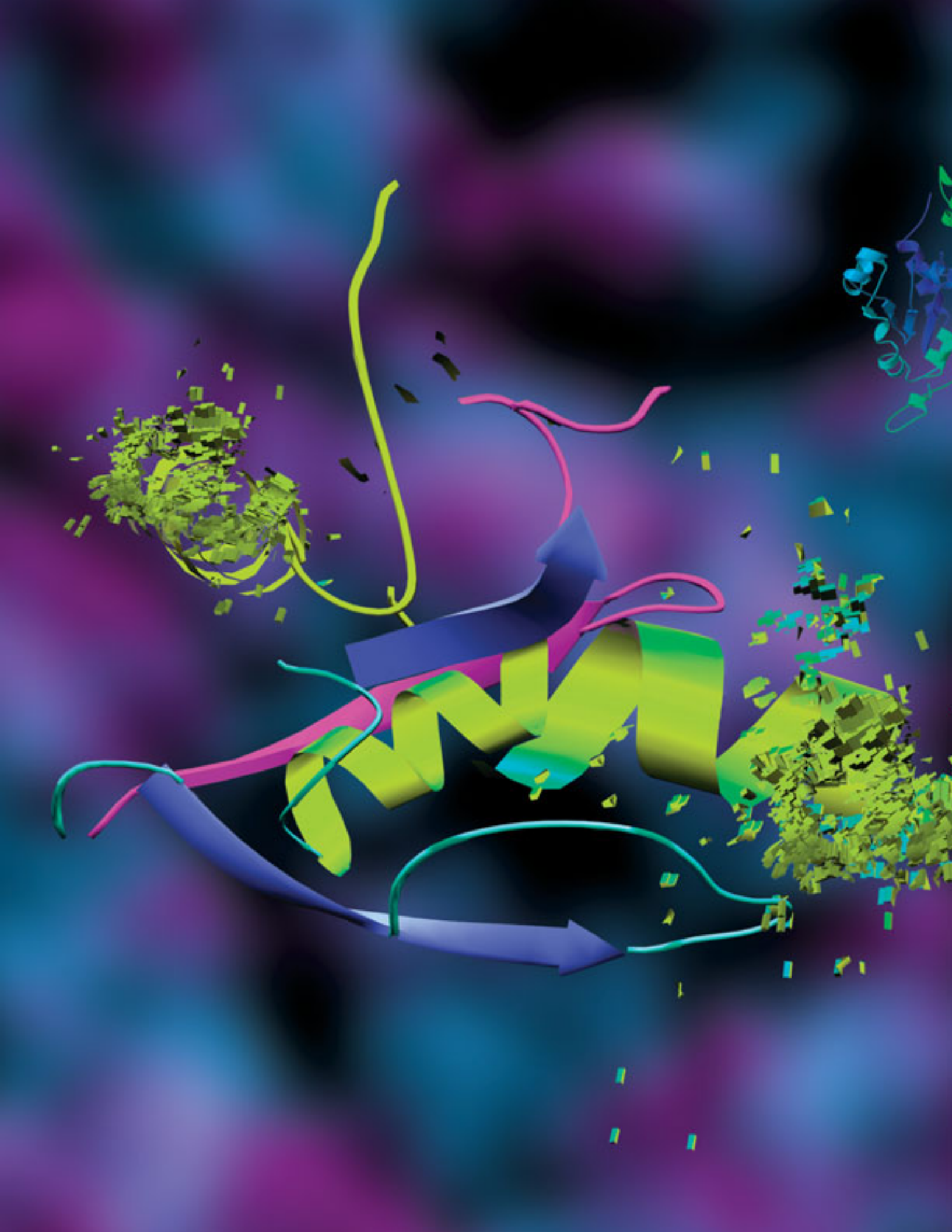

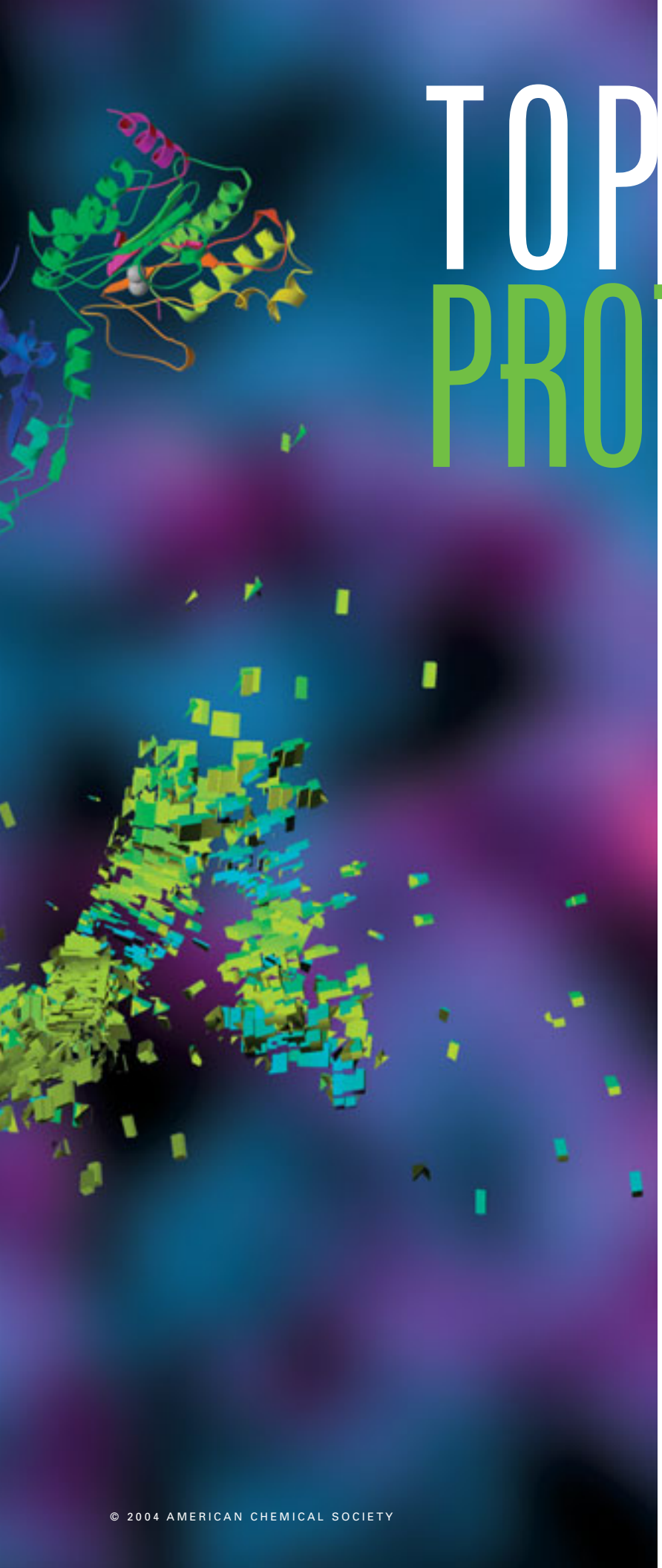

TOP-DOWN PROTEOMICS

A growing number of labs are using many types of mass spectrometers to directly analyze intact proteins and to improve conversion of MS data into biological knowledge.

The timeline for maturation of a new measurement approach is difficult to predict but often accelerates as more laboratories focus on lowering barriers to its progress. Such is the environment of a small but growing number of research teams developing and applying MS for interrogation of intact proteins. This is an extension of tandem MS from small molecules to small peptides (carved from whole proteins by proteolysis), which ushered in the era of proteomics.

