Internal Fragments Generated from Different Top-Down Mass Spectrometry Fragmentation Methods Extend Protein Sequence Coverage

Muhammad A. Zenaidee,[1] Benqian Wei,[1] Carter Lantz,[1] Hoi Ting Wu,[2] Tyler R. Lambeth,[2] Jolene K. Diedrich,[3] Rachel R. Ogorzalek Loo,[1] Ryan R. Julian,[2] Joseph A. Loo [1,4]

[1] Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, CA 90095

[2] Department of Chemistry, University of California Riverside, Riverside, CA 92521

[3] Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA 92037

[4] Department of Biological Chemistry, University of California Los Angeles, Los Angeles, CA 90095

Corresponding Author

Joseph A. Loo

University of California-Los Angeles, Los Angeles, CA, United States

Email: jloo@chem.ucla.edu

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ABSTRACT

Top-down mass spectrometry (TD-MS) of intact proteins results in fragment ions that can be correlated to the protein primary sequence. Fragments generated can either be terminal fragments that contain the N- or C- terminus, or internal fragments that contain neither termini. Traditionally in TD-MS experiments, the generation of internal fragments have been avoided because of ambiguity in assigning these fragments. Here, we demonstrate that in TD-MS experiments, internal fragments can be formed and assigned in collision-based, electron-based, and photon-based fragmentation methods and are rich with sequence information, allowing for a greater extent of the primary protein sequence to be explained. For the three test proteins cytochrome *c*, myoglobin, and carbonic anhydrase II, the inclusion of internal fragments in the analysis resulted in approximately 15-20% more sequence coverage, with no less than 85% sequence coverage obtained. By combining terminal fragment and internal fragment assignments, on average the sequence information obtained can be between 1-2 amino acids, i.e., a cleavage site is observed between 2 amino acids, which results in near complete protein sequence coverage. Hence, by including both terminal and internal fragment assignments in TD-MS analysis, deep protein sequence analysis, allowing for the localization of modification sites more reliably, can be possible.

INTRODUCTION

Top-down mass spectrometry (TD-MS) has become a prominent technique for the analysis of intact proteins and their proteoforms.[1-2] In TD-MS, an intact protein is transferred into the gas-phase to form a multiply charged protein ion distribution.[3-4] These multiply charged proteins can then be subsequently activated in order to generate fragment ions.[5] Fragment ions result in singly and multiply charged mass spectral signals that can be assigned to piece together the amino acid sequence of the protein. For these types of experiments, the ability to generate protein ions in higher charge states has been shown to enhance the fragmentation efficiency of intact protein ions.[6-9] TD-MS has recently been shown to be effective for the analysis of intact proteoforms and large proteins,[10-20] with recent studies utilizing TD-MS for the analysis of intact monoclonal antibodies.[21-22] However, in TD-MS, the efficiency for fragmentation of the intact protein and accurate assignment of fragment ions to the primary protein sequence is a key limiting factor in the efficiency and precision of TD-MS.

In TD-MS, fragmentation along the protein backbone can generate terminal fragment ions, where only one cleavage event occurs to generate N-terminal-containing *a, b, c* fragments or C-terminal-containing *x*, *y, z* fragments. Traditionally, collision-based fragmentation methods such as collisionally activated dissociation (CAD)[23-24] or surface induced dissociation (SID)[25] have been utilized for the fragmentation of small proteins and peptides. These methods can be efficient at generating protein fragments, generally cleaving the peptide-amide bond to give *b* and *y* fragment ions. Electron-based methods, where an electron is utilized to generate fragments, is also a commonly utilized fragmentation method. In electron-based methods such as electron capture dissociation (ECD),[9, 26-27] electron ionization dissociation (EID),[13, 28-29] and electron transfer dissociation (ETD),[30] the amino-alkyl bond can be broken, which results in the formation of *c* and

z fragment ions. These electron-based fragmentation methods are useful, as these methods preferentially cleave the backbone of the protein, hence conserving labile modifications. Despite the advantages that are conferred by electron-based methods, these methods can be inefficient due to other interactions between residues of the protein, which limits the extent of fragmentation. Recently, the use of ultraviolet photodissociation (UVPD) has shown to be an efficient fragmentation method for large proteins and protein complexes.[31-32] In UVPD fragmentation, the alkyl-carbonyl bond is generally broken resulting in *a* and *x* fragment ions. Although terminal fragments have been well characterized for each of these dissociation methods, there are still many mass spectral signals that can go unassigned, limiting the information obtained from the experiment.

