ion. In the case of a b2-ion, this corresponds to a six-membered diketopiperazine ring. Over the past decade and a half, infrared multiple-photon dissociation (IRMPD) spectroscopy has contributed significantly to the establishment of these CID product ion structures.

Focusing on b2-ions, theoretical studies have shown that the diketopiperazine structure is lower in energy than the oxazolone structure but that the reaction mechanism leading to this structure is kinetically disfavored, as it requires a trans-to-cis isomerization of the peptide bond. The competition between the formation of oxazolone and diketopiperazine b2-ion structures is influenced by many factors including the length of the peptide and the identity of the residues involved. Alternative b2-ion structures have also been established, particularly where functional groups in the residues become involved in the reaction pathways. Early on, O’Hair and co-workers suggested several alternative structures for peptides containing Arg, His, Lys, Met, Gln, Ser, or Asn residues. Wysocki and co-workers utilized IRMPD spectroscopy to scrutinize the behavior of His-containing peptides. Furthermore, the amide moieties in the side-chains of Asn and Gln may lead to alternative b2-ion structures, as was also established by IR ion spectroscopy.

In CID of protonated peptides, small neutral losses are often encountered, with water and ammonia detachment being

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common loss channels. Indeed, for larger peptides, small neutral losses can be a dominant dissociation pathway that can limit the reliability and coverage of top-down proteomics by CID. Understanding the sequence dependence of these processes should be useful in enhancing these methods. Ordinarily, water loss from a protonated n-residue peptide yields a fragment ion that formally, i.e., in terms of m/z, corresponds to the b$_n$ sequence ion. However, MS$^{+}$, H/D exchange, and later also spectroscopic investigations, indicated that protonated oligoglycines do not expel a water molecule from the C-terminal free acid but via an entirely different pathway. The combined work of different groups$^{35–40}$ showed that the water loss from protonated oligoglycines results from the interaction of two backbone amide linkages within the peptide, resulting in a dihydromidazolone moiety.$^{37}$ IRMPD spectroscopic investigations$^{25}$ of the dehydration product ions were decisive in the determination of the correct structure.

Water loss from protonated dipeptides—possessing only a single amide linkage—must follow a different path that has been the subject of numerous studies as well.$^{35,41–46}$ Several studies employing IRMPD spectroscopy have focused particularly on the question of the structure of this formal b$_n$-ion. The groups of Armentrout and of Polfer showed that the water loss from ArgGly and AsnGly results in oxazolone formation, while dehydration of GlyArg results partly in diketopiperazine formation.$^{37,47}$ Wysocki and co-workers established dehydration pathways for protonated HisAla bifurcating into oxazolone and diketopiperazine products.$^{22}$ (Here, we use the term bifurcate to indicate multiple pathways for decomposition, without implying anything regarding the mechanisms of these pathways.) Our group recently investigated dehydration pathways of protonated dipeptides containing Asn and Gln.$^{50}$ Whereas AlaAsn and AsnAla follow a bifurcating mechanism leading to oxazolone as well as diketopiperazine product ions, AlaGln exclusively produced oxazolone fragments. In contrast, for GlnAla, expulsion of water mainly occurs from the Gln side-chain, resulting in a five-membered imino-substituted prolinyl structure, in line with earlier studies on the dehydration and deamidation of peptides with an N-terminal Gln or Glu residue.$^{50}$

For peptides containing Ser and Thr residues, water loss from the hydroxyl moiety in the side-chain may be an alternative to water loss from the C-terminal carboxylic acid.$^{45,51–53}$ Very recently, we used ion spectroscopy to investigate deamidation and dehydration of protonated AsnThr, which suggested that water loss indeed does not occur from the C-terminus, leading to a product ion containing an oxazoline—not oxazolone—moiety (Schemes 1 and 2).$^{55}$ Computational investigations of the potential energy surface (PES) suggested the participation of the Asn side-chain amide moiety so that the question of whether the formation of an oxazoline fragment is a generic pathway for Thr- and Ser-containing peptides is relevant. Therefore, we address here dipeptides with an aliphatic side-chain at the first residue (AlaSer, AlaThr, GlySer, GlyThr, PheSer, PheThr, ProSer, and ProThr) and compare with results for AsnSer and AsnThr.$^{53}$

A few earlier studies, based mainly on MS$^{+}$, have investigated the influence of Ser and Thr residues on peptide dissociation and have reported on possible oxazoline formation. Harrison studied the dehydration of longer, doubly protonated peptides containing Ser and Thr residues and suggested formation of product ions containing either aziridine or oxazoline moieties.$^{52}$ Several studies have proposed oxazoline ring formation in the CID-driven loss of phosphoric acid from phosphoserine residues.$^{53,54}$ Most relevant to the present report is perhaps a study by Reid and O’Hair involving N-acetylated serine (as well as several other derivatives of this amino acid),$^{51}$ providing evidence for an oxazoline dehydra

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**Scheme 1. Potential Nucleophilic Attack Reactions That Lead to the Dehydration of the Protonated Dipeptides XxXSer (Left) and XxXThr (Right)$^{40}$**

![Scheme Image](https://dx.doi.org/10.1021/jacs.0c00239)

$^{40}$H$_2$O is assumed to be expelled from the C-terminus with concomitant nucleophilic attack on the C-terminal carbon or from the Ser/Thr side-chain with concomitant nucleophilic attack on the β-carbon atom. Arrows indicate nucleophilic attacks resulting in five- and six-membered ring structures, which are considered to be most likely. Arrow colors correspond to resulting fragment ions in Scheme 2.