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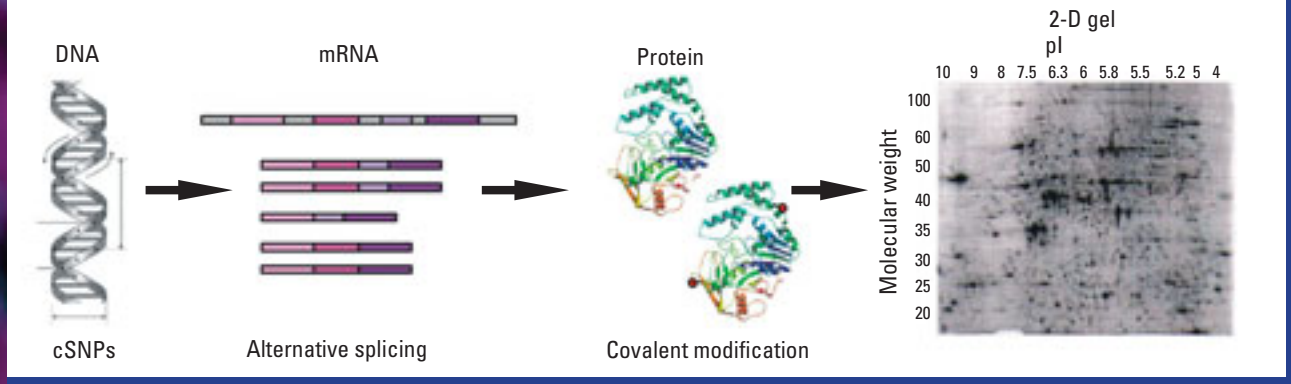


FIGURE 1. From one gene, many protein forms.

(left to right) DNA, RNA, and protein levels are depicted with a low-resolution top-down view of the proteome displayed via (right) a 2-D gel.

The tectonic collision of biology with separation science, MS, and informatics occurred over the past 15 years and was driven by contributions from more than 100 laboratories. Like budding yeast, MS is sprouting emergent approaches for the direct profiling and MS/MS analysis of heterogeneous proteins in ever more complex mixtures. Such approaches promise to determine molecular indicators of complex diseases and deepen our understanding of dynamic regulatory mechanisms in cell biology.