Protein TD-MS product ions arising from multiple cleavage events can result in the formation of an internal fragment ion, generating *ax, ay, az, bx, by, bz, cx, cy, cz* fragment ions depending on the type of cleavage that occurred.[29, 33-36] As the number of theoretical internal products that can be generated is significantly greater than the number of theoretical terminal fragments that can be generated, the probability of matching a mass spectral signal to an internal fragment is much larger than the probability of matching it to a terminal fragment. Because of the large number of possibilities, internal fragment assignment can be difficult and has largely been ignored. The ambiguity of assigning internal fragments likely scales significantly as the size of the protein increases, thus increasing the false discovery rate. Agar and coworkers classified the ambiguity of assigning internal fragments as three categories: arrangement ambiguity, frameshift ambiguity, and mass accuracy ambiguity.[37] A more complete understanding of the mechanism of the formation of internal fragments would help to increase the confidence for assigning internal fragments. Although there are caveats to assigning internal fragment assignments currently, the

utility and advantage of including internal fragments to improve sequence coverage should not be ignored.

Recently, our lab demonstrated the use of Clips-MS, a computer algorithm that can be utilized in order to assign internal fragments generated from TD-MS experiments.[35] Here, we demonstrated that the inclusion of internal fragments can significantly enhance the information obtained from a single mass spectrum. In addition, they demonstrate that the inclusion of internal fragments can be beneficial for identifying sites of modifications on the protein backbone. The computer algorithm presented demonstrates a method for precisely and efficiently assigning internal fragment mass spectral signals. This can be beneficial for extracting extensive protein information from a single mass spectrum.

Here, we utilize Clips-MS for the analysis of intact cytochrome *c* (12 kDa), myoglobin (17 kDa), and carbonic anhydrase II (29 kDa) fragmented by CAD, ECD, EID, and UVPD at varying precursor charge states. Interestingly, for these proteins, an increase in the isolated precursor charge results in more assigned fragments in the spectrum. For all cases, internal fragments could be generated, and the inclusion of internal fragments results in greater protein sequence information that can be extracted. On average, the extent of protein information is enhanced by ~ 20-30%. Surprisingly, for the proteins we have studied to date, internal fragments only account for < 20% of the total ion current (*vide infra*); however, if they are included in TD-MS analysis, they can provide rich information on the sequence of the protein.

EXPERIMENTAL

Materials. Cytochrome *c* and apo-myoglobin from equine, carbonic anhydrase II from bovine, yeast enolase, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. LC/MS grade water and methanol were obtained from Fisher Chemical (Hampton, NH, USA).

Sample preparation. Unless stated otherwise, all solutions were prepared with 20 μM of protein dissolved in water/ methanol/ formic acid (49.5:49.5:1, v/v/v).

Mass spectrometry. ECD, EID, and CAD experiments were conducted on a 15-T Bruker SolariX Fourier transform ion cyclotron resonance (FTICR)-MS instrument equipped with an infinity ICR cell (Bruker Daltonics, Billerica, MA, USA). Protein solutions were loaded into in-house pulled capillaries coated with gold, and electrosprayed by applying a voltage between 0.8 and 1.2 kV on the ESI capillary. Each individual charge state was isolated at a window of 10 *m*/*z*. For CAD fragmentation, the CAD energy was adjusted between 10-30 V until the precursor charge state was ~5% of the mass spectral signal. For ECD fragmentation, the pulse length was set at 0.1s, with a lens voltage at 50 V and bias voltage at 2V. For EID fragmentation, the pulse length was set at 0.1s, with a lens voltage at 50 V and bias voltage at 26V. For each spectrum, 200 scans were obtained.