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**EXPERIMENTAL METHODS**

**IRMPD Spectroscopy.** A modified Bruker quadrupole ion trap mass spectrometer (AmaZon Speed ETD) coupled to the Free Electron Laser for Infrared Experiments (FELIX) was utilized to record IRMPD spectra of the peptide fragment ions.$^{35–37}$ Protonated peptides were generated by electrospray ionization (ESI) using solutions of 10$^{-6}$–10$^{-7}$ mol L$^{-1}$ of the peptide in 50:50 acetonitrile/water with 0.1% formic acid and mass isolated in the ion trap. Collisional activation of the protonated precursor ions was effected with helium background gas for approximately 40 ms and an amplitude parameter of ~0.3 V, optimizing the intensity of the [M + H − 18]$^+$ fragment ion.

FELIX produces tunable infrared radiation in the form of 6 μs long macropulses of approximately 10−60 mJ at a 10 Hz rep rate and a bandwidth of 0.5% of the center frequency. The IR beam was focused in the center of the ion trap and is estimated to overlap nearly 100% of the trapped ion cloud. Resonant absorption of the infrared radiation by the ion population leads to high vibrational excitation of the molecule mediated by intramolecular vibrational redistribution (IVR).
Scheme 2. Potential Isomeric Fragment Ion Structures Formed by Dehydration of \([\text{XxxSer} + \text{H}]^+\) and \([\text{XxxThr} + \text{H}]^+\)

<table>
<thead>
<tr>
<th>structure name →</th>
<th>1. diketopiperazine</th>
<th>2. oxazolone</th>
<th>3. monoketopiperazine</th>
<th>4. oxazolone</th>
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</thead>
<tbody>
<tr>
<td>(\text{H}^+) site</td>
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<td>optimizes to 2.4</td>
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<td>33 (13)</td>
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<tr>
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<td>104 (108)</td>
<td>175 (161)</td>
<td>115 (129)</td>
<td>optimizes to 4.1</td>
</tr>
<tr>
<td>2</td>
<td>63 (54)</td>
<td>118 (114)</td>
<td>153 (150)</td>
<td>214 (201)</td>
</tr>
<tr>
<td>3</td>
<td>88 (89)</td>
<td>101 (87)</td>
<td>0 (0)</td>
<td>55 (50)</td>
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<tr>
<td>4</td>
<td>39 (22)</td>
<td>33 (26)</td>
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</tbody>
</table>

The potential energy surface for the dehydration of protonated GlySer was investigated at the same level of theory: B3LYP/6-31++G(d,p) followed by MP2(full)/6-311+G(2d,2p) single-point calculations. Transition states (TSs) were located and optimized using one of the TS, QST2, or QST3 protocols in Gaussian16 or by doing relaxed potential energy surface (PES) scans, which generally connect the TS with intermediates on either side. In all cases, it was verified that the TSs correspond to first-order saddle points. In any cases where the PES scans were ambiguous, intrinsic reaction coordinate (IRC) scans were performed for selected, more complex TSs to verify that they correspond to barriers between the intended minima.

The dashed line indicates the additional methyl group of the Thr residue. Colors relate to cyclization reactions indicated in Scheme 1. Potential protonation sites are indicated with numbers.

Table 1. Calculated Relative 298 K Gibbs Energies (in kJ/mol)\(^a\) for the Possible Structures of the Dehydration Product Ions of the Dipeptide \([\text{AlaSer} + \text{H}]^+\)^b

<table>
<thead>
<tr>
<th>structure name</th>
<th>1. diketopiperazine</th>
<th>2. oxazolone</th>
<th>3. monoketopiperazine</th>
<th>4. oxazolone</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{H}^+) site</td>
<td>optimizes to 1.5</td>
<td>optimizes to 2.4</td>
<td>73 (77)</td>
<td>33 (13)</td>
</tr>
<tr>
<td>1</td>
<td>104 (108)</td>
<td>175 (161)</td>
<td>115 (129)</td>
<td>optimizes to 4.1</td>
</tr>
<tr>
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<td>63 (54)</td>
<td>118 (114)</td>
<td>153 (150)</td>
<td>214 (201)</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>39 (22)</td>
<td>33 (26)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For selected structures that were particularly promising, further conformational fine-tuning was performed using Molecular Mechanics/Molecular Dynamics (MM/MD) with AMBER 12.^29 Geometries obtained from the initial DFT calculations were minimized in a simulated annealing MD procedure of up to 1000 K with a 1 fs step size. A total of 500 conformational structures were generated and grouped based on geometrical similarity to obtain between 20 and 35 different conformers. These structures were then optimized with the above DFT protocol. Alternatively, chemical intuition was used to design input configurations with optimal H-bonding. Single-point calculations were performed at the MP2(full)/6-311+G(2d,2p) level using the B3LYP/6-31++G(d,p) structures and zero-point vibrational and thermal corrections. Unless noted otherwise, we present in the tables and figures the lowest-energy conformer identified for the selected isomer/protomer; this restriction is necessary to maintain readability of this text, although we have inspected computed spectra for higher-energy conformers. Also, because there are several isomers of each product ion (different connectivities of the backbone heavy elements), we use the term “protomer” to distinguish the differently protonated forms of each of them.