From the top

Before it had a name, proteomics used 2-D gels to fractionate a complex cellular lysate into intact protein spots visualized by staining (Figure 1, right). This "top-down" molecular perspective focused largely on intact protein molecules (albeit at low chemical resolution) expressed by cells and revealed many, though certainly not all, analytical targets for identification. As methods improved and were combined with genome sequencing, a large-scale understanding of protein heterogeneity emerged along with the realization that multiple protein products can come from a single gene (Figure 1). The importance of this theme in higher organisms has only grown as more minds wrap around the implications of the Human Genome Project.

Both the number of proteins modified and the number of modifications per protein increase in multicellular organisms versus typical bacteria or extremophiles. For eukarya such as humans, the main sources of protein heterogeneity are highly similar genes (gene families); coding polymorphisms (different amino acids among individuals in a population); variable processing of messenger RNA; and posttranslational modifications (PTMs), which can involve proteolytic trimming or decoration with any of more than 100 known chemical groups (*1*). It is this very heterogeneity that is so insidiously difficult to measure, yet it is required for changes in protein subcellular location, complexation, degradation, signal transduction, and regulatory control of enzymatic function. These biological events also change the molecular weight of intact proteins.

As impatient proponents of systems biology meet with technological roadblocks, developers are increasingly motivated to improve PTM analysis, including those developers who focus on the top-down approach to protein analysis (Figure 2b). Interest lies in increasing both the efficiency of protein identification (knowing which gene encodes the protein in question) and the characterization of protein primary structures (including PTMs). To improve efficiency and characterization, either specific PTMs are targeted for detection or a high percentage of "sequence coverage" is obtained. Sequence coverage simply means accurately measuring a mass that is or is not consistent with the underlying chemical composition (e.g., a predicted DNA sequence). Sequencing by MS requires cleaving between every backbone position to pro-

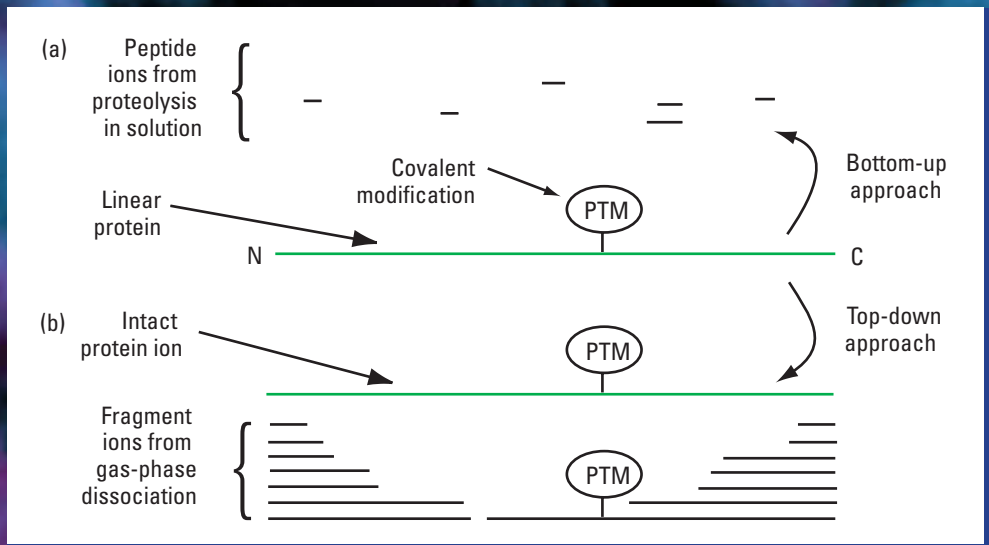


FIGURE 2. (a) Bottom-up and (b) top-down approaches for DNA-predicted protein sequence analysis. The sequence coverage is typically 5–70% for bottom-up and 100% for top-down.

