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Review

Structure specific chromatographic selection in targeted proteomics

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Abstract

The whole proteome of any organism is too complicated to be analyzed in a simple one-step process and direct attempts for the entire proteome analysis normally lead to considerable loss of information. A practical approach is the targeting of the specific structural feature of interest using chromatography. This approach simplifies the proteome while preserving most of the vital information necessary for analysis. Selection of peptides with specific amino acids (cysteine, histidine and methionine) or N- or C-terminal peptides is an accepted procedure for proteome simplification when general analysis is desired. While selection of enzymatically and non-enzymatically modified proteins and peptides is used when post-translational modifications are targeted. Protein interaction with small molecules as well as other proteins also has been studied using chromatographic selection methods.

Keywords: Proteomics; Amino acid; Chromatography; Affinity selection; Glycosylation; Phosphorylation; Oxidation; Nitration; Glycation; Specific amino acid selection

Contents

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1. Introduction

Proteomics was conceived as a technique that would examine a proteome in a single, integrated set of analytical operations. With accumulating experience we are learning this probably is not possible at present. Beyond the 10,000–30,000 proteins coded by the genome of many cells there are splicing variants, post-translational modifications (PTMs), and genetic variations between individuals. This makes the proteome far more complex than the genome. Glycoproteins alone can have 10–50 glycan variants at a single site in one protein. The fact that the proteome is dynamic adds another level of complexity. Unique patterns of change in PTMs and protein expression are hallmarks of cellular differentiation, regulation, and disease progression.

The complexity of a proteome can far exceed the capacity of analytical systems. We must accept that with higher plants and animals, proteomics methods do not at present, and will not in the immediate future, define more than a small portion of a proteome on a routine, high throughput basis, i.e. within 24 h. Thus, all proteomics methods are a priori targeted in some way, either intentionally or by limitations of the analytical system being used.

2. Chromatographic targeting of structural features

How then do we decide which part of the proteome to analyze and which targeting strategies provide the most meaningful data? A variety of chromatographic and electrophoretic methods, sometimes enabled by protein or peptide derivatization, have been accumulating over the past three decades that target structural features common to multiple proteins and peptides. The presence of histidine, cysteine, methionine, phosphorylation, glycosylation, glycation, nitration, and specific types of oxidation along with the propensity of proteins to interact with substrates, cofactors, allosteric effectors, and other proteins (Fig. 1) are examples of properties that are associated with cellular regulation and disease, and can be targeted. Selection of proteins and peptides from complex mixtures on the basis of common structural features or chemical behavior can reduced sample complexity 80% or more in a single separation step [1]. Moreover, identification is greatly facilitated by knowledge derived from structure-based selection. This is very important in proteome samples (and their proteolytic digests) that potentially contain 10^4–10^6 components.

Analysis of post-translational modification (PTM) provides one of the strongest cases for targeted selection. Although many proteins are post-translationally modified during their biological lifetime, perhaps only one in 20–50 of their tryptic peptides will be modified, depending on the PTM. The number of peptides that must be examined to find a PTM will be reduced at least an order of magnitude by PTM specific selection.

Finally there is the issue of using separation behavior to predict structure. It is possible with both reversed phase chromatography [2] and ion mobility separators [3] to predict the elution or migration time of a peptide to within a few percent. When a search of a database for potential peptide candidates finds multiple possibilities, it is possible to differentiate between them by correlating predicted migration behavior of candidates with that of the unknown. Molecular weight and separation behavior are frequently adequate to identify a peptide without sequence data.

3. Targeting change

It is assumed above that a compelling case can be made for examining a particular type of structure. That is not always true. In the case of many diseases, one has no idea which proteins are associated with the disease. How then is it possible to target and select relevant peptides or proteins on the basis of structure? Very important proteins associated with the onset and progression of either a regulatory event or disease. These proteins generally change in concentration, and sometimes structure, during the event. This means that samples taken before and after the biological event will be different. Two-dimensional gel electrophoresis and quantification by staining have been used for decades in such comparative analyses, even before the arrival of proteomics [4–6]. Recently developed isotope coding methods allow multiple complex mixtures to be globally searched and compared for any component that differs in concentration more than 15–20% between samples [7]. Detailed discussions of these methods can be found in recent reviews [8,9]. A unique feature of some new coding methods is that struc-

