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COMMENT

Comment on:
"Quantum Chemical Mass Spectrometry: Verification and Extension of the Mobile Proton Model for Histidine" by Julie Cautereels and Frank Blockhuys, *J. Am. Soc. Mass Spectrom*. 28, 1227-1235 (2017)

In a recent article [1], a newly developed computational method is applied to a series of histidine containing tripeptides (sequences XHS, where X varies). Amongst a number of bold statements, the authors claim to provide both the first quantum mechanical verification of the mobile proton model for histidine and to extend the model in general based on these findings. A single proton mobilization mechanism is stated to be responsible for the purely theoretical fragmentation behavior that produces a lactam b_2 ion and complement neutral S residue. In my opinion there is insufficient evidence to justify these claims.

Product Ion Structure(s)

The first questions I ask any student drawing putative fragmentation products are: (1) How do you know the products have the structures you claim? (2) Which *other structures* could contain this elemental composition? (3) Can you think of *other pathways* that would theoretically enable generation of each structure? (4) How would you test each of these putative assignments or pathways? Typically as mass spectrometrists, we do not know, but infer (or speculate on) structures based on prior experience and chemical knowledge. If we wish to have greater confidence in our suppositions, we need to investigate further. Additional isotopic labeling, gas-phase H/D exchange, ion-mobility or infra-red action spectroscopy, or computational data are necessary to systematically test our structural hypotheses. We can only disprove these structural hypotheses. To communicate the acquired evidence effectively, we also need to think about it from the standpoint of the typical reader of the journal to whom our results are submitted (*J. Am. Soc. Mass Spectrom.* in the present case). The arguments presented need to be robustly supported with experimental and/or computational evidence.

Based on the evidence presented in this manuscript [1], the authors do not know the structure(s) of the ions or neutrals formed in any of the reactions they study. Nor do they know if these ions are even formed experimentally from these protonated peptides. With this in mind, it would seem sensible to cast as wide a net of structural possibilities as possible, to avoid missing potentially relevant structural data points. The authors are to be commended for looking at 10 protonated peptides (sequences XHS, where X varies), rather than just one. However, a single proton mobilization mechanism is stated to be responsible for the theoretical fragmentation behavior that produces a **lactam b_2 ion** and complement neutral S residue. The authors do not mention the possibility of the b_2 ion having an oxazolone [2] or diketopiperazine [3] structure, or a more complex mixture [4–9]. The potential for isomerization between the oxazolone and lactam forms [6] is not investigated either. Prior energy-resolved MS^n experiments [6] performed on histidine-containing protonated peptides provided evidence that leaving group identity directly influences the structure of the b_2 ion structure(s) formed. Spectroscopic and statistical data provide evidence that this phenomenon is general [8, 10, 11]. In summary, the overwhelming majority of experimental [4, 7, 9] and theoretical data [4–7] on histidine-containing b_2 ions generated from protonated peptides discredit the lactam structure and instead support the presence of oxazolone or diketopiperazine structures, or mixtures of these two isomers.

Proton Mobilization Mechanisms in Histidine-Containing Peptides

Energetically feasible mechanisms are necessary to generate specific product structures. In keeping with the mobile proton hypothesis, the present article discredits some charge-remote mechanisms. This has also been previously addressed by other authors, though often not discussed in detail, i.e., publications are inherently biased towards what would be considered the most competitive fragmentation pathways (usually based on evidence) at the expense of those that are less so. Aside from some egregious errors in the Figures (see Section 3), this is an excellent test to perform. However, in the introduction the authors discuss the early computational data from the Wysocki group [12]. These data indicate that **amide oxygen protonation strengthens the amide bond** by increasing the double bond character**,** whereas **amide nitrogen protonation weakens the amide bond** by removal of the partial double bond character. Higher level, density functional and ab initio data from the Paizs, Siu, Hopkinson, Irikura, and other groups provided further evidence for this phenomenon, including in peptides with limited proton mobility ([13], reference 19 of the original manuscript, cited as an example of "(statistical) analysis of mass spectra" for reasons unknown). Additionally, the proposed mechanism, as illustrated, involves a 4-center proton transfer; a transition structure that effectively forms a square. Typically 5- or 6-membered proton transfers are substantially less energetically demanding. Consequently, 4-center proton transfers are often the rate-determining step in peptide fragmentation mechanisms in which they are invoked (recent mass spectrometry examples: [14–18]).