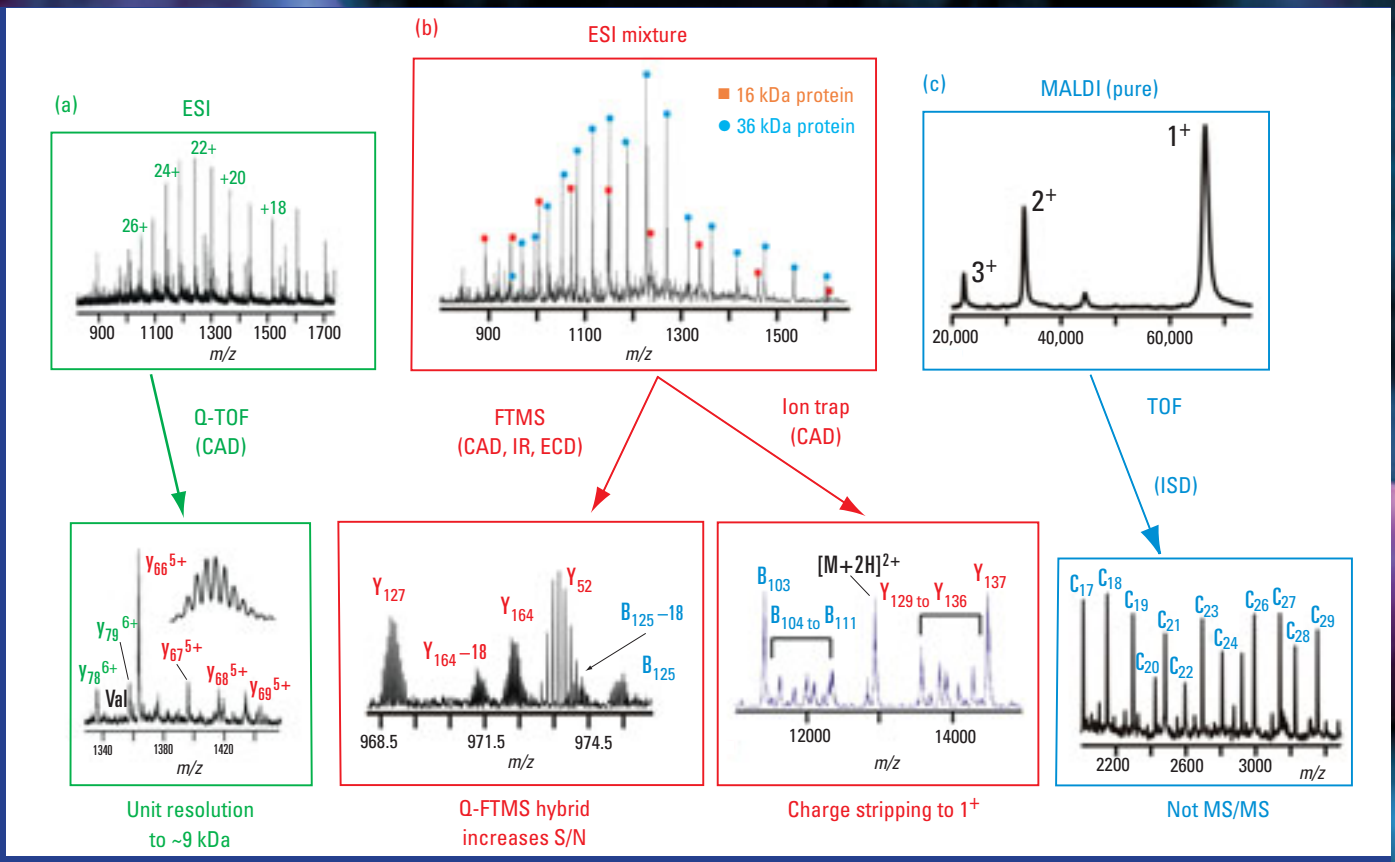


FIGURE 3. Examples of top-down MS with representative types of fragmentation data taken from (a) an ESI/Q-TOF; (b, left) ESI/Q-FTMS and (right) ESI/ion trap; and (c) a MALDI/TOF instrument.

(Adapted from Refs. 12, 15, 23, and 28.)

vide a mass ladder (composed of mass differences between fragment ions) and allows sequence determination de novo.

Defined and combined

The most rigorous definition of a top-down experiment involves high-resolution measurement of an intact molecular weight value M_r and direct fragmentation of protein ions in the gas phase (Figure 2b; *2*). The accuracy of an M_r value can vary on different instruments, but it is most useful if the value is <2 Da in order to better match the accuracy obtained for smaller protein fragments (often in the low parts-per-million range). Better M_r accuracy reduces uncertainties associated with stating that a protein is completely characterized.

The term "bottom-up" refers to the dominant experiment in proteomics today wherein digestion to small peptides occurs without determining an accurate M_r value of the intact protein (Figure 2a). The analysis of <3-kDa peptides produced by tryptic digestion allows high-throughput protein identification but typically prevents interrogation of the complete DNA-predicted sequence retrieved from the database. Efficient surveying of an entire protein sequence with 100% coverage at the intact and fragment ion levels (e.g., a complementary pair) is the hallmark of a top-down experiment. The fragment ions generated from direct MS/MS enable protein identification by database retrieval, quick positioning of the N- and C-termini, confirmation of large sections of DNA-predicted sequence, and partial or exact localization of polymorphisms or modifications. "Bottom-uppers" continue method development to increase sequence coverage, mine data, and selectively purify or detect targeted PTMs (e.g., phosphorylation). "Top-downers" work on the next generation of advances in separations, hardware, and software to solve the general problems of PTM detection, localization, and quantitation.

