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Deciphering combinatorial post-translational modifications by top-down mass spectrometry

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Abstract

Post-translational modifications (PTMs) create vast structural and functional diversity of proteins, ultimately modulating protein function and degradation, influencing cellular signaling, and regulating transcription. The combinatorial patterns of PTMs increase the heterogeneity of proteins and further mediates their interactions. Advances in mass spectrometry-based proteomics have resulted in identification of thousands of proteins and allowed characterization of numerous types and sites of PTMs. Examination of intact proteins, termed the top-down approach, offers the potential to map protein sequences and localize multiple PTMs on each protein, providing the most comprehensive cataloging of proteoforms. This review describes some of the dividends of using mass spectrometry to analyze intact proteins and showcases innovative strategies that have enhanced the promise of top-down proteomics for exploring the impact of combinatorial PTMs in unsurpassed detail.

Keywords

Proteomics; Top-down; Ion activation; Internal ions; Ion-ion reactions; Proteoform

Introduction

The construction of proteins by the ribosome results in thousands of protein sequences with unique function-dependent structures. Following translation, proteins are modulated by addition of post-translational modifications (PTMs) as well as further shaped by the key processes of folding and assembly into multimeric macromolecules to create functional structures. Many of the PTMs are reversible and dynamic, offering a means to regulate protein activity and subcellular localization while also contributing to dysfunction of pivotal biological processes in diseases [1–3]. The vast array of PTMs, each endowing proteins with different chemical properties, result in an immense diversity in the proteome with different modified proteins known as proteoforms [4–6]. Hundreds of types of PTMs are known, ranging from common ones such as phosphorylation, glycosylation and acetylation, to less common and even rare ones like sumoylation, cholesterolyation, and S-nitrosylation, among

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others [3]. Identification and localization of PTMs is a challenging task, exacerbated by the broad dynamic range of proteins, both in terms of sizes and abundance, in addition to the variations in lability of the PTMs and other chemical properties that influence the ability to detect proteoforms [1,2].

Mass spectrometry has proven to be one of the most versatile and powerful methods for qualitative and quantitative identification of proteins and their interactions with other molecules [7–9]. Numerous advances in sample preparation, fractionation, enrichment, MS/MS methods, quantification strategies, and data acquisition and processing methods have contributed to the ability to profile thousands of proteins and decipher variations in PTMs in a high throughput manner [10–15]. There have also been monumental inroads in functional characterization of proteins based on mass spectrometry approaches [16]. Mapping PTMs is made even more challenging by the fact that individual proteins may harbor a combination of modifications that further regulate their functions and activities as well as interactions with other proteins [17–19]. The combinatorial pattern of modifications affords an extraordinary way to fine-tune protein function and govern downstream signals while at the same time introducing far more heterogeneity into the potential protein repertoire. Understanding the co-dependence of PTMs is an enormous unsolved problem that demands even more advanced analytical strategies for accurately deciphering combinatorial modifications and their functional outcomes and elucidating how PTMs influence structure. This perspective focuses on the newest mass spectrometry-based strategies to advance the characterization of PTMs, particularly emphasizing those methods that have the greatest potential for mapping combinatorial PTMs.

Overview of the proteomics workflow

The major workflows for protein identification by mass spectrometry are categorized as “bottom-up” and “top-down” (with the intermediate “middle-down” strategy often considered a sub-category of bottom-up method) (Figure 1). Conventional “bottom-up” proteomics involves enzymatic digestion of proteins into peptides prior to LC-MS/MS analysis. Bioinformatics software is utilized to stitch together the original proteins based on matching the identified peptides to a proteome-scale database.

Bottom-up proteomics, allowing analysis of thousands of peptides in a single run, has become a routine procedure owing to robust nanoscale separation methods, high performance MS/MS instrumentation, and development of sophisticated bioinformatics tools designed for analysis of LC-MS data [7–9]. Bottom-up proteomics have advanced tremendously owing to improvements in enrichment methods, the widespread availability of high resolution/high accuracy mass spectrometers, and innovative data acquisition methods that enable more peptide identifications. These bottom-up methods excel for quantitative applications as well as for achieving unprecedented levels of protein identifications. Localizing specific PTMs has also advanced considerably owing to development of innovative fractionation and enrichment methods. However, the ability to map multiple PTMs, mutations, truncations, and polymorphisms of individual proteins is impeded by the analysis of peptides which cover only short sequence sections, often leaving gaps and blurring the context of combinatorial modifications.

