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Mixture analysis

Tandem mass spectrometric analysis of complex biological mixtures

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Abstract

Proteomics is suddenly one of the hottest research areas of science, inspired by the completion of the sequencing of the human genome and of genomes of a number of other key species. The genome's DNA sequence predicts those of its expressed proteins, and mass spectrometry (MS) is already playing a dominant role in the qualitative and quantitative analysis of these complex protein mixtures. Tandem mass spectrometry (MS/MS) for targeted compound analysis, pioneered by Cooks, is now a key technique for such problems, and this paper tries to show how it will be even more important in the future. The most used current MS approach to proteome analysis is applied after enzymatic degradation produces a complex mixture of peptides that provide MS or MS/MS sequence information. In the top down MS approach, ionization of protein mixtures yields mixtures of molecular ions that can be separated by MS for MS/MS sequence characterization of the selected "purified" protein. All posttranslational modifications and errors will be represented if MS/MS yields a complementary set of fragment ions, something not usually achieved with the smaller pieces of the bottom up approach. Here the new technique of electron capture dissociation (ECD) shows great promise, providing for more backbone cleavages than conventional methods and designating N- and C-terminal fragment ions. ECD specifically dissociates backbone bonds without appreciable loss of glycosylation, phosphorylation, or other posttranslational modifications, in contrast to energetic dissociation methods. This appears especially applicable for a proteome problem that involves quantitative determination of the extent of phosphorylation at two of 16 phosphorylation sites in the 50 kDa Lyn kinase. (Int J Mass Spectrom 212 (2001) 81–87) © 2001 Elsevier Science B.V.

1. Introduction

The instrumentation of physicists has revolutionized analytical chemistry. As forerunners, emission spectroscopy revolutionized elemental analysis, and x-ray diffraction provided identification of crystalline powders (mainly inorganic) as well as fundamental structural data such as bond distances and angles. The entrance of mass spectrometry (MS) into analytical chemistry 60 years ago was not only in its general application to organic molecules (notably, light hydrocarbon gases), but also through the quantitative

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Dedication: The outpouring of key contributions to mass spectrometry by Graham Cooks continues unabated, even with the approach of his 60th birthday. Continuing unabated also with this are his enthusiasm, energy, friendliness, and cooperative spirit that have been such an important part of the renaissance of mass spectrometry and analytical chemistry. The editors had suggested that I write this as a "contribution bearing on the topical area of mixture analysis." It is a special pleasure to do this, in that it allows me to pay tribute to Graham's unique contributions in this area. He not only recognized the importance of quantitative analysis for a targeted compound in a complex mixture, such as those encountered in important biological problems, but he also pioneered the basic techniques that enabled tandem mass spectrometry to provide targeted analyses of unusual specificity, sensitivity, speed, and simplicity [1].

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analysis of complex mixtures. Even today an analytical chemist would be proud of 1% accuracy in the quantitative determination of 10 and even 20 components in a mixture; this was achieved on a routine basis by the famous Model 21-102 instrument from the Consolidated Electrodynamics Corporation. The author remembers the mass spectrometry sessions of the Pittsburgh Analytical Conferences of the early 1950s in which exciting developments in quantitative targeted-compound analysis, almost all by petroleum companies, dominated the newer applications of MS to structural characterization. The story is well known of how this latter field grew rapidly; among many others, Beynon showed that high resolution MS could give direct determination of elemental composition [2], Rylander et al. attracted the attention of organic chemists by demonstrating that the tropylium ion can be formed in the gas phase [3], and Biemann characterized peptides and alkaloids [4]. However, the true culprit in the demise of quantitative mass spectrometry was the gas chromatograph (GC), followed by the liquid chromatograph (LC). The resolving power of chromatographs was competitive with that of mass spectrometers, and of course chromatographs cost far less. For more difficult problems, the answer was to combine these techniques, with GC/MS becoming a standard tool for many scientific research areas and commercial problems [5]. A special advantage of the mass spectrometer is that isotopically labeled internal standards could give accurate quantitative results even with complicated low yield extraction processes.

However, some mixtures, mainly those that are naturally occurring, are so complex as to be challenging to even the best GC/MS or LC/MS analysis. Crude oil is a first outstanding example, and MS "type analysis" has been a very important analytical problem for the petroleum industry for many years [6,7]. The hydrocarbons can be aliphatic, unsaturated, cyclic, aromatic, and so forth, as well as containing multiple heteroatoms such as oxygen, nitrogen, and sulfur. High-resolution mass spectrometry has played a key role in this [7], although the number of literature references poorly reflects the commercial importance of this analysis, with the best methods remaining unpublished for obvious competitive reasons.