The potential energy surface for the dehydration of protonated GlySer was investigated at the same level of theory: B3LYP/6-31++G(d,p) followed by MP2(full)/6-311+G(2d,2p) single-point calculations. Transition states (TSs) were located and optimized using one of the TS, QST2, or QST3 protocols in Gaussian16 or by doing relaxed potential energy surface (PES) scans, which generally connect the TS with intermediates on either side. In all cases, it was verified that the TSs correspond to first-order saddle points. In any cases where the PES scans were ambiguous, intrinsic reaction coordinate (IRC) scans were performed for selected, more complex TSs to verify that they correspond to barriers between the intended minima.

RESULTS AND DISCUSSION

\([\text{AlaSer} + \text{H} – \text{H}_2\text{O}]^+\). In Scheme 1, we present a selection of suggested (net) dehydration reactions, in which water loss is accompanied by ring formation through a nucleophilic attack. We have assumed that water loss occurs either from the C-terminus or from the hydroxyl group in the Ser side-chain. Only reactions leading to product ion structures incorporating a five- or six-membered ring were considered, because noncyclic and larger/smaller ring structures are often higher in energy, and numerous reports have shown that they are less common as (peptide) CID product ions. With these considerations in mind, Scheme 2 presents the four most likely isomeric product ion structures, where colors correspond to the arrows in Scheme 1. For each of the possible product ion structures, multiple protonation sites are conceivable as...
indicated by the numbers in Scheme 2. The relative Gibbs energies at 298 K of the lowest-energy conformer of each of these protomers are listed in Table 1. Protonation at the ring oxygens of the oxazolone and oxazoline structures typically leads to spontaneous ring opening, and these structures were not further considered.

Figure 1 shows the calculated spectra for structures 1.5, 2.4, 3.4, and 4.1, i.e., the lowest-energy protomers for each of the likely isomers. The experimental IRMPD spectrum of [AlaSer + H − H_2O]^+ is overlaid onto the computed spectra, which clearly suggests that the product ion possesses an oxazoline structure (4.1) and not one of the isomeric alternatives. Four bands in the experimental spectrum are especially diagnostic: 1800 cm\(^{-1}\) (C-terminal C=O stretch of 4.1), 1625 cm\(^{-1}\) (unresolved NH_2 scissor and C=N stretch of 4.1), 1500 cm\(^{-1}\) (oxazoline CH_2 scissor of 4.1), and 1140 cm\(^{-1}\) (C−OH bend of the carboxylic acid group). Only the computed spectrum of oxazoline structure 4.1 simultaneously reproduces these four bands, in particular, the most intense IR absorption in the spectrum at 1140 cm\(^{-1}\). Observation of the free-acid C=O stretch and C−OH bend at 1800 and 1140 cm\(^{-1}\) indicates directly that water loss leaves the C-terminal carboxylic acid intact, such that H_2O must be expelled from elsewhere in the dipeptide.

The dissociation channels that are observed upon IRMPD of [AlaSer + H − H_2O]^+ are also suggestive of an oxazoline structure. The m/z 116 product ion (neutral loss of 43, ethanimine, CH_3CH=NH) suggests detachment of the ethylamine side-chain,52 which is only plausible for the oxazolone and oxazoline structures. Neutral loss of the N-terminus as an alkylamine, rather than neutral loss of CO, has been shown to be diagnostic for b-type ions that are not oxazolones protonated on the oxazolone N.36,60 Here, further evidence for a non-oxazolone structure is provided by the absence in the experimental spectrum of the main predicted IR band of oxazoline structure 2.4 near 1930 cm\(^{-1}\) (oxazoline ring C=O stretch). Again, this leaves the oxazoline structure as the only plausible product ion structure. The m/z 71 IRMPD dissociation product (neutral loss of 88) likely corresponds to expulsion of ethanimine and HOCH=NH groups from the oxazoline structure. Hence, [AlaSer + H − H_2O]^+ possesses the oxazoline structure 4.1, which does not correspond to the thermodynamically most favorable structure, the monoketopiperazine 3.4, Table 1. The oxazoline product ion identified suggests that water loss occurs from the Ser side-chain and the amide oxygen acts as the nucleophile in the reaction. A more detailed investigation of the reaction pathway is presented below.

[AlaThr + H − H_2O]^+. Table 2 lists the product ion structures for [AlaThr + H − H_2O]^+ that we consider to be most likely, along with computed relative Gibbs energies for different protomers. The candidate product ion structures are analogous to those discussed above for the AlaSer system, except for the additional methyl group; the energetic ordering of the different isomers and protomers is also largely analogous.