The alternative workflow, top-down proteomics, offers several distinct advantages as well as notable challenges. The most compelling advantage is the potential to uniquely characterize each protein and its modifications (known as a proteoform) in its entirety [20–26]. A top-down approach uses the intact mass to identify differing proteoforms based on exact molecular compositions and then subsequent fragmentation (MS/MS) to confirm sequences and localize modifications. This method has resulted in identification of thousands of proteoforms from complex biological samples, such as the over 5000 proteoforms reported from human H1299 cells [27] and close to 2000 proteoforms from human fibroblasts with over 400 above 30 kDa [28]. This top down workflow has also been adapted for clinical-type human samples, such as human tissues and human peripheral blood mononuclear cells (from blood draws) to facilitate more translational applications [29–36]. Efforts to further expand the number of proteins and proteoforms identified range exploration of methods to improve solubility of certain classes of proteins [23]; development of more strategic enrichment, fractionation and separation methods to capture low abundance proteoforms and mitigate sample complexity [37–40]; and design of elevated informatics to provide more robust, user-friendly data processing tools [41,42]. Many of these challenges and concepts have been addressed in recent reviews or perspectives [20–26].

One impressive example illustrating the power of the top-down approach is illustrated in Figure 2 for a bis-phosphorylated, N-acetylated cardiac troponin proteoform, cTnI, which is one key protein in the cardiac troponin complex that regulates contraction and relaxation of cardiac muscle [36]. The phosphorylation patterns modulate cardiac contractility and thus are critical harbingers of heart disease. After enrichment and purification, cardiac troponin proteoforms from cardiac tissue were characterized by a combination of two MS/MS methods, electron capture dissociation (ECD) and collisionally activated dissociation (CAD), using Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry to localize the PTMs based systematic analysis of the fragment ion assignments via high accuracy measurements and isotope patterns [36].

Owing to unsolved limitations in instrumentation necessary to routinely analyze larger proteins (typically ones greater than 30–40 kDa) or those with low abundances, top-down methods have yet to surpass bottom-up methods in terms of the breadth and throughput of proteome analysis. However, the top-down strategy allows multiple modifications to be pinpointed on individual proteins if the fragmentation patterns are sufficiently detailed, and this access to mapping combinatorial patterns of modifications is arguably the most powerful attribute that remains inaccessible by any bottom-up proteomics approach. Capitalizing on and magnifying this asset requires the ability to generate the richest fragmentation patterns of proteins and fully dissect those fragmentation patterns to maximize the information content. The following sections will consider the evolution of ion activation methods utilized for characterization of intact proteins as well as auxiliary methods to glean more information from the fragmentation patterns.

Ion activation and MS/MS for intact proteins

An essential keystone in characterizing proteins is the production of extensive series of fragment ions that allow confirmation of sequence and PTM information. The MS/MS

aspect arguably poses one of the most significant challenges for several reasons. A protein must be activated with sufficient energy to generate meaningful fragment ions that map the sequence and bracket modifications. Attaining this goal requires generation of hundreds of fragment ions, and confident assignment requires valid isotopic patterns and high mass accuracy. MS/MS spectra with insufficient assigned fragment ions may allow protein identification but not comprehensive characterization of sequence variations or site-specific localization of PTMs.

Collisional-based methods (collision-induced dissociation (CID) or collisionally activated dissociation (CAD)) remain the mostly popular activation methods in tandem mass spectrometry and mesh well with high throughput workflows [43,44]. CID has been used for analysis of intact proteins, allowing identification of proteins via database searches that match the fragment ions to in silico-generated collections of fragment ions for each protein. The relatively low energy deposition of collisional activation, a feature that is more pronounced for larger proteins, the dependence on mobile protons to facilitate the mechanisms of fragmentation, and the notable preferential backbone cleavages that occur adjacent to specific amino acids (such as Pro, Asp, Glu) often results in limited sequence coverage [45–47]. These factors have contributed to the development of other activation methods, ones that enable more extensive fragmentation of proteins.

Electron-based activation methods (electron capture dissociation (ECD) and electron transfer dissociation (ETD)) are currently the most widely used for top-down analysis [48–55]. In general, these methods have yielded higher levels of sequence coverage than collisional activation methods for intact proteins, with the significant benefit of preserving labile PTMs which facilitates site localization. The scope of ECD, originally restricted to FTICR systems, has been extended to other platforms owing to the development of a modular electromagnetostatic cell that enables ECD on both Orbitrap and TOF platforms [56–59].