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The preceding evidence and associated argument certainly do not guarantee that the sole mechanism advocated in this manuscript [19, 20] is incorrect either here or elsewhere. Perhaps the proton transfer is catalyzed by the adjacent serine alcohol in this particular example. This certainly would have been an interesting finding. The authors do not state that this is the case, nor do they provide any means of checking this. No transition structures are presented either pictorially in the manuscript, nor are any XYZ coordinates provided in the supporting information. However, extensive prior literature indicates that amide nitrogen protonation should at the very least be considered, i.e., those structures previously shown/hypothesized to lead to the oxazolone, diketopiperazine, and also the lactam structure [4–7, 9, 21].

No discussion of the separated product ion and neutrals relative energies is provided. Proton-bound dimer separation is the final step in the vast majority of protonated peptide fragmentation mechanisms (Figure 1 for a generic example). It is assumed that all reactions examined are transition structure-limited: "Considering that the three steps in the MPM are kinetically controlled, we focus on activation energies," i.e., the product energies are assumed to be lower than at least one transition structure (TS). Prior data on histidine-containing peptides indicates substantial differences in product favorability as a function of structure formed [6]. If the reaction is product-limited, interpretation of experimental data is more difficult and is strongly influenced by experimental conditions (degree of activation, type of activation, experimental timescale). Extremely detailed protonated peptide examples were discussed in a recent *J. Am. Soc. Mass Spectrom.* article [22].

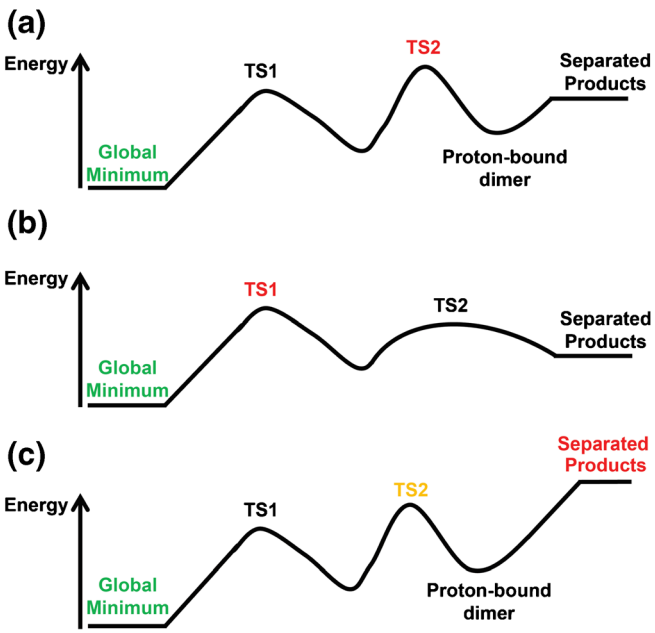

Figure 1. Multistep endothermic reaction energy plots. **Top**: the second transition structure (**TS2**, highlighted in red) is the rate-determining barrier because it requires far more energy to overcome than **TS1** and is less energetically favorable than the separated products. **Middle**: the first transition structure (**TS1**, highlighted in red) is the rate-determining barrier. Here the comparatively facilely overcome **TS2** is followed by direct product separation (i.e., requires no intermediate proton-bound complex). **Bottom**: the rate-determining barrier is separation of the proton-bound dimer to products following **TS2**. That process requires far more energy than either of the preceding transition structures. This situation is typical for species that form strong, efficient intermolecular bonds between the two pieces of the fragmented ion