UVPD experiments were conducted on an LTQ-Orbitrap Velos-Pro (Thermo, San Jose, CA, USA) with a 213 nm laser to achieve UVPD. Protein solutions were loaded into in-house pulled capillaries coated with gold, and electrosprayed by applying a voltage between 0.8 and 1.2 kV on the ESI capillary. Each individual charge state was isolated at a window of 10 *m*/*z*. UVPD energy was until the precursor charge state was ~5% of the mass spectral signal. For each spectrum,

200 scans were obtained. Carbonic anhydrase UVPD data was collected on a Orbitrp Fusion Lumos (Thermo).

Data analysis. Data was saved to an xy file and opened by mMass for peak picking and deconvolution.[38-40] Peaks were picked with a minimum of 5:1 signal:noise ratio. Each isotopic distribution was then deconvoluted with the highest charge being set as the isolated precursor. Deconvoluted mass lists were generated with $[M + H]^+$ masses.

Deconvoluted mass lists were searched with ClipsMS against the protein sequence. The error (ppm) was set to 2 ppm, with the smallest internal fragment size set at 5. No modifications were set and all fragments were searched for all proteins with the biased version being searched that will assign terminal fragments over internal fragments that may match at a given ppm error.

RESULTS AND DISCUSSION

To investigate the formation of terminal and internal fragments from different activation methods, denatured myoglobin was electrosprayed from acidified solutions and fragmented by CAD, ECD, EID, and UVPD (**Figure S1**). For all cases, extensive fragmentation was achieved, with many fragments deconvoluted and assigned to either terminal or internal fragment ions. To investigate the location of internal fragments and relative abundance of internal fragments compared to terminal fragments for myoglobin, the fragment location maps were plotted for all fragmentation methods (**Figure 1**). On average, internal fragments are lower in abundance than terminal fragments with smaller markers than terminal fragments. In general, internal fragments show complementary fragmentation to terminal fragments with much more sequence information being accessed within the interior of the protein compared to the exterior of the protein, which are

in good agreement with previous studies of internal fragments for the elucidation of protein primary sequences and demonstrates that internal fragments can provide complementary information to terminal fragments.[29, 34-35] As such, it would be beneficial to utilize internal fragment analysis on larger proteins as conventional sequence information can be missing from the interior of the protein. The data shown here suggest that internal fragments can account for many of the mass spectral peaks observed in a mass spectrum regardless of fragmentation method and more information of the primary protein amino acid sequence could be extracted from a single experiment if internal fragments are included in the analysis enhancing the efficiency of these types of experiments.