The most complex, and by far the most important, mixtures of organic compounds are those generated by living organisms. These are key problems in the medical, pharmaceutical, environmental, drug enforcement, and even the sports industries. The importance of tandem mass spectrometry (MS/MS) in these and future problems is examined here in the context of the pioneering contributions of Cooks nearly 25 years ago.

2. Principles of mixture analysis by tandem mass spectrometry

The 1983 review article on this theme by Busch and Cooks [8] still serves, in my opinion, as a bible for anyone wishing to understand the basic advantages of MS/MS for mixture analysis [1,9]. Even with unit resolution, separating out all of the mixture ions of a single value (e.g. mass 329) will reduce the mixture complexity by a factor of hundreds; fragmentation of these selected ions from the targeted compound yields a new set of products for which the most unique (e.g. mass 226) provides further selectivity of hundreds. Cooks also pioneered in using the triple quadrupole mass spectrometer for these applications, in which the targeted compound mass was selected in the first quadrupole, fragmented in the second, and the selected product ions separated in the third [8].

The impact of this development is wonderfully illustrated by a story told me by a former postdoctorate, Dr. Senn of the Boehringer-Mannheim pharmaceutical company. He visited the Finnigan Corporation early in the 1980s when they first started selling the triple quadrupole. He brought with him a sample representing the extraction of drug metabolites from a 1 mL sample of human blood; the identification and quantification of a metabolite was a necessity for the approval of this new drug for humans. As I remember the story, in less than an afternoon's visit, he was completely convinced that the triple quadrupole would solve the problem. GC/MS selecting the mass of the desired metabolite still found more than a dozen components of blood of that mass, but in MS/MS of these selected ions the metabolite uniquely produced

an abundant fragment ion making possible quantitative analysis at the low picogram level. Without haggling, Martin on the spot bought the instrument used for his sample, but insisted on taking it back with him to Germany.

3. Proteomics

Of the many important types of complex biological mixtures [10], let us skip forward to a current example whose analysis holds a truly high potential for expanding our understanding of human biochemistry and thus alleviating important health problems. The sequence of the human genome, completed so recently, predicts the amino acid sequence of proteins that provide the basic machinery for the body's functions [11–18].

By far the most widely used MS methodology that has been developed to identify these proteins [11] is what we call the "bottom up" approach [12]. Separation of a protein mixture by two-dimensional sodium dodecylsulfate (SDS) gel technology can result in a thousand or more spots that represent the individual proteins. Impressive developments from many key laboratories have given a general methodology that involves proteolysis of the separated spot, with MS analysis of the resulting peptide mixture to provide their accurate molecular weights. In some cases these mass values are sufficient to identify the protein from its predicted DNA sequence. If this is not possible, MS/MS of some of the peptides can provide their sequence information that is then far more definitive for matching against the possible DNA-predicted sequences. Successful identification can be achieved even if posttranslational modifications or DNA sequence errors have occurred, but a substantial proportion of SDS spots usually remain unidentified. However, with the obvious enormity of this total problem, combined with its equally obvious high importance, the development of these fast routine methodologies to take advantage of the genome sequence information represents a tremendous advance in the proteome field.

Identification problems arise from several sources. Although DNA sequencing accuracy approaching 0.1% is claimed, even this means that there will be one error, on average, in a one thousand-base DNA sequence, making incorrect the sequence predicted for a 333 amino acid protein produced by the three-base code; if this causes a frame shift, of course far more than a single amino acid will be predicted incorrectly. Posttranslational modifications will also change the mass of any affected peptide, although protein identification by the bottom-up approach still may be possible using the masses and/or sequences of the unaffected peptides. After identification of the genome location that codes for this protein, the next problem is to confirm its complete sequence and locate its posttranslational modifications [11–18].

3.1. The "top down" approach

This is an alternative methodology that we have pursued [12] that not only provides identifications of proteins but also direct characterization of posttranslational modifications and sequence errors [18]. Further, this approach should be especially important for quantitative analysis of targeted proteins in such complex mixtures. As a special problem, a high degree of heterogeneity can be encountered; partial posttranslational modifications of even a single protein at several sites will produce a mixture of products, for which quantitative analysis is necessary to characterize the protein function.

The standard procedure for characterization of a small molecule from its electron-ionization mass spectrum is to determine its molecular weight from the molecular ion and then to find "complementary fragment ions," those whose masses sum to that of the molecule [19]. Structural identification of the pieces could then lead to their combinations that limit the possible structures of the molecule. The earliest general application of tandem mass spectrometry was to fragment further these ionized pieces for their structural characterization, with a near quantitative "fingerprint" mass spectrum obtained by high-energy collisionally activated dissociation (CAD) [20–22].