On Reviewing and Writing Papers on a Particular Chemical Class

If you are either an author or a reviewer of a manuscript on tripeptides, it is incumbent on the authors to ensure that the Figures and Schemes actually show tripeptides. Polypeptides contain multiple amide bonds. Figure 1 [1] lacks an amide NH in the first two structures. Worse still, Figure 4 contains multiple reactions, which are neither size consistent with the theoretical calculations, nor with each other. Thus the product of ΔE_{frag3} is apparently either doubly charged, or a radical cation with *m/z* 1.0078 u greater than the precursor ion. Everyone makes typographical errors (including me), but when a paper is littered with them it's hard to escape the impression that neither the authors nor reviewers took sufficient care.

Article Slant and Conclusions

The article goes to substantial lengths to justify its existence early on:

Page 2: "Despite the efforts described above, the mechanism of the MPM [mobile proton model] has not yet been evaluated as a whole and in sufficient detail using quantum chemical calculations, although, considering its importance, it deserves such a treatment."

Page 2: "Considering the importance of obtaining more detailed insight into the fragmentation mechanisms of peptides in order to improve the above mentioned computational tools for protein identification, we have performed a detailed analysis of the *full* mechanism of the MPM for histidine."

Clearly a better understanding of how and why a given protonated peptide fragments to produce diagnostic product ions is one route to an improved peptide sequence identification. I don't think anyone disputes this. The basic idea though is far from new. For example, the Paizs' Pathways in Competition model [23] of peptide fragmentation advocated this approach in 2005. Unlike the present work, that proposal includes the product energies and proton-bound dimer gas-phase ion chemistry too.

In the section entitled "Extension of the Proposed Mechanism", the authors claim:

"Now that the MPM [mobile proton model] for histidine as presented in the literature has been confirmed computationally, it seems that there is room for extension of the model."

The authors' calculations appear to have shown that this mechanism is *a possible means* of b_2 ion formation. What they have failed to determine is if it is a remotely important mechanism in the fragmentation of these 10 protonated peptides.

Additionally the authors argue: “Furthermore, the results of the calculations suggest that when interactions occur between the mobile proton in the imidazole moiety of histidine and the oxygen atoms of the carbonyl groups of the other amino acids in the backbone, the mechanism should be extended to include larger-ring intermediates.”

This argument for larger ring system is even less likely to be correct as according to the authors’ own calculations the pertinent transition structures require substantially more energy to access. As previously, there’s no spectroscopic evidence to support the existence of these product ion structures either.

In conclusion, the authors claim that: “...the formation of the cyclic intermediates is an energy-favorable process, and (3) the fragmentations of the cyclic intermediates via rearrangements are also energy-favorable.” Formation of the cyclic-intermediate requires the initial proton transfer to occur first. Thus formation of the initial cyclic, intermediate (alleged “reactive structure”) requires on average at least 143 ± 25 kJ/mol. It is unclear how this can be described as energetically favorable. The subsequent amide bond cleavage requires on average at least 181 ± 17 kJ/mol to complete. With no calculation of the separated product energies, how is this “energy-favorable”?

I appreciate that when new to a field one is certainly more likely to miss some of the literature. Occasionally, this can even be an advantage. This can only be true, however, if a thorough examination of the potential processes affecting the results is undertaken. In the present article, there is no evidence that this was done.

Synopsis

The article “Quantum Chemical Mass Spectrometry: Verification and Extension of the Mobile Proton Model for Histidine” argues for a particular mechanism of proton mobilization for histidine-containing protonated peptides utilizing a new computational approach. The proposed b_2 product ion lactam structures are neither consistent with, nor tested against, much of the prior literature (spectroscopy, hydrogen deuterium exchange, density functional theory, etc., which currently support oxazolone and diketopiperazine structures or mixtures of the two). The authors entirely ignore the possibility of amide nitrogen protonation [12, 13, 24] being a source of amide bond fragmentation, despite documented evidence for this weakening the amide bond. The authors’ subsequent claims of broader applicability of their findings are thus highly questionable.

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