For years, mass spectrometrists fought to achieve ever softer ionization methods to produce intact ions of small molecules that could be directly characterized by tandem MS, a top-down approach to molecular analysis (*3*). Thus, the notion of degrading a molecule without an M_r value is the newer approach. Indeed, aspects of top-down were described as early as 1987 (*4*). However, proteolysis was used for controlled degradation because MS/MS was not possible at the "high" mass of 7 kDa. Several groups have revived and modernized this experiment as a top-down/bottom-up hybrid and applied it to proteins from bacteria (*5*), yeast (*6*), and even humans (*7*). Hybrid experiments are likely to become quite popular for identification of biomarkers and better characterization of wild-type proteins, recombinant proteins, and large-molecule therapeutics.

Direct fragmentation of intact protein ions

Though most often associated with FTMS, top-down fragmentation was initially demonstrated with electrosprayed ions of ribonuclease A (14 kDa) using a triple-quadrupole instrument (*8*). Shortly thereafter, Feng and Konishi demonstrated the same approach on a 150-kDa antibody also using electrospray ionization (ESI) with a triple-quadrupole instrument (*9*). Initial reports of protein ion analysis with high resolution (i.e., isotopic resolution of fragment ions) came via FTMS/MS (*10*, *11*). In the years

Table 1. Instrumental configurations for interrogation of intact proteins.

Ionization method	Analyzer	Ion fragmentation methods	MS/MS on mixtures?	Approximate resolving power for fragment ions	References
ESI	Triple quad	CAD	Yes	10^3	8
MALDI	TOF	ISD[a]	No	10^2	15, 33, 43
ESI	Q-TOF hybrid	CAD	Yes	10^4	12
ESI	Ion trap	CAD[b]	Yes	10^3; 10^4 possible	13, 14, 23
ESI	FTMS	CAD, IRMPD, ECD	Yes	10^5; 10^6 possible	2, 11, 21, 22, 41
ESI	Q-FTMS hybrid	CAD, IRMPD, ECD	Yes	10^5; 10^6 possible	28

CAD, collisionally activated dissociation; IRMPD, IR multiphoton dissociation; ECD, electron capture dissociation.
[a] MALDI with ISD has been extended very recently by using a TOF/TOF for MS/MS of *c* and *y* ions (*52*).
[b] IRMPD has been demonstrated on peptides in an ion trap.

since, expanded demonstrations of top-down on a variety of MS instruments have been reported using MALDI/TOF, MALDI TOF/TOF, ESI/Q-TOF, ESI/ion trap, and ESI/quadrupole-FTMS (Q-FTMS; Table 1 and Figure 3).

For methods that target the multiply charged ions produced by ESI (e.g., 30+ from a 30-kDa protein), determination of fragment ion charge states is critical for obtaining reliable mass measurement. Working on a quadrupole–TOF hybrid (Figure 3a), Nemeth-Cawley and colleagues directly assigned mass by resolving isotopic peaks for fragment ions up to ~9 kDa (*12*). Since 1995, McLuckey and colleagues have been developing a quadrupole ion trap with fast ion–ion reactions to reduce fragment ion charge states to 1^+. This charge reduction allows easy conversion of m/z to mass without resolving isotopic peaks (Figure 3b, right; *13*, *14*).

In the mid-1990s, MALDI/TOF with in-source decay (ISD; Figure 3c) came into practice when it was noticed that long series of *c*- and *y*-type ions could be produced at high laser powers and sample loads (*15*). Although ESI/FTMS remains the high-resolution option for direct fragmentation of intact protein ions in mixtures, it has not undergone many instrument hardware advancements (except bigger magnets) until recently. The construction of Q-FTMS hybrids by academic laboratories (*16*, *17*) and recently by commercial FTMS vendors has improved the dynamic range of MS/MS for high-mass ions (Figure 3b, left).

Applications

As early as 1994, ESI/FTMS was applied to recombinant protein analysis. A heterogeneous 42-kDa protein was found to have a ragged N-terminus and an alkylated Cys residue in its active site (*18*). Analysis of a cluster of recombinant gene products revealed a set of covalent modifications on enzymes involved in thiamin biosynthesis (*19*). More recently, 3–20 wild-type proteins have been identified and characterized by ESI/FTMS in studies of *Shewanella oneidensis* (a metal-reducing organism; *5*), *Methanococcus jannaschii* (grows best at 85 °C; *20*), *Mycobacterium tuberculosis* (causes TB; *21*), and *Bacillus cereus* (relative of anthrax; *22*). McLuckey and co-workers identified several proteins from *E. coli*, including an overexpressed one directly from a cell lysate, by using their charge stripping approach on an ESI/ion trap instrument (Figure 3b, right; *23*, *24*). Of the mass discrepancies detected and characterized to date, many are incorrectly predicted translational start sites (errors in genome annotation), with the rest dispersed among N-terminal and internal acetylations, signal peptide cleavages, disulfide bonds, phosphorylations, methylations, and simple glycosylations.

The ability of the top-down technique to characterize eukaryotic proteins with more complicated combinations of PTMs is progressing rapidly, especially as improved hardware and software become widely available (*25*). One clear advantage of top-down is that the quantitation of multiple protein forms with combinations of PTMs is straightforward when the mixture is measured intact. Further, correlation of multiple PTMs with one another is best done by direct MS/MS of a multiply modified species (given sufficient S/N). Two assumptions are made in this type of semiquantitative PTM analysis: Prior fractionation steps do not perturb their relative concentrations before analysis, and ionization efficiencies among the various protein forms are similar.