Extension of these techniques by orders of magnitude in mass to proteins of course demanded a similar

advance in instrumentation capabilities of mass range, resolving power, mass accuracy, and MS/MS (even MS^n). Here the invention of the Fourier-transform ion cyclotron resonance mass spectrometer by Marshall et al. [23] has provided the most appropriate capabilities to date, especially with sample introduction by electrospray ionization (ESI) [18,24].

Fourier-transform mass spectrometry (FTMS) measures simultaneously the ion cyclotron resonance frequencies of the multiply-charged ESI ions trapped in the cell, recording those from, for example, mass 500 to over 100 000; taking the Fourier transform of this combined frequency signal then gives the mass spectrum over this unusually broad mass range. In a premier example, a single FTMS mass spectrum of a complex proteolysis mixture yielded 585 molecular weight values, most with six-figure accuracy for peptides as large as 30 kDa [18]. With the ppm resolving power and mass accuracy possible with FTMS, the main limitation to molecular weight accuracy is in the matching of the measured isotopic abundance distribution to that calculated theoretically; on average, this only causes a ± 1 Da inaccuracy starting at mass 20 000, with ± 3 Da found for the molecular weight determination of two 112 kDa proteins [25]. FTMS sensitivity is impressive, with subattomole ($<10^{-18}$ mol) samples yielding accurate molecular weights, and identification of 10^{-17} mol of carbonic anhydrase (29 kDa) from its MS/MS spectrum [26]. The nondestructive FTMS measurement of spectra is an obvious advantage for MS^n. After measurement of their molecular weights, the protein ions are still trapped inside the FTMS, so that all ions except those selected can be ejected from the cell, selected waveform inverse Fourier transform (SWIFT) [27], to obtain their MS/MS spectrum.

For the fragment ions in this MS/MS spectrum, the process can be repeated to obtain an MS/MS/MS (MS^3) spectrum of one of its product ions. As an example of FTMS utility in MS/MS characterization, ESI of a commercial polyethyleneglycol/polypropyleneglycol (PEG/PPG) block copolymer mixture gave 135 molecular ion compositions representing different combinations of PEG and PPG. MS/MS of four of these molecular ions, each representing less than 1% of the total mixture, then gave MS/MS spectra; these were mainly binary block copolymers, not ternary as indicated by manufacturer [28].

For proteomics, this top down strategy [12] can be applied directly to the initial identification of proteins, as an alternative to the current powerful bottom up method [11], as well as to their extensive characterization, such as of posttranslational modifications. Here the first problem is the removal of the intact protein from the SDS gel; transfer to the mass spectrometer becomes increasingly difficult for proteins larger than ~20 kDa [29]. However, Martin et al. have shown that mixtures containing thousands of nonapeptides presented by Class I MHC molecules to trigger the attack of killer T-cells can be separated by nano-high-performance liquid chromatography to give fractions containing possibly six peptides; MS/MS of the biologically active fractions (even 10^{-17} mol) then gives peptide sequences that make possible synthesis and biological testing [30]. Capillary electrophoresis separation of only 10^{-17} mol of a protein gave an ESI spectrum from which the measured molecular weight did not match any in the protein database. However, MS/MS gave accurate masses of 9 fragment ions, of which a "sequence tag" successfully matched masses and their mass differences from the C-terminal end of the protein; the correctly matched carbonic anhydrase in the protein database was not N-acetylated, so its molecular weight was less by 42 Da [31]. Thus after protein identification, the molecular weight differences can indicate the extent of posttranslational modification, and the mass differences in individual fragment ions can then provide the location of these modifications.

3.2. Electron capture dissociation [32–34]

Linear molecules such as proteins have a great advantage for structural characterization by MS/MS; product ions from direct fragmentation (without rearrangement) of the chain must represent a partial sequence containing one end or the other of the protein. (This end-identification ambiguity is said [11] to be "the basic problem . . . for de novo interpretation".) The other main difficulty for protein sequenc-

ing is that all MS/MS conventional dissociation methods cleave the weakest backbone bonds, as all add energy to the ion. However, the new technique electron capture dissociation (ECD) involves a nonergodic dissociation ($<10^{-12}$ s), so that the strength of a backbone bond has little effect on its cleavage probability. Thus for carbonic anhydrase with 259 amino acids, CAD cleaved bonds between 66 of its amino acids [12]. However, ECD cleaved 116 different bonds, with a total of 138 cleavages between different pairs of amino acids due to all methods, including infrared multiphoton dissociation [35] and blackbody infrared dissociation [36,37]. Critical for this, ECD cleaves the amide backbone bond to produce a · and y fragment ions, as well as cleaving the N–C_α bond to produce c, z · fragments; thus a y, z · pair ($\Delta m = 16.02$ Da) in the ECD spectrum shows that these contain the C−terminus. This nonselectivity leads to much more complete sequence data; for cytochrome *c*, the combined methods gave cleavages between all but nine of the residue pairs [33]. For ubiquitin, 76 amino acids, the combined MS/MS methods gave sufficient data that a computer program could de novo generate the correct total sequence as the most probable, without prior sequence data [38].