Ultraviolet photodissociation (UVPD) has offered another alternative to collisional activation for analysis of intact proteins [60,61]. The peptide bond in proteins exhibits strong absorption around 190 nm, making it a chromophore well-suited for photoactivation using 193 nm [62–66] or 213 nm [67–69] photons (two wavelengths that have been utilized for UVPD of proteins) and resulting in high energy deposition (6.3 eV or 5.9 eV per photon, respectively). Because UV photo-absorption results in excitation of electrons to excited electronic states, the fragmentation pathways observed for proteins are rather unique in that the most prominent fragment ions are typically *a/x* ions originating from cleavage of the C – C_α backbone bond, in contrast to the cleavage of C-N amide bonds that are dominant for collisional activation (*b/y* ions) or N – C_α bonds for electron activation (*c/z* ions). Proteins may dissociate directly from the excited states or after internal conversion to the ground electronic state along with intra-molecular vibrational energy re-distribution, leading to the diverse array of product ions that are the hallmark of UVPD [60]. An equally important facet of UVPD is the preservation of labile PTMs, facilitating their localization (akin to electron activation methods and in contrast to collisional activation).

The widespread availability of high performance mass spectrometers has allow successful adoption of the top-down approach for solving a wide range of biological problems. A few

specific representative (but by no means comprehensive) examples are briefly summarized here to illustrate the growing impact of proteoform analysis by top-down MS/MS methods [70–84]. CID and ETD were used to facilitate differentiation of hemoglobin variants in blood, pinpointing single amino acid differences and providing diagnostic markers for thalassemia screening [72]. ECD and CID were used to identify variations in profiles of sarcomeric proteins extracted from septal myectomy tissues from patients with hypertrophic cardiomyopathy, revealing alterations in splicing and phosphorylation patterns among FHL2, ALP-H, elfin, cypher-5, cypher-6, and calsarcin-1 [75]. Histone proteoforms (H2A, H2B, H3, H4) extracted from CD8 T cells obtained after in vivo influenza infection of mice were characterized in detail using ETD and CID, unveiling numerous increases in PTMs linked to transcriptional activation and correlating with the stage of T cell differentiation [76]. The heterogeneity of O-glycan proteoforms of the spike protein receptor-binding domain of severe acute respiratory syndrome coronavirus 2 (SAR-CoV-2) was determined using ECD and CID, identifying eight O-glycoforms as well as a new 2-fucosylated glycan [81]. A large scale top-down study reported 30,000 proteoforms expressed from 1690 human genes spanning 21 cell types from human blood and bone marrow, representing an impressive compilation of a blood proteoform atlas [83].

Although these examples have highlighted the exciting attributes of the top-down workflow for protein characterization, particularly in the context of elucidating proteoforms, the compelling fruits of top-down methods for complete characterization of combinatorial PTMs have not been fully harvested. As observed for all activation methods for characterization of intact proteins, performance (primarily in terms of sequence coverage) falters for the mid-section of proteins and tends to degrade with the size of the protein. A number of factors contribute to the deterioration in performance, but the root cause largely originates with the decreasing signal-to-noise as the precursor ion decomposes into a great array of fragment ions (sub-division of the finite ion current) and the inability to assign many fragment ions. The latter problem arises from congestion of the MS/MS spectra containing hundreds of fragment ions in multiple charge states, resulting in overlap in the isotope patterns that are essential for confirming fragment ion identities. In addition, assignment of fragment ions is typically achieved by matching accurate *m/z* values to in silico product ions referenced to the N-terminus or C-terminus of each protein of interest. Those fragment ions that don’t retain the N- or C-terminus, classified as “internal ions”, are generally discarded. Owing to these reasons, typical MS/MS spectra of proteins may reveal hundreds of assignable fragment ions, in addition to an even greater array of non-assigned ions. The loss of information content is particularly critical for advancing the ability to characterize combinatorial patterns of PTMs which rely on comprehensive fragmentation to localize sites. Four inroads are briefly described to underscore some of the ongoing efforts to expand the opportunities of top-down methods for solving the combinatorial PTM puzzle.