Table 2. Calculated Relative 298 K Gibbs Energies (in kJ/mol)^a for Possible Structures of Dehydration Reaction Product Ion of Protonated AlaThr

<table>
<thead>
<tr>
<th>Structure^b name →</th>
<th>1. diketopiperazine</th>
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<th>4. oxazoline</th>
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<td>4</td>
<td>110 (105)</td>
<td>91 (73)</td>
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<td>64 (51)</td>
</tr>
<tr>
<td>5</td>
<td>30 (12)</td>
<td>32 (25)</td>
<td></td>
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</tbody>
</table>

^a At the MP2(full)/6-311+G(2d,2p)//B3LYP/6-31++G(d,p) level. Relative B3LYP/6-31++G(d,p) energies are in brackets. ^b See Scheme 2 for structures and numbering of protonation sites.
Figure 2 presents the IRMPD spectrum of the [AlaThr + H − H₂O]⁺ product ion together with the calculated spectra of structures 1.5, 2.4, 3.4, and 4.1. It is again immediately obvious that the computed spectrum for the lowest-energy oxazoline ion (4.1) matches the experimental spectrum far better than any of the other candidates. Thus, the [AlaThr + H − H₂O]⁺ product ion has an oxazoline structure, protonated at the oxazoline ring nitrogen (4.1).

The diagnostic bands and their normal mode assignments are also analogous to those for [AlaSer + H − H₂O]⁺, except for the 1500 cm⁻¹ band, which has predominantly oxazoline NH bending character for [AlaThr + H − H₂O]⁺. Structure 4.1 is clearly the only candidate that can simultaneously account for all diagnostic bands in the observed spectrum. The MS³ fragments that are observed upon IRMPD include the same neutral-loss channels (43 and 88) as observed for [AlaSer + H − H₂O]⁺. These observations suggest that the dehydration reaction pathways for protonated AlaThr and AlaSer are the same, i.e., H₂O loss does not occur from the C-terminus but rather likely involves the Thr side-chain hydroxyl moiety.

H₂O-Loss Product Ions of Other Protonated XxxSer and XxxThr Dipeptides. Water-loss product ions from protonated AlaSer and AlaThr are thus assigned to possess an oxazoline ring structure on the basis of the spectral comparisons in Figures 1 and 2, which clearly rule out alternative isomeric product ion structures. We now quickly screen several other XxxSer and XxxThr peptides to investigate whether this behavior is generic or whether the identity of the first residue influences the reaction pathway and hence the [M + H − H₂O]⁺ product ion structure. We first consider peptides with Gly, Phe, and Pro as the first residue, which are significantly different, but have in common that they do not possess additional nucleophiles. We therefore expect analogous nucleophilic attack reactions to be likely so that analogous product ion structures incorporating a five- or six-membered ring are adopted as the most likely candidates.

The top two panels of Figure 3 present IRMPD spectra for the H₂O-loss product ion of GlySer and PheSer, along with the calculated spectrum that was found to provide the best match; additional comparisons with predicted spectra for alternative structures.
structures are shown in the Supporting Information (Figures S1 and S2). The corresponding structures and 298 K Gibbs energies compared to the lowest-energy isomer for that species are given in the respective panels. Tables S1 and S2 list relative energies of all investigated structures.

For each of the two ions, we observe that the oxazoline structure presents the best match with experiment, just as for AlaSer discussed above. The four bands near 1800, 1625, 1500, and 1140 cm$^{-1}$ are again diagnostic for the oxazoline structure and rule out any of the other isomers (see Figures S1 and S2). Protonation on the oxazoline nitrogen atom corresponds to the lowest-energy protonation site, in good agreement with the observed spectra. The overall lowest-energy structure for [PheSer + H – H$_2$O]$^+$ and [GlySer + H – H$_2$O]$^+$ is a monoketopiperazine structure (3.4), as for [AlaSer + H – H$_2$O]$^+$, but this structure can unambiguously be excluded on the basis of the clear mismatch of diagnostic bands in the 1400–1800 cm$^{-1}$ range.

Interestingly, the situation for [ProSer + H – H$_2$O]$^+$ is somewhat different. In the range between 1400 and 1900 cm$^{-1}$, we observe more absorption bands than for the XxxSer peptides discussed above, which may suggest that there is more than one structure contributing to the observed spectrum. One may expect that the higher proton affinity of the N-terminal secondary amine of Pro may become a competitive protonation site. Inspecting the relative energies of the oxazoline isomer with protonation at the oxazoline nitrogen, 4.1, and with protonation at the N-terminus, 4.4, we observe that this is indeed the case: at the MP2 level, 4.4 is 13 kJ mol$^{-1}$ lower in free energy than 4.1, whereas at the B3LYP level, 4.1 is lower by 3 kJ mol$^{-1}$. Note, too, that these structures are linked by simple motion of the proton and occupy a double-well potential. The transition state for interconverting these two structures lies 30 kJ mol$^{-1}$ above 4.4 and 16 kJ mol$^{-1}$ above 4.1, such that the TS lies below the zero-point motion of the NH stretch in 4.1 (harmonic frequency of 3136 cm$^{-1}$).