On the front end

Regardless of which instrumental engine is used, top-down proteomics faces the same challenges as those of existing proteomic platforms—front-end sample handling, data acquisition, and computer-aided data reduction.

At the front end, Rose and Opitek sought to combine chromatographic and electrophoretic methods to directly fractionate complex protein mixtures (*26*). Work has continued on coupling sampling methods for intact proteins to MS in both on- and off-line modes. In a process that is analogous to a 2-D gel (from which intact proteins are difficult to retrieve), isoelectric focusing in a column followed by reversed-phase LC (RPLC) provides tractable mixtures for off-line analysis by ESI/TOFMS (*27*). Gel electrophoresis using an acid-labile surfactant has been combined with RPLC to provide intact proteins within a ~5-kDa range for analysis by MALDI/TOF or ESI/Q-FTMS/MS (*28*). Whitelegge and Faull developed HPLC with tailored solvents for sticky membrane proteins (*29*). Most of their work has been performed using HPLC on-line with an ESI/triple-quadrupole instrument.

Although the speed of MS/MS data acquisition is not yet on a chromatographic time scale, CE/MS and LC/MS have been used successfully to detect intact proteins. However, McLafferty

and co-workers demonstrated proof-of-principle for the low-attomole analysis of protein standards with optional fragmentation in the ESI source for standards up to 30 kDa (*30*). Also, Marshall et al. demonstrated capillary HPLC/ESI/FTMS with targeted fragmentation of protein standards up to 30 kDa (*31*). The Smith group used capillary isoelectric focusing to detect hundreds of *E. coli* proteins without fragmentation (*32*). Cohen and co-workers have commercialized a 2-D cation-exchange system with RPLC and integrated it with on-line ESI/TOF for protein detection (*6*). Identification is achieved using off-line bottom-up digestion.

Life above 5 kDa

The core aspect of bottom-up proteomics is data acquisition—10^4 to 10^6 MS/MS spectra can now be collected. Yet many of these spectra are poor because of differences in fragmentation behavior among peptide ions. Such analyte-dependent properties also present difficulties for proteins >5 kDa. Even without the temporal complication of LC, the data acquisition challenge for intact protein ions has not been thoroughly addressed. Hundreds of intact *E. coli* proteins separated on an isoelectric focusing gel have been imaged through the use of MALDI-based analysis (*33*). ISD has been used successfully for manual protein identification (Figure 3c) with detection of fatty acid acylation.

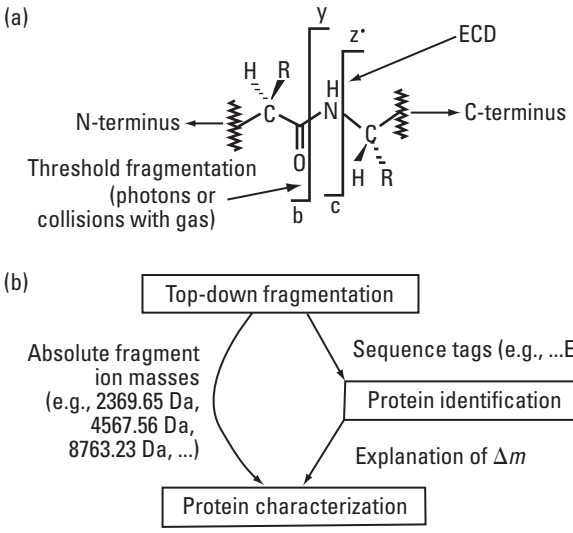


FIGURE 4. Fragmented ions.

(a) Nomenclature for fragment ions observed after dissociation of backbone bonds in a protein. Zig-zag brackets indicate backbone cleavage sites. (b) Two methods for identifying intact proteins from databases. (left) The absolute mass of fragment ions can be used if they match those in a database above statistical levels. If the correctly modified protein form is in the database, it will receive the most matches. (right) The sequence tag approach needs only the relative mass difference between adjacent fragment ions to read a small stretch of sequence (e.g., five amino acids) to identify a protein. Such series of adjacent fragment ions are observed in spectra from high-quality threshold dissociation, or those obtained using ECD or ISD.

For ESI-based work, a better grasp of the effect of the protein charge state on "threshold" dissociation pathways (Figure 4a) has assisted the understanding of the gas-phase chemistry of these multiply charged ions. From the combined work of Williams, McLafferty, and McLuckey, three general classes of protein charge states have emerged (*34–36*). For disulfide-reduced proteins in general, higher charge states fragment more readily, but into fewer dissociation pathways, than intermediate charge states, with ~50% of fragmentations overall occurring adjacent to Pro, Asp, and Glu residues. Intermediate charge states yield a maximum number of fragmentation channels because of a greater degree of proton mobility and/or greater diversity of precursor ion structures (*36*). Low charge states tend to yield more uninformative product ions such as neutral losses of H_2O and NH_3. Such fundamental knowledge is helping to both automate the MS/MS process of data acquisition (*37*) and raise confidence in protein identification by database retrieval (*23*). Within the next decade, instruments capable of unattended MS/MS at >5 kDa will become available to enable efficient analysis of ever higher mass components in increasingly complex mixtures.