Hybrid MS/MS methods

Notable gains in performance are obtained by combining ion activation methods, either in tandem or by integrating different MS/MS strategies [85–91]. For example, electron, collisional, and photoactivation methods can be combined in several formats, performed simultaneously to create a broader range of fragment ions or in series to maximize

conversion of non-dissociated precursors into informative fragment ions. Combining different activation methods may also be used to decrease the density of fragment ions by dispersing them among a greater range of charge states (encompassing a broader *m/z* range). For example, ECD has been combined with UVPD (ECuvPD) or collisional activation (EChcD) in order to increase the sequence coverage of subunits of monoclonal antibodies (mAbs) as well as intact mAbs, including mapping the key complementarity determining regions [91]. 213 nm UVPD has been integrated with ETD and EThcD to expand the coverage of monoclonal IgG1, illustrating the reciprocity of orthogonal activation methods [88]. In another study, 266 nm UVPD was used to preferentially cleave disulfide bonds in combination with ECD for characterization of the resulting proteins [86]. These types of hybrid MS/MS methods are generally straight-forward to implement and have motivated other efforts to glean insight from top-down spectra.

Ion-Ion reactions

A hugely promising frontier for extending the performance of top-down methods entails the integration of ion-ion reactions into the top-down workflow [92]. Ion-ion reactions allow manipulation of charge states of ions based on fast and efficient charge reduction processes, primarily via gas-phase proton transfer charge reduction (PTCR) reactions as originally introduced ~25 years ago [93] and since expanded to other types of reactions [92]. For proteins dispersed among a broad range of charge states, PTCR may be used to consolidate them into single charge states and significantly increase the signal-to-noise ratios [93–95]. Alternatively, PTCR may be used to shift fragment ions to lower charge states, thereby decreasing spectral crowding in regions of the MS/MS spectra that are densely congested with ions of similar *m/z* values [97–101]. As illustrated in Figure 3, the fragmentation pattern generated by UVPD of carbonic anhydrase (25+ charge state) shown in the uppermost trace is dense and packed with fragment ions, many with overlapping isotope patterns that impede determination of molecular compositions and prevent ion assignment [98]. The ability to alleviate overlap in isotopic distributions of ions is critically important for effective MS/MS analysis and identification of proteoforms. By subjecting *m/z*-selected slices of the fragment ion population to reactions with a proton-scavenging reagent, fragment ions are shifted to higher *m/z* regions to alleviate congestion, as shown in the four lower traces in Figure 3. Significant improvements in proteoform analysis have been obtained by incorporating PTCR, either to fractionate very congested regions of MS1 spectra or to disperse fragments over a broader *m/z* landscape in MS/MS analysis in top-down workflows, on high performance Orbitrap or Fourier transform ion cyclotron resonance mass spectrometers [94–101]. The impressive performance gains by strategic manipulation and concentration of charge states in the gas phase offers one of the most compelling opportunities for advancing proteoform analysis.

Internal Ions

As underscored in the examples above, improvements in mass accuracy and resolution, ion activation methods, and search algorithms have elevated the characterization of intact proteins by top-down approaches. However, careful curation of MS/MS spectra reveal that numerous fragment ions are not assigned. Many of these unassigned ions correspond to

internal fragment ions, products that do not contain either the N- or C-termini of proteins [102–109]. Internal ions are often ignored owing to the substantial computational demands to search for them and the corresponding high rate of false positives related to the potential to match the mass of an internal ion to others with the same elemental composition that are not uniquely anchored to the N- or C-terminus of a protein. The recent introduction of the ClipsMS (Comprehensive Localization of Internal Protein Sequences) algorithm facilitates searches and assignment of internal fragments [107]. One graphical example of the striking array of internal fragment ions identified upon electron ionization dissociation of myoglobin (16+ charge state) is shown in Figure 4, indicating numerous fragment ions that span sections of the protein [105]. Since these internal ions are not anchored by the N- or C-terminus, these ions are typically not assigned in most database search methods. As reported in a series of recent studies [103–109], assignment of internal ions offers a means to increase the coverages and localization of PTMs, particularly in the mid-sections of proteins which are frequently less thoroughly mapped by top-down MS/MS strategies.