In the bottom two panels of Figure 3, we therefore compare the experimental spectrum with computed spectra for the oxazoline structures 4.1 and 4.4. The two strongest peaks in the experimental spectrum near 1140 and 1800 cm$^{-1}$ match with intense predicted features for both structures, corresponding to the COH bending and C=O stretching modes of the C-terminal free acid, respectively. In the range between 1550 and 1700 cm$^{-1}$, there are three features in the experimental spectrum, and the theoretical spectra for 4.1 and 4.4 are significantly different here. We suggest that a combination of 4.1 and 4.4 could explain the three bands in this range, although the predicted band positions, especially for 4.4, appear to be slightly off. This hypothesis assigns the band near 1650 cm$^{-1}$ as the oxazoline C=N stretch of 4.4, the band near 1620 cm$^{-1}$ as the C=N stretch of the protonated oxazoline of 4.1, and the band near 1560 cm$^{-1}$ as the NH$_2$ scissor mode of the protonated N-terminus of 4.4. These normal mode characters are schematically indicated in Figure 3. For the remainder of the spectrum, the broad and partially resolved feature between 1000 and 1400 cm$^{-1}$ can be rationalized as representing a sum of the predicted spectra of 4.1 and 4.4, perhaps with the contribution of the lower-energy protonomer 4.4 being dominant. A noticeable deviation between experiment and theory is the high experimental intensity of the band near 910 cm$^{-1}$, which is not reproduced theoretically. The weak bands predicted in this range for both 4.1 and 4.4 are assigned to modes with delocalized C–C, C–O, and C–N stretch character in the proline and oxazoline rings.

Spectral comparisons with alternative isomeric structures are shown in Figure S3. We conclude that most other structures can be excluded. An exception is the global minimum monoketopiperazine structure 3.4, whose contribution cannot be excluded on the basis of spectral comparison. It is clear, however, that this structure alone cannot explain the observed spectrum and that the oxazoline structures give a better overall match with all bands in the 1400–1800 cm$^{-1}$ range. We therefore conclude that protonated GlySer, PheSer, and ProSer all form oxazoline-type fragment ions upon dehydration.

Figure 4 presents IRMPD spectra for the analogous series of [XxxThr + H – H$_2$O]$^+$ fragment ions produced from protonated GlyThr, PheThr, and ProThr. Again, we only show the computed spectrum that provides the best match with experiment, which in all cases is the oxazoline isomer. Spectral comparisons with alternative isomers give a far less convincing match, as shown in Figures S4–S6, providing further support for the assignment of oxazoline structures. Each panel in Figure 4 gives the energy of the assigned structure relative to the lowest-energy isomer for each species. Relative energies for alternative (protonation) isomers are listed in Tables S4–S6.

The overall lowest-energy isomer identified for [GlyThr + H – H$_2$O]$^+$, [PheThr + H – H$_2$O]$^+$, and [ProThr + H – H$_2$O]$^+$ is the monoketopiperazine structure protonated on one of the piperazine nitrogen atoms (3.4). The diketopiperazine structure protonated on one of the keto-oxygen atoms (1.5) and the oxazoline structure protonated at the ring nitrogen atom (4.1) are competitive in energy; the B3LYP level even places these structures lower than 3.4 for some of the systems. In all cases, however, computed spectra for these low-energy alternatives are in clear disagreement with the experimental spectra, particularly in the diagnostic frequency range between 1400 and 1800 cm$^{-1}$. Some examples are shown in Figures S4–S6. For the [ProThr + H – H$_2$O]$^+$ fragment ion, protonation at the secondary amine N-terminus (4.4) is competitive with protonation at the oxazoline nitrogen (4.1), and both computed spectra are shown in Figure 4. As discussed above for [ProSer + H – H$_2$O]$^+$, the experimental spectrum suggests that both structures contribute, with 4.1 perhaps being the dominant protonation isomer. We note that although 4.1 is 5 kJ mol$^{-1}$ higher than 4.4 at the MP2 level, it is predicted to be 10 kJ mol$^{-1}$ lower in energy at the B3LYP level. Again, these two species reside in a double-well potential coupled by a transition state associated with proton motion.

The diagnostic bands in the spectra for the XxxThr-derived fragments have mode characters that are entirely analogous to their XxxSer counterparts. The band near 1800 cm$^{-1}$ is the carboxylic C=O stretch, the band near 1625 cm$^{-1}$ results from two unresolved modes with NH$_2$-scissoring and oxazoline C=N stretching, the 1500 cm$^{-1}$ band combines C=O stretching and NH bending in the oxazoline ring, and the 1140 cm$^{-1}$ band is C–OH bending of the carboxylic acid moiety. The experimental spectrum for [ProThr + H – H$_2$O]$^+$ deviates from that for [ProSer + H – H$_2$O]$^+$ in that the anomalously strong band near 910 cm$^{-1}$ is not reproduced; we leave this as an experimental observation.

In conclusion, entirely analogous to the three XxxSer dehydration products shown in Figure 3 and the AlaSer- and AlaThr-derived fragment ions, we observe exclusively oxazoline formation in the dehydration of these protonated XxxThr
peptides. This result appears generic and provides spectroscopic confirmation for various studies that proposed oxazoline formation upon dehydration of Ser- and Thr-containing peptides. As compared to the dehydration of protonated N-acetylated serine, reported to form an oxazoline species in ref 51, it is interesting to note that the monoketopiperazine isomer, which is the lowest-energy product ion for the dipeptides in most cases, is not an alternative for the N-acetylated amino acids, as they do not possess an N-terminal amine.