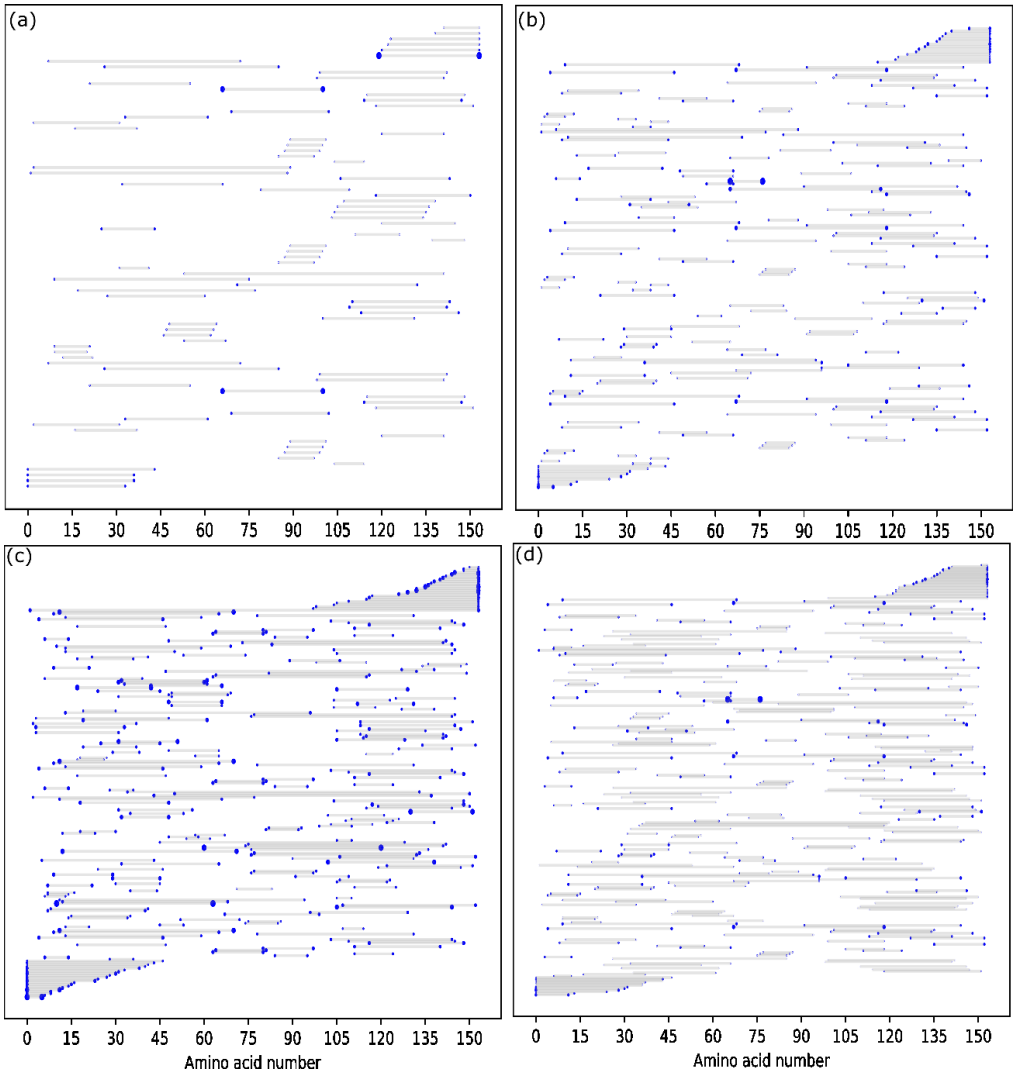


Figure 1. Fragment location map showing regions of the myoglobin protein sequence covered by terminal and internal fragments assigned from [myo, 16H]$^{16+}$ fragmented by (a) collisionally activated dissociation, (b) electron capture dissociation, (c) electron ionization dissociation, and (d) ultraviolet photodissociation. Terminal fragments are denoted by fragments that start or end on the maxima of the x-axis and are clustered on the bottom left and top right of each panel, and internal fragments are located sporadically along the x-axis.

The types of assigned terminal and internal fragments for myoglobin formed by different dissociation methods are plotted (**Figure 2**). For CAD, the fragments that are assigned are *b* and *y* fragment ions and minor *a* and *x* fragment ions, which agree with previously reported observations (**Figure 2a**).[24] The fragment types observed for CAD do not change dramatically with increasing charge state, however more fragments are observed at higher charge states. For ECD, the majority of the fragments are *c* and *z* fragment ions with some minor *b* and *y* fragment ions observed (**Figure 2b**).[9, 26-27, 41] Interestingly for ECD, at higher charge states, more fragments are observed as well as more *b* and *y* fragment ions compared to lower isolated charge states. This observation agrees with previous reports for ECD mechanisms of protein ions, and can be attributed to higher charge states reducing the barriers of dissociation leading to the formation of more *b* and *y* fragment ions at higher charge states.[8-9, 26-27, 41] In contrast for EID, the fragment types observed are predominantly *c, z, a,* and *x* fragments with minor *b* and *y* fragments formed (**Figure 2c**). As EID utilizes higher energy electrons for dissociation, the higher energy deposited into the protein ion can result in the different fragment types forming compared to ECD.[28] However, the mechanism of EID fragmentation is not well understood and requires further work. For EID, an increase in the precursor charge results in more fragments formed and identified but not to the same extent for

ECD. For UVPD, all *a, x, b, y, c,* and *z* fragment types are observed with little difference in the number of fragments formed at higher charge states (**Figure 2d**). This agrees with previously reported observations for protein fragmentation with UVPD. Similar observations are also observed for dissociation of cytochrome *c* and carbonic anhydrase II (**Figure S2 and S3**). The observations here are crucial for understanding the types of internal fragments that can be generated by different dissociation methods in order to enhance the accuracy of internal fragment assignments.