Dissecting protein complexes

Advances in top-down methods have inspired efforts to use mass spectrometry to characterize protein complexes. This growing field, “native mass spectrometry”, utilizes electrospray ionization to transfer intact protein assemblies into the gas phase in a manner that preserves quaternary structures [26,110–112]. High resolution mass spectra can reveal the stoichiometries of complexes, and subsequent activation can disassemble the complexes in a manner that reveals the arrangement of subunits (Figure 5) [113–118]. For example, the fast, high energy deposition of surface-induced dissociation (SID) causes disassembly of protein complexes without significant unfolding and in a manner that preferentially disrupts the weakest interfaces, thus providing direct insight about subunit connectivity [119,120]. The possibility of analyzing supramolecular complexes, disassembling them into their constituent proteins, and then characterizing each protein in a step-wise top-down scheme offers a compelling opportunity to directly explore the compositions of protein assemblies in a mode that harmonizes proteomics and structural biology. In this scenario, the ability to characterize PTMs of the individual proteins in the complexes has the potential to allow construction of structure/functional relationships at an unprecedented level of detail.

Conclusions

Numerous innovative advances in mass spectrometry technologies have accelerated the understanding of the interplay between protein structure and function. Integral to extending the depth of the structure/function synergy is the ability to exhaustively map combinatorial patterns of post-translational modifications. The development of new strategies aimed at extracting more information from fragmentation patterns of intact proteins have cemented the dividends of the top-down approach, now being applied to increasingly complex biological problems such as ribosomes, proteasomes and viruses [26]. Moreover, transformative breakthroughs in the development of multiplexed individual ion/charge detection mass spectrometry methods offer the potential for unsurpassed gains in sensitivity and harvesting information from complex samples [121–123]. The next step requires integrating the established methods and upcoming breakthroughs to enable high throughput

analysis of all proteins and assemblies in cells, further capitalizing on the emerging field of single-cell proteomics [124,125]. This will require inroads in separation methods compatible with native mass spectrometry and even more sophisticated data analysis for a systems biology approach to understanding structure, function, interactions, and assembly.

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Abbreviations

CAD	collisionally activated dissociation
ECD	electron capture dissociation
ETD	electron transfer dissociation
FT-ICR	Fourier-transform ion cyclotron resonance
HCD	higher energy collisional dissociation
PTM	post-translational modification
UVPD	ultraviolet photodissociation

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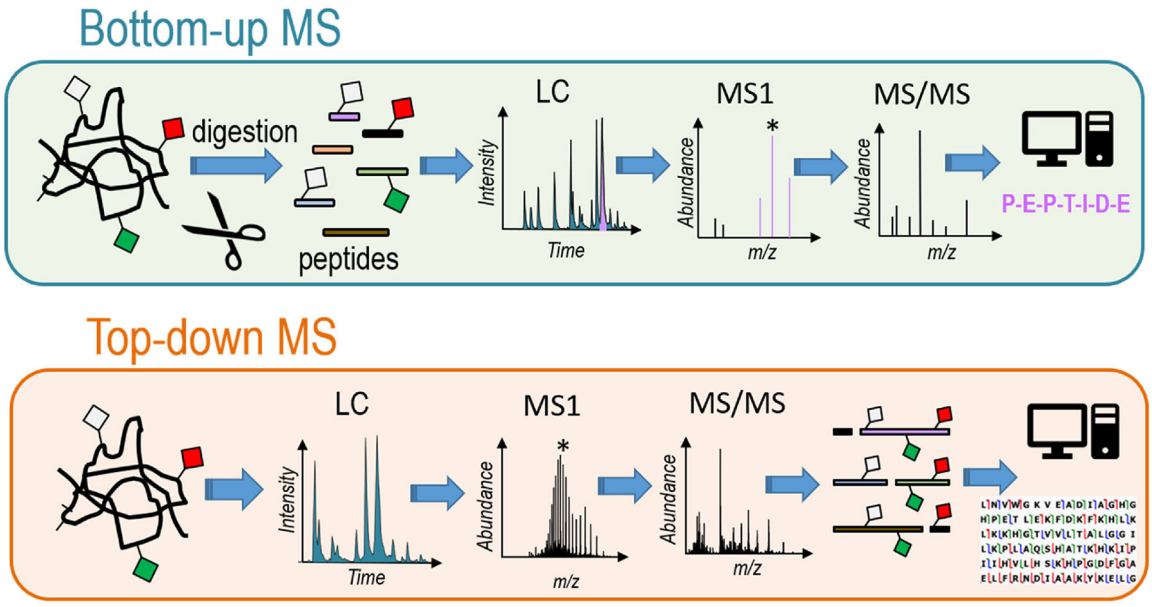

Figure 1.