**Dehydration of Protonated AsnSer and AsnThr.** In contrast to the XxxSer and XxxThr peptides discussed above, AsnSer and AsnThr contain additional nucleophiles that may influence the dehydration reaction and the resulting product ion structure. Deamidation of these peptides was shown to expel NH3 from the amide moiety in the Asn side-chain, forming a furanone ring product ion, which is rationalized by ring closure between the Asn amide carbon atom and the backbone amide nitrogen. If dehydration follows a parallel mechanism, expelling H2O from the Asn side-chain with the backbone amide nitrogen acting as the nucleophile, a pyrrolidone ring-containing product would be formed (structure 5 in Scheme 3). For protonated AsnThr, however, the [AsnThr + H − H2O]+ spectrum reported in ref 53 indicates that an oxazoline structure is formed, consistent with the other [XxxThr + H − H2O]+ systems presented here. Below, we investigate the dehydration of protonated AsnSer. We assume as candidate product ions the four isomeric structures considered for XxxSer plus the pyrrolidone-containing product ion (Scheme 3); moreover, we include the Asn side-chain nitrogen and oxygen atoms as potential protonation sites.

As compared to the systems discussed above, the spectrum observed for [AsnSer + H − H2O]+, shown in Figure S5, is more difficult to match with computed spectra for likely candidate structures. Figure S7 shows comparisons with low-energy versions of each of the isomeric candidate structures; relative energies are listed in Table 3. The IRMPD spectrum obtained from all fragment mass channels, shown in the top panel of Figure S5, features three IR bands in the 1675–1850 cm⁻¹ region (labeled A, B, and C), whereas not one of the computed spectra presents three vibrational bands in this region. In contrast to the other XxxSer dipeptides, we suspect that dehydration of protonated AsnSer proceeds along a bifurcating m/z 220 → m/z 202 + H2O reaction pathway, producing at least two isomeric [AsnSer + H − H2O]+ product ions. Analyzing each of the IRMPD fragment channels from these ions individually reveals clearly distinct IR spectra, consistent with more than one isomer in the [AsnSer + H − H2O]+ ion population generated upon CID.

The IR spectrum imprinted into the major dissociation channel at m/z 185 ([AsnSer + H − H2O − NH3]+, not shown) displays a spectrum similar to the IRMPD spectrum from all IRMPD fragment channels. This suggests that all [AsnSer + H − H2O]+ isomers undergo loss of NH3 as their main IRMPD dissociation channel, which is plausible, because all likely candidate structures feature a primary amine moiety, and such a process parallels what was observed for [AsnThr + H − H2O]+.53

A clearly distinct spectral response is imprinted into IRMPD channel m/z 87, corresponding to neutral loss of 115 mass units from the [AsnSer + H − H2O]+ precursor ion. This spectrum is shown in the bottom panel of Figure S5 and corresponds closely to the spectrum computed for oxazoline structure 4.1. In the high-frequency range, one observes that both the C-terminal carboxylic C=O stretch near 1800 cm⁻¹ and the amide C=O stretch near 1700 cm⁻¹ match the experimental bands A and C, while band B is absent in this fragment channel. With the oxazoline structure assigned, we rationalize the m/z 87 fragment as the...
The third main dissociation channel is observed at m/z 143, [AsnSer + H − H2O − 59]⁺. The IR spectrum imprinted into this channel is shown in the middle panel of Figure 5 and is clearly distinct from the spectrum observed in m/z 87. In the high-wavenumber range, this spectrum displays bands A and B, while band C is (nearly) absent. Matching this spectrum with one of the computed spectra is more speculative, and we tentatively suggest monoketopiperazine structure 3.4, which provides the best matching frequencies for bands A and B. The band near 1750 cm⁻¹ is assigned to unresolved C=O stretches of the strongly H-bonded carboxylic acid and the ring carbonyl; the 1700 cm⁻¹ band is the Asn amide C=O stretch. Consistent with this assignment, the loss of 59 amu can be identified as NH2C(=O)CH3, the side-chain on the monoketopiperazine structure. This assignment fails to account for observed bands near 1135 and 1790 cm⁻¹ (weak), which may be explained by the fractional presence of an alternative conformation of 3.4, shown in lighter gray color, although this is speculative. Computed spectra for alternative low-energy isomers are shown in Figure S7.

Combining the calculated spectra of 4.1 and 3.4 in a 2:1 ratio gives the spectrum overlaid onto the IRMPD spectrum obtained from all channels in the top panel of Figure 5. This ratio has been chosen to roughly match the observed spectrum. It is thus proposed that the dehydration reaction pathway of protonated AsnSer bifurcates toward the thermodynamically favored monoketopiperazine product and the (probably) kinetically more favorable oxazoline ion. Both pathways can be rationalized by H2O expulsion from the Ser side-chain but induced by different nucleophiles: attack by the N-terminal amino nitrogen produces the monoketopiperazine product and by the backbone amide oxygen leads to the oxazoline ion. In contrast, for dehydration of protonated AsnThr, no evidence for formation of a monoketopiperazine reaction was found.53 A

H2NC(=O)CH2CH=NH2⁺ tail expelling the oxazoline ring carboxylic acid as a neutral.