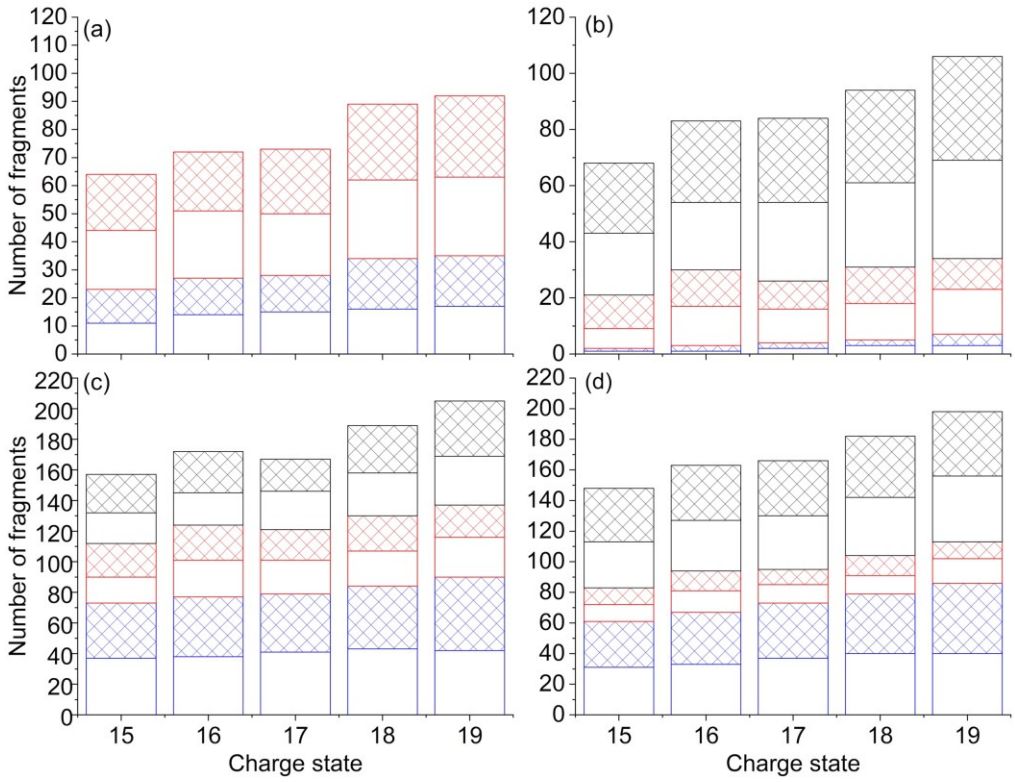


Figure 2. Fragment types assigned for $[Myo, zH]^{z+}$, where z = 15, 16, 17, 18, or 19 fragmented using (a) collisionally activated dissociation, (b) electron capture dissociation, (c) electron ionization dissociation, and (d) ultraviolet photodissociation. The black bars represent *c* and *z* fragment ions, red bars represent *b* and *y* fragment ions, and blue bars represent *a* and *x* fragment ions.