Schematic representation of bottom-up and top-down strategies. Bottom-up methods include a proteolytic digestion step undertaken on a mixture of proteins of interest, and the resulting mixture of peptides is separated and analyzed by LC-MS/MS in which MS1 and MS/MS spectra are acquired for the eluting peptides, then evaluated using database search methods to identify proteins. Top-down approaches examine intact proteins in which a mixture of proteins is separated and analyzed by LC-MS/MS in which MS1 and MS/MS spectra are acquired for the intact proteins as they elute, thus maintaining the entire context of modifications for each proteoform.

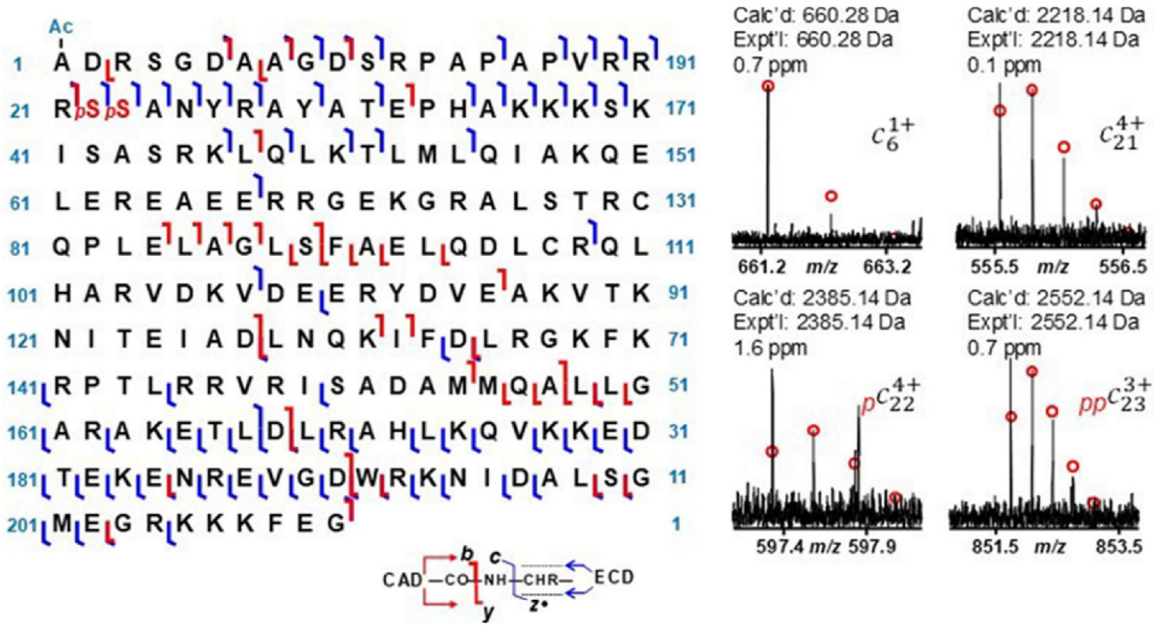

Figure 2.

Sequence map showing backbone cleavages caused by ECD (resulting in *c/z* fragment ions) and CAD (yielding *b/y* fragment ions) of bis-phosphorylated swine cTnI proteoform (with Met excision and N-terminal acetylation) and isotopic profiles of four c ions including c_6 ion with acetylation, c_{21} with no phosphorylation, c_{22} with one phosphorylation, and c_{23} with two phosphorylations which localized phosphorylation sites at Ser22 and Ser 23. The red "p" represents phosphorylation; Ac, acetylation. Reproduced from [36].