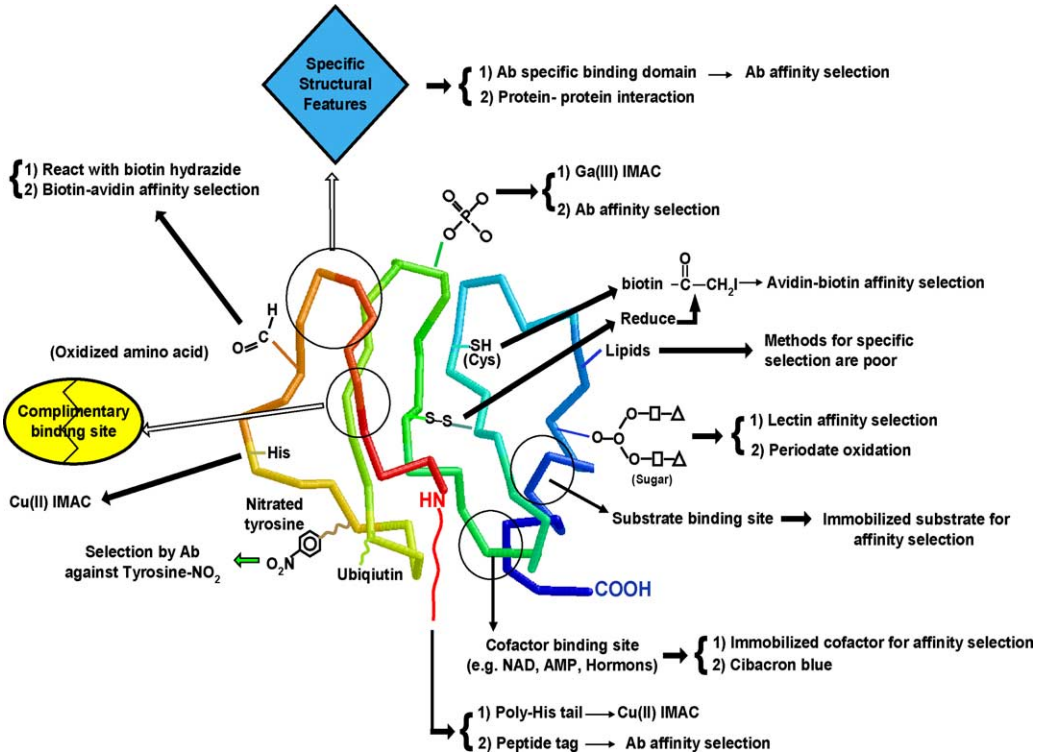

Fig. 1. A general structure specific selection scheme. One mode of selection is on the basis of structural features such as complimentary to another protein, a cofactor binding site, or a specific structural feature such as a substrate binding site. Post-translational modifications such as phosphorylation, glycosylation, and oxidation can be also target based on unique properties of the moieties added during modification. Sometimes specific amino acids, such as histidine and cysteine, in peptides derived from proteins are targeted using affinity selection strategies, either directly or after covalent modification with a tagging agent such as biotin that can later be selected with avidin.

tural features of the proteome are targeted and selected first and then a search is made for changes in concentration or structure [10]. Protein expression [11], phosphorylation [12], and glycosylation [13,14] have all been examined in this way.

4. Integrating proteolysis into the proteomics process

The proteomics revolution is based on the fact that mass spectrometers can readily determine partial sequence and molecular weight of peptides. With data from only a few peptides and DNA sequence from a DNA database it is possible to identify proteins. But before this can happen, proteins must first be converted to peptides. This seemingly trivial task can present substantial problems. Disulfide cross-linking, glycosylation, and the presence of hydrophobic domains reduce the rate at which some proteins can be digested. Reduction of disulfide bridges makes it easier for proteolytic enzymes to penetrate proteins but glycosylation and aggregation still reduce the rate of proteolysis in some cases. This leaves two options; further alter protein structure or increase the rate of proteolysis. Immobilized enzymes provide a relatively simple route to accomplish the later.

Based on decades of research, it is known that in immobilized enzyme reactors packed with high surface area support media that enzyme concentrations of up to 100 mg/mL can be obtained. The enabling feature of immobilization in the case of proteolytic enzymes such as trypsin is that it allows very high enzyme densities without auto-proteolysis and sample contamination with enzyme fragments. This means (1) it is possible to have enzyme to substrate ratios 10–1000 times higher than the 1/50 ratio (w/w) normally used in solution and (2) the absolute concentration of enzyme can be orders of magnitude higher. When the enzyme to substrate ratio exceeds 10 there will be an acceleration of proteolysis, especially when reactor temperature is elevated as well [15,16]. As will be discussed below, 100-fold acceleration is common.

The effectiveness of immobilized trypsin columns has evaluated with *Escherichia coli* lysates using size-exclusion chromatography (SEC) to examine the proteolysis products [17]. Trypsin columns were operated in both a continuous-flow and stopped-flow mode at temperatures ranging from ambient to 37 °C with incubation times of 0–2 h. Total digestion of reduced and alkylated protein mixtures was achieved in 20 min at 37 °C judging from the size of the cleavage fragments and the absence of peptides retained beyond the total inclusion volume. A useful feature of the silica based SEC column used was that miscleaved hydrophobic peptides were retained beyond the total inclusion volume of the column and easily recognized. Solution based digestion in contrast left more miscleaved peptides, even after 36 h of digestion.

Beyond the obvious time advantage of immobilized enzyme columns they are easily incorporated into multidimensional separation systems for automated proteomics [18,19].

One of the problems alluded to above is that in some proteins proteolysis can produce fragments that are more hydrophobic than the parent protein. They can even aggregate and precipitate. When this occurs in an immobilized enzyme column there is the potential for (1) reducing the proteolytic efficiency of the reactor by blocking sorbent pores; (2) increasing the backpressure across the column; and (3) contamination of future samples. There are at three ways to deal with this problem. One is to gradient elute the reactor with a mobile phase used in reversed phase chromatography (RPC) after each sample has been processed. RPC mobile phases effectively solublize most hydrophobic cleavage fragments. This intermittent use of organic solvents and weakly acidic mobile phases seems not to limit immobilized enzyme column life. A second alternative is to execute the reaction in high concentrations of urea or guanidinium hydrochloride. These reagents have been widely used to deal with this problem in bulk proteolysis, but do not completely circumvent it. In addition, urea can carbamylate proteins at high concentration. A third alternative is to introduce organic solvent into the proteolysis buffer. The reaction rate of trypsin is actually enhanced with some peptides in solvents of lower dielectric constant [20]. Notably, methanol-based digestions produced fewer missed cleavages while acetonitrile-based digestions produced the most