Table 3. Relative 298 K Gibbs Energies for Different (Proto)isomers of [AsnSer + H − H2O]⁺ 

<table>
<thead>
<tr>
<th>Structure name →</th>
<th>1. diketopiperazine</th>
<th>2. oxazoline</th>
<th>3. monoketopiperazine</th>
<th>4. oxazoline</th>
<th>5. pyrrolidone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>optimizes to 1.2</td>
<td>120 (132)</td>
<td>49 (39)</td>
<td>131 (144)</td>
<td>133 (144)</td>
</tr>
<tr>
<td>2</td>
<td>134 (140)</td>
<td>231 (227)</td>
<td>49 (39)</td>
<td>131 (144)</td>
<td>257 (281)</td>
</tr>
<tr>
<td>3</td>
<td>97 (89)</td>
<td>116 (110)</td>
<td>70 (76)</td>
<td>131 (144)</td>
<td>70 (76)</td>
</tr>
<tr>
<td>4</td>
<td>114 (112)</td>
<td>110 (99)</td>
<td>0 (0)</td>
<td>139 (144)</td>
<td>139 (144)</td>
</tr>
<tr>
<td>5</td>
<td>80 (67)</td>
<td>94 (88)</td>
<td>226 (235)</td>
<td>226 (235)</td>
<td>226 (235)</td>
</tr>
<tr>
<td>6</td>
<td>100 (88)</td>
<td>183 (174)</td>
<td>75 (71)</td>
<td>120 (111)</td>
<td>120 (111)</td>
</tr>
<tr>
<td>7</td>
<td>237 (238)</td>
<td>82 (83)</td>
<td>137 (141)</td>
<td>137 (141)</td>
<td>137 (141)</td>
</tr>
</tbody>
</table>

See Scheme 3 for structures and protonation sites.
Mechanistic Aspects. The oxazoline product structure identified for the dehydration reactions of the investigated XxxSer and XxxThr dipeptides suggests a reaction mechanism as summarized by the red arrow in Scheme 1: collisional activation induces proton transfer toward the hydroxyl oxygen, and nucleophilic attack by the amide oxygen leads to H2O expulsion and concomitant formation of the oxazoline product. This pathway (see Scheme 4a) was suggested and computationally investigated for the dehydration of protonated Ac−Ser−OH by Reid et al., although a complete potential energy surface going from reactants to products was not provided. At the present level of theory, the TS for dehydration of protonated GlySer shown in Figure 6a lies at 140 kJ mol−1 above the reactant, where we define GlySer protonated at the N-terminus (shown in Figure 6, lower left) as the zero of energy. (Notably, MP2 calculations assign this N-terminally protonated structure as the ground structure, whereas B3LYP prefers protonation the amide oxygen. These two structures lie within 7 kJ mol−1 at either level of theory. An IRMPD spectrum of the protonated parent ion suggests that the N-protonated form is the dominant structure, see Figure S8.) Our more detailed computational characterization of the Reid−O’Hair reaction path reveals another, higher-energy TS to arrive at the protonation isomer that leads to dehydration in this mechanism. This rate-limiting step in the reaction, with a TS lying 158 kJ mol−1 above the ground level, corresponds to a proton transfer that yields a geminal diol complex, as shown in Figure 6a. After this step, the molecule has to rearrange to position the first carbonyl for backside attack at the side-chain to eliminate water via the 140 kJ/mol TS shown in Figure 6a. The full PES is shown in Figures S9 and S10. Because there are two relatively high-energy TSs along this reaction coordinate, other pathways were also investigated.

An alternative dehydration mechanism has been suggested for [AsnThr + H]+ along with a detailed computational investigation of the entire PES. Rate-limiting TSs leading to the oxazoline product ion were found to be competitive with those leading to the oxazolone product by water loss from the C-terminus. In these pathways, sketched in Scheme 4b, the added proton migrates to the carbonyl oxygen of the backbone amide linkage. The electrophilic amide carbon is then susceptible to attack by the Thr (or Ser) hydroxyl oxygen, transferring its proton to a nearby nucleophile. In AsnThr, the Asn side-chain oxygen acts as the proton accepting nucleophile. In peptides that do not possess a nucleophile in the first residue, the N-terminal nitrogen must act as the nucleophile accepting a proton from the Ser or Thr side-chain. The complete PES for this mechanism in [GlySer + H]+ is shown in Figure S11, with the key TSs summarized in Figure 6b. Again, a relatively complicated series of dihedral angle torsions position the side-chain hydroxyl to attack the amide carbon while transferring the proton to the N-terminus, thus generating a tetrahedral intermediate. This is the rate-limiting step in the reaction for [GlySer + H]+, 97 kJ mol−1 above the ground conformer. (In the [AsnThr + H]+ system, the availability of the Asn side-chain reduces the energy of the analogous TS.) The protonated N-terminus then needs to reposition itself—see also Scheme 4b—to transfer its proton to the nearby hydroxyl group (the protonated carbonyl of the first residue), leading to water expulsion. This TS lies submerged at 77 kJ mol−1 for [GlySer + H]+. Note that in this reaction mechanism, it is actually the backbone amide oxygen atom that is expelled as a water molecule. A nearly equivalent pathway involving a tetrahedral intermediate was identified (not shown), in which the carboxylic acid group lies...
on the other side of the five-membered ring such that it cannot assist in water formation. As a consequence, the two key TSs lie 112 and 97 kJ mol\(^{-1}\) above the ground conformer, 15–20 kJ mol\(^{-1}\) above the first pathway described.