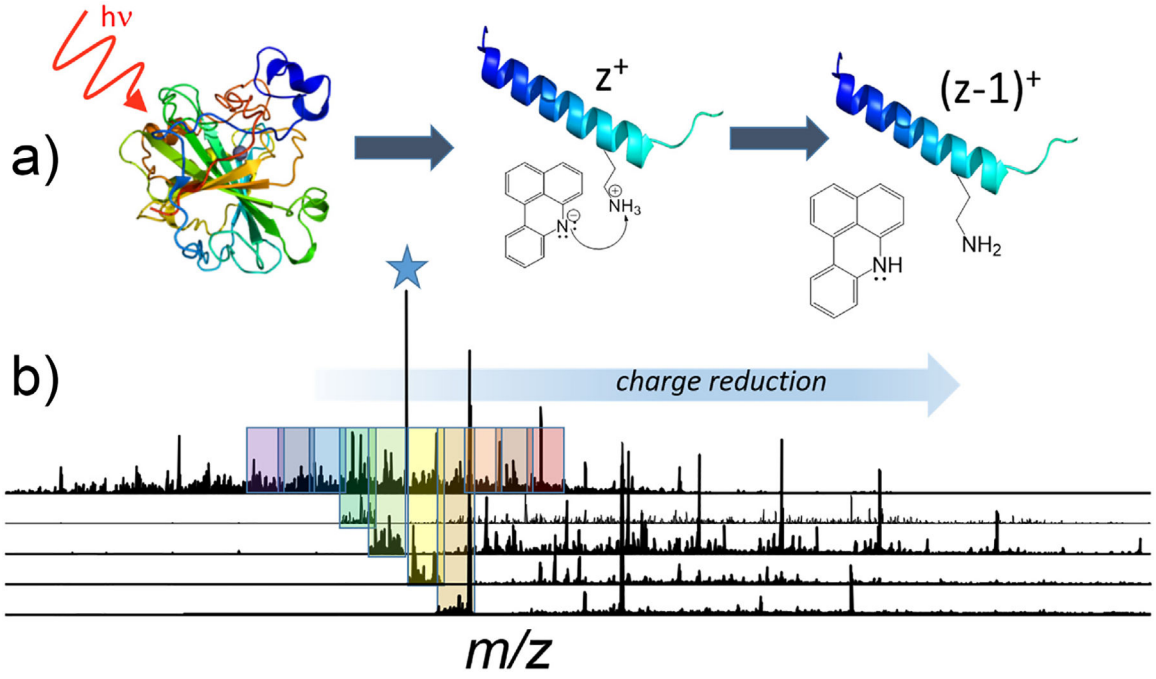

Figure 3.

Proton transfer charge reduction (PTCR) reactions may be undertaken on fragment ions produced from top-down MS/MS characterization of proteins. A) UVPD of a protein generates many fragment ions (F) in a variety of charge states. PTCR using a suitable proton-scavenging reagent ion reduce the charge states of fragment ions (charge state z^+), thus shifting them to lower *m/z* values (i.e., retaining the same mass *m* (aside from loss of a proton) and a lower charge state $(z\text{-}1)^+$) and consequently dispersing the fragment ions over a broader *m/z* range. B) A protein, such as carbonic anhydrase (25+), creates many fragment ions upon UVPD. PTCR reactions undertaken on the groups of fragment ions outlined in the shaded boxes alleviates congestion in the MS/MS spectra and allows confident assignment of a much larger portion of the fragment ions. Adapted from [98].