The protein sequence information for cytochrome *c*, myoglobin, and carbonic anhydrase II with and without internal fragments were investigated (**Figure 3**). For CAD, the inclusion of internal fragments enhanced the sequence information from ~30% to ~75% (**Figure 3a**). For ECD, the sequence information that can be obtained for myoglobin increased from ~55% to ~82% when internal fragments are included in the analysis (**Figure 3b**). Although there is an increase in the sequence information obtained when considering terminal fragments only, the enhancement observed when internal fragments are included do not increase as significantly. For EID, similar trends are observed where the inclusion of internal fragments enhanced the sequence information obtained for myoglobin from ~57% to ~ 90% (**Figure 3c**). Similarly, for UVPD, the inclusion of internal fragments enhanced the sequence information from ~ 62% to ~88% (**Figure 3d**). However, for UVPD the enhancement of sequence information at higher charge states is not as significant as by electron-based dissociation methods. On average, the extent of ion signal attributed to internal fragments was less than 20% (**Figure S4**). For CAD, the ion signal assigned to internal fragments was approximately ~ 20% (**Figure S4a**). Similarly, for ECD, EID, and UVPD, the average signal assigned to internal fragments were 13%, 15%, and 18%, respectively. The average internal fragment peak intensities for the different fragmentation methods were approximately half to a magnitude lower than the terminal fragments (**Table S1**). Despite this, internal fragments are rich with sequence information that can be beneficial for TD-MS experiments. The data shown here demonstrates that internal fragment analysis can enhance the sequence information obtained from a mass spectrum despite dissociation methods utilized.

The results suggest that internal fragments are formed in TD-MS experiments with commonly utilized activation/dissociation methods. Although internal fragments are considerably

lower in ion abundance than terminal fragments, internal fragments are rich with sequence information. For the three test proteins cytochrome *c*, myoglobin, and carbonic anhydrase II, the inclusion of internal fragments in the analysis resulted in approximately 15-20% more sequence information, with no less than 85% sequence information obtained. This suggests the utility of internal fragments for deep protein sequence analysis as well as for localizing sites of modifications. High sequence coverage for proteins greater than 30 kDa has been observed using UVPD, ETD, and other methods.[42-43] Our preliminary data for 46 kDa enolase using ECD is promising, showing 40% sequence coverage with inclusion of internal fragments (**Figure S5**). As more mass spectral signals can be assigned, less information is lost, which can be helpful for applying TD-MS on larger proteins.