Another difference between bottom-up and top-down lies in the response of covalently modified precursor ions dissociated at the threshold using either IR photons or collisions with gas. PTMs such as phosphorylation and glycosylation, which are labile during MS/MS fragmentation at the peptide level, can be stable to MS/MS fragmentation at the protein level. It appears that PTM stability is a function of the precursor ion size (*38*). For example, serine phosphorylation is stable to MS/MS on a 9-kDa phosphoprotein (*20*), but not so for typical phosphopeptides. An N-linked sugar moiety was stable during collisional MS/MS on the 15-kDa ribonuclease B (*39*), but other data at 25 kDa suggest that some glycosylations can be ejected preferentially (*12*).

Regardless of PTM stability when fragmented by standard methods, any PTM that survives the process of ionization can be localized using the gentle MS/MS process of electron capture dissociation (ECD; *40*; Figure 4a). Further, McLafferty's extensive studies of 8.5–29-kDa protein standards have shown that ECD has the remarkable ability to induce cleavages at the majority of backbone sites (*41*). Thus, the use of ECD allows pinpoint localization of even labile PTMs by extensively sequencing the entire protein. Though the sample requirements can be high for such experiments, sequencing the entire protein is simply not possible with other ion fragmentation methods for precursors >5 kDa. Because many aspects of ISD for MALDI-generated ions are similar to ECD (*42*), it is predictable that labile PTMs stable to ECD would also be stable through the ISD process (*43*).

Informatics

The first protein identification using top-down data based on protein standards was published in 1996 (*44*). Recently, the specificity of the top-down approach was tested with 2–5 proteins fragmented at once inside an FTMS system and a statistical model to retrieve probability-based data (*20*, *37*). Such fragmentation in parallel works well for proteins from bacteria but will prove

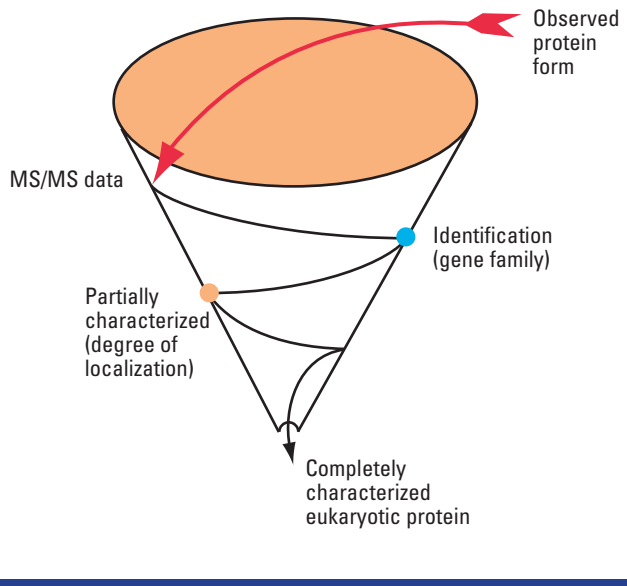


FIGURE 5. Top-down analysis funnel for identifying and characterizing eukaryotic proteins.

difficult to use in a "first pass" analysis of eukaryotic proteomes, in which the top-down process can be conceptualized as a funnel. The complete characterization of a protein purely by top-down fragmentation sometimes requires a multistep approach. Upon identification of a protein from a database, mass discrepancies Δm may be detected. "Follow up" fragmentation using ECD can improve the degree of localization for Δm to the extent desired (Figure 5). Alternatively, exact PTM localization can come from prior analyses, if that information is housed in a database.

With well over 1 million different protein forms expressed in the human body, through all life stages and tissues, the protein complexity that drives much of mammalian biology maps a vast chemical space. Soon, most abundant proteins and their PTMs will be detected and localized in model organisms using a variety of MS technologies. Treating PTMs as a necessary part of database entries for protein sequences is an emerging trend in proteomics (*45*). This practice will make PTM analysis efficient by avoiding the need to detect and localize every PTM every time. Indeed, including PTMs in databases has begun (e.g., by SWISS-PROT in their Human Proteome Initiative), but embedding this information into MS search engines is not yet widespread. Providing this information is especially critical for top-down because the entire protein can be interrogated at once, making it possible to identify and characterize multiple PTMs in parallel (Figure 4b, left arrow; *25*).