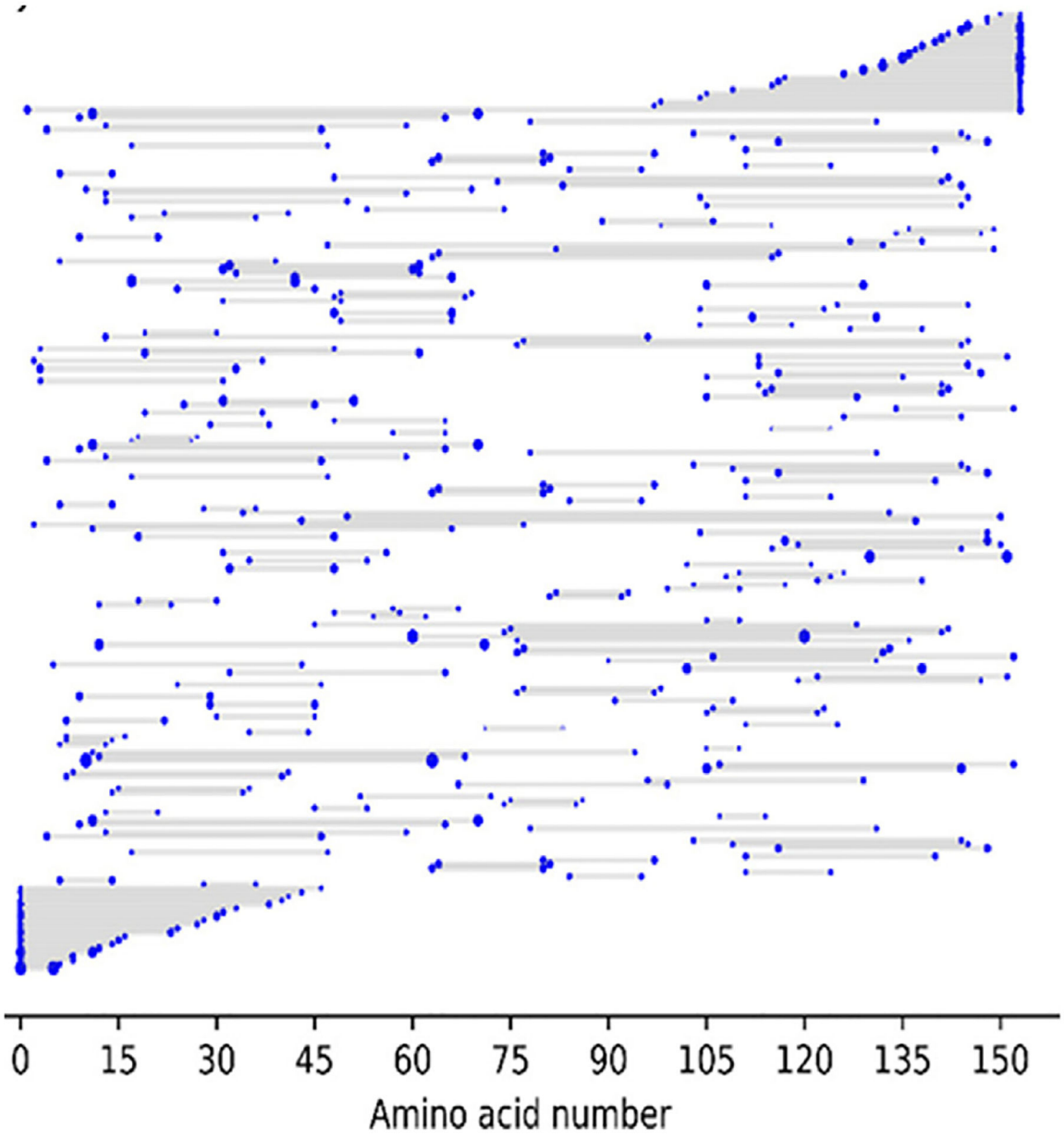

Figure 4.

Electron ionization dissociation of myoglobin (16+ charge state) results in production of a large array of fragment ions originating from cleavages of the protein backbone The fragment location map indicates the region of the protein sequence covered by terminal and internal fragments. The solid gray contoured regions represent those fragment ions that include the N-terminus (lower gray contour) or C- terminus (upper gray contour) of the protein. The numerous horizontal bars in the middle region of the map represent the segments spanned by internal ions, indicating many different sizes of internal ions, none of which contain the N-terminal or C-terminal amino acid. Reproduced from [108].

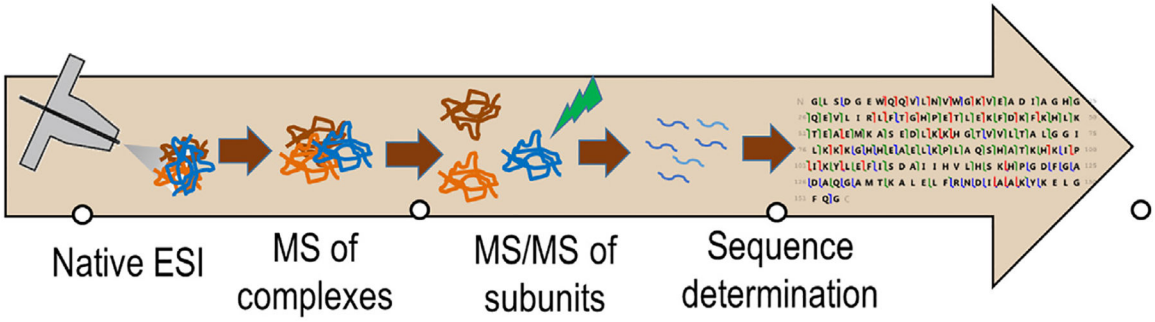

Figure 5.

Intact protein complexes transferred to the gas phase are disassembled into protein subunits and characterized by MS/MS to allow identification of the proteins within the complexes.