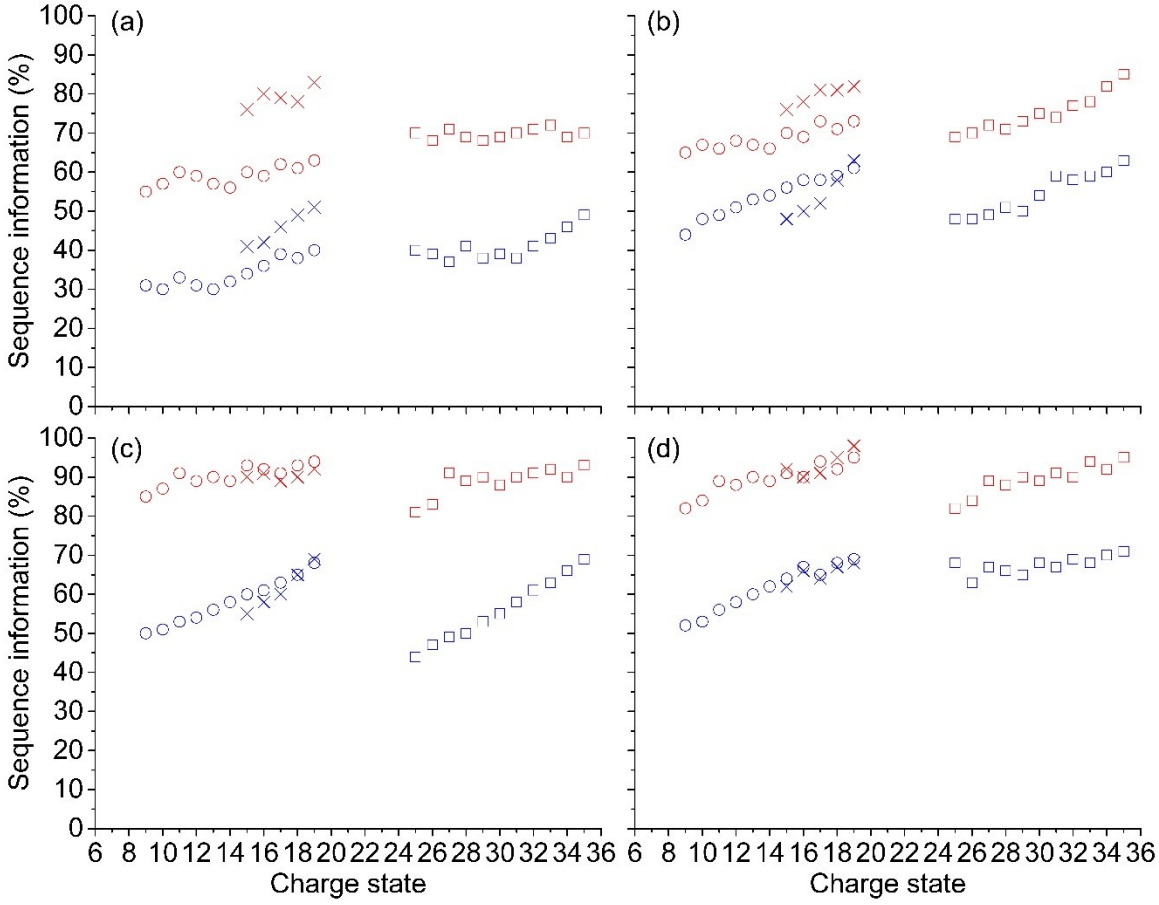


Figure 3. The extent of sequence information obtained for cytochrome *c* (open circles), myoglobin (crosses), and carbonic anhydrase II (open squares), with red symbols indicating the internal fragments included and blue symbols indicating assignments with only terminal fragments using different fragmentation methods, (a) collisionally activated dissociation, (b) electron capture dissociation, (c) electron ionization dissociation, and (d) ultraviolet photodissociation.

ASSOCIATED CONTENT

Supporting Information

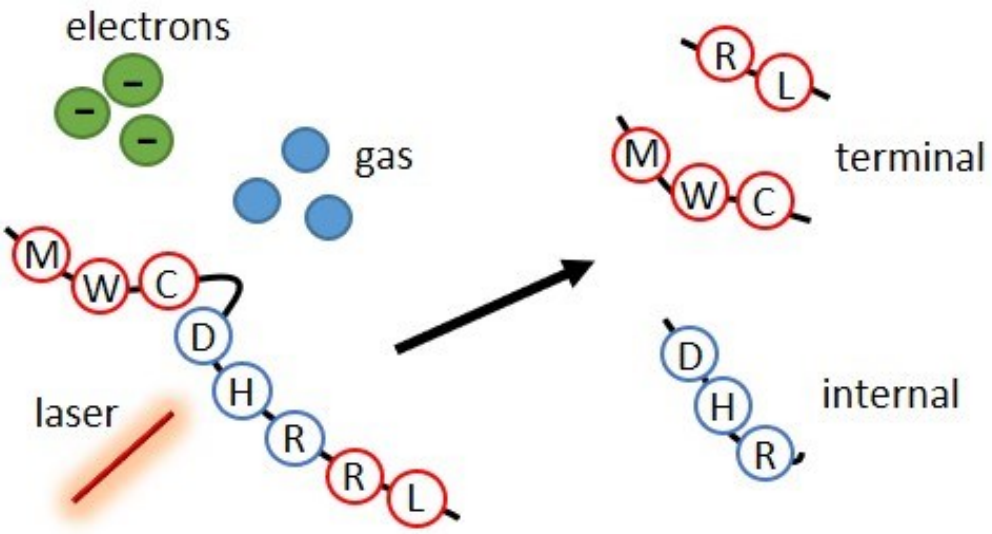
The Supporting Information is available free of charge at (…).

Tandem mass spectra of myoglobin generated by CAD, ECD, EID, and UVPD (Figure S1); fragment ion types assigned for tandem mass spectra of cytochrome c (Figure S2) and carbonic anhydrase II (Figure S3) generated by CAD, ECD, EID, and UVPD and as a function of precursor charge; relative fraction of terminal and internal fragments generated for the proteins by CAD, ECD, EID, and UVPD and as a function of precursor charge (Figure S4); ECD mass spectral data for yeast enolase (Figure S5); average fragment ion intensities for proteins (Table S1)

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