A new website called ProSight PTM (https://prosightptm.scs.uiuc.edu) uses known PTM information during a top-down database query (*46*). PTMs predicted from function or distinctive sequence motifs are also included, and the user decides how much of this expanded chemical space to search. In unannotated databases, the success rate for protein identification by top-down is >80%, even using sparse MS/MS data of marginal quality (*20*). However, including many PTMs in a database can actually improve retrieval scores for modified proteins because more absolute mass values of fragment ions match those predicted from annotated database entries. Therefore, appropriately annotated databases and embedded software tools, combined with the information uncovered through both bottom-up and top-down, will make subsequent proteomic analyses more efficient and biologically meaningful, regardless of the measurement approach.

Biomarker identification without digestion

The direct profiling of tissue or quickly prepared proteins has been developed to modernize the classic notion of a biomarker. The hope is to increase the number of "data channels" and molecular information using new technology. To the extent that PTMs or a particular form of a degraded protein (~5–30 kDa) harbor critical information that correlates to disease states, this information is obscured by shotgun digestion of a complex protein mixture, such as serum or urine.

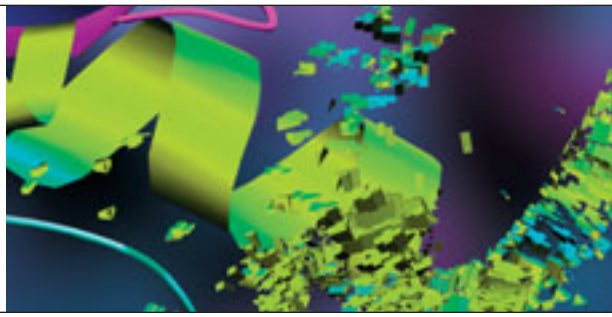
For biomarker discovery and validation, many researchers have advocated intact protein profiling using MALDI/TOF to detect hundreds of protein signals (*47*). Chaurand et al. have established MALDI/TOF for tissue imaging with spatial resolution of ~50 µm (*48*). Though biomarker validation does not need protein identification, eventually understanding marker biogenesis is highly desirable. For such identifications, a limitation of MALDI/ISD studies is that the fragmentation of precursor ions occurs in the MALDI source. Without any ion isolation, it is not true MS/MS. Fragmentation approaches that dissociate ions all together in the ion source (e.g., nozzle-skimmer in ESI and ISD in MALDI) execute a "multiplexed" version of top-down if the sample being interrogated is not pure (*20*). Therefore, other top-down methodologies using electrospray will increasingly be used to identify validated or putative biomarkers. For bacteria, Fenselau's team showed that identification of small *B. cereus* proteins could act as potential markers for infectious spores (e.g., anthrax; *22*). Lubman and co-workers have applied isoelectric focusing and RPLC, ESI/TOF, and off-line tryptic digestion to identify proteins and PTMs from human cancers (*49*). Together, these approaches hold the promise of nearly 100% reliability (high predictive value) for earlier detection of complex diseases or drug efficacy.

Limitations as challenges

Top-down is not as widespread as bottom-up for many reasons, but there are fewer obstacles every year. FTICR instrumentation has had limited availability, and intact proteins present challenges with sensitivity and throughput. In ESI and MALDI, protein standards can be analyzed with ultrahigh sensitivity with fast acquisition of fragmentation data (*30*), whereas mixtures of wild-type proteins are proving harder to handle. However, projects on the scale of analyzing several hundred intact proteins will be feasible shortly (*50*) and will detect moderately abundant proteins <50 kDa.

As many of the basic issues limiting top-down are resolved, the more intrinsic challenges such as protein size and dynamic range will be left. As for all proteome analysis approaches using MS, the

As many of the basic issues limiting top-down are resolved, the more intrinsic challenges such as protein size and dynamic range will be left.



~10^6 range of protein abundances in cells presents a major hurdle. For ESI in particular, signal suppression within complex mixtures exacerbates the challenge of multiple charge states and a large number of isotopic peaks that dilute the signals of larger proteins. Localization by direct MS/MS is also challenging when PTMs are present at very low relative abundance. For larger proteins, controlled or limited digestion to 10–50-kDa peptides has been demonstrated for complete coverage of a 159-kDa protein (*51*) and will soon be extended to proteomics. For mammalian proteins, discerning mutations and allelic variation versus actual modifications in new samples may prove difficult in some cases. For large-scale proteome projects, such ambiguities can be resolved by parallel application of detailed fragmentation (e.g., ECD) or targeted sequencing of PCR products from specific loci.

Meeting society's expectations for "postgenomic" bioengineering and biomedicine requires further insight into biology at the protein level. With so much signaling and regulatory logic encoded at the PTM level, acquiring a protein "parts list" and measuring PTM dynamics are critical to determining molecular indicators and causes of multigenic disease phenotypes. The technology development focusing on intact proteins surveyed above is similar to the process that peptide-based proteomics went through a few years ago. Better detection and understanding of protein-level heterogeneity is one promise of top-down proteomics. As more research groups gain access to new MS instruments and work to remove barriers, this promise will be increasingly fulfilled.

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