Yet, an alternative dehydration mechanism, one that appears most straightforward, is suggested in Scheme 4c for [GlySer + H\(^+\)]. Proton transfer from the N-terminus to the backbone amide oxygen is facile,\(^{28,61}\) but rotation of the proton toward the hydroxyl moiety of the Ser side-chain encounters a higher barrier (+76 kJ/mol), as seen in the PES in Figure S10. This gives access to a conformation with OH···OH and H\(_2\)N···HN hydrogen bonds that is virtually isoenergetic with the N-terminally protonated starting point (+4 kJ/mol at MP2 but −3 kJ/mol at B3LYP).\(^{62}\) Two slightly different conformers of this structure lead to two nearly equivalent TSs for proton abstraction by the Ser hydroxyl oxygen. Concomitant nucleophilic attack by the amide oxygen onto the Ser \(\beta\)-carbon corresponds to the rate-limiting TS at 231 and 242 kJ/mol (Figures 6c and S12), leading to the incipient, oxazoline-ring-containing product ion. This dehydration mechanism is reminiscent of that proposed for protonated GlyGly,\(^{28,63}\) where the carboxylic hydroxyl takes the role of the Ser hydroxyl group (note that both the Ser \(\beta\)-carbon and the C-terminal carbon are \(\varepsilon\) relative to the amide oxygen, leading therefore to five-membered ring product ions, oxazoline and oxazolone, respectively).

The pathway proceeding via the tetrahedral intermediate, Scheme 4b, for expulsion of H\(_2\)O from the backbone amide oxygen is calculated to be the most favorable pathway. The mechanism suggested in Scheme 4c, which may be attractive for its simplicity, is disfavored energetically by a large margin. The significantly lower rate-limiting TS energies for the other two pathways can qualitatively be attributed to a more favorable backside attack in the TS geometry. The pathway in Scheme 4b distinguishes itself from the other two in that the expelled water molecule carries the amide oxygen atom and not the Ser hydroxyl oxygen. \(^{18}\)-O-isotope labeling or methylation of the Ser hydroxyl could therefore verify whether the pathway via the tetrahedral intermediate is indeed operative. Threshold CID studies could also differentiate between possible pathways by providing accurate experimental values for the TS energies. Water loss involving amide oxygens has been experimentally established in deprotonated peptides,\(^{63,64}\) but the mechanism had not been explored.

Various (spectroscopic) studies on the dehydration of protonated dipeptides not containing a Ser or Thr residue showed that water is expelled from the C-terminal carboxylic acid group.\(^{28,47,48,50,65}\) This reaction is suggested to proceed via a nucleophilic attack on the C-terminal carboxylic acid carbon generating a \(b_2\)-type sequence ion, which can have either an oxazoline or a diketopiperazine structure. In contrast, for the Thr and Ser terminal peptides studied here, an alternative mechanism is at work. The monoketopiperazine structure is in most cases the thermodynamically most favorable structure, but it is the higher-energy oxazoline fragment ion that is actually formed. This observation appears to be analogous to the diketopiperazine/oxazolone dichotomy in the formation of \(b_2\) sequence ions: although diketopiperazine is usually lower in energy, oxazolone structures are in most cases formed. Qualitatively, this has often been attributed to the unfavorable trans-to-cis isomerization around the peptide bond required to arrive at the diketopiperazine structure, although this is not the rate-limiting TS, and more detailed simulations of the PES are needed to quantitatively explain the preference for oxazolone products. Such investigations may then also provide more insight into the prevalence of oxazoline over monoketopiperazine products for the dehydration of XxXSer and XxXThr dipeptides.

In addition to the lower reaction energies for the formation of oxazoline versus oxazolone reported by Boles et al. for protonated AsnThr,\(^{23}\) the relative energies of oxazoline versus oxazolone reported by Boles et al. for protonated AsnThr,\(^{23}\) the relative energies of oxazoline versus oxazolone structures and monoketopiperazine versus diketopiperazine structures appear to be consistently favored toward the structure resulting from water expulsion not involving the C-terminus. This thermodynamically more stable product could further add to the preference of water-loss mechanisms alternative to C-terminal water loss.

**CONCLUSIONS**

The dehydration products of several protonated XxXSer and XxXThr dipeptides have been characterized with the use of...
infrared action spectroscopy and quantum-chemical calculations, giving insight into the reaction mechanisms at play under low-energy CID conditions. The experiments indicate that an oxazoline product ion is formed for all [XxxSer + H]+ and [XxxThr + H]+ precursor ions investigated (Xxx = Gly, Ala, diketopiperazine product ions).49

The potential energy surface for the dehydrogenation of [GlySer + H]+ was computationally investigated. Several pathways leading to the oxazoline product ion were probed at the MP2(full)/6-311+G(2d,2p)//B3LYP/6-31++G(d,p) level. A reaction mechanism similar to that reported for protonated AsnAla, where H2O expulsion from the C-terminus forms isomeric oxazolone and diketopiperazine product ions.49

The authors declare no competing financial interest.

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