The complementarity of ECD and other MS/MS methods is especially valuable for characterization of posttranslational modifications. The energy introduced into the ion by the latter methods can cause loss of the side chain; thus the glycosylation heterogeneity obvious in the molecular ion spectrum of a 38 kDa heavy chain construct of IgE could be traced by MS^3 to a single "high mannose" glycosyl side chain [39]. CAD of the 7 kDa peptide containing the glycosylation gave a 42 amino acid fragment as its smallest product, but MS/MS/MS of this showed the sequential loss of six 162 Da (hexose) units followed by loss of a 203 Da (N-acetylglucosamine) unit providing specific characterization as a high mannose glycosylation. However, as shown by Mirgorodskaya and coworkers [40], ECD does not fragment glycosyl side chains, so that the extensive ECD backbone fragmentation can locate these accurately in the protein sequence. The interested reader is referred to a recent ECD review for more details [41].

3.3. Quantitative mixture analysis for future proteomics

Cooks showed us how tandem mass spectrometry can provide quantitative analysis of important small molecules such as drug metabolites in highly complex mixtures such as body fluids. To illustrate how this might be extended to proteomics, probably its most important problem is the identification and location of post-translational modifications. Here, phosphorylation (and dephosphorylation) is among the most important for key biological functions [15,16]. Conventional isotopic ^{32}P labeling and autoradiography of the tryptic peptides provides only semiquantitative analyses. Oda and coworkers [15] have developed a general bottom up MS approach to compare the quantitative phosphorylation of sites in wild type and mutant systems. One is labeled by growing the cells on an ^{15}N substrate, and then the isolated enzymes are mixed, subjected to proteolysis, and the LC-separated labeled and unlabeled peptides compared directly by MS or MS/MS to determine the difference in the degree of phosphorylation. ECD produces negligible loss of phosphorylation [34], so that we are attempting to apply the top down approach to the following problem brought to me by my colleagues, Prof. Baird and Dr. Holowka, that is now under study by postdoctorate, Frank, and graduate student, Young. The 50 kDa Src family Lyn kinase is a critical protein in signal transduction that has an unusual effect; the degree of phosphorylation at tyrosine residue 397 up regulates the activity of the enzyme, but the degree of phosphorylation at Tyr 508 down regulates the activity [42]. In addition to these two tyrosine residues, many other sites on the protein also have the potential to be phosphorylated on tyrosine, serine, or threonine residues to some degree as determined by sequence pattern recognition with functional consequences. At least five sites are likely to be phosphorylated based on our knowledge of other signaling kinases, and as many as 16 sites have significant phosphorylation possibility. Phosphorylation on tyrosine compared to that on threonine or serine corresponds to different functional effects, so that a complete understanding of

this system could demand quantitative analyses of all 16 sites.

Using the Oda system of isotopic labeling [15], proteolysis-produced peptides containing only the 397 site would give four mass values, corresponding to the wild and mutant species, each without and with phosphorylation. ECD fragmentation in the same labeling experiment should provide the same quantitative data directly without proteolysis and separation, presumably requiring less time and sample. However, isotopic labeling is required by the effect of phosphorylation on LC separation efficiencies; this should have little effect on MS separation efficiencies. Thus quantitative data should be possible without isotopic labeling, with the protein molecular ions indicating all degrees of overall phosphorylation. All of these, or ions of a specific degrees of phosphorylation, can be SWIFT [27] selected for ECD, which produces a negligible loss of phosphorylation [34]. Thus there is even the possibility that the enzymatic activity of Lyn kinase can be correlated with the degree of phosphorylation at combinations of the various sites. For this proposal, the ECD spectrum of 24 kDa casein with phosphorylation at five of its 12 Ser residues is an encouraging example [34].

4. Conclusion

The pioneering development of MS/MS techniques for targeted small molecule analysis by Cooks [1,7] has provided the vision for MS^n extension to much larger molecules. Here the just-recognized critical importance of proteomics should provide the incentive for applications of great benefit to critical problems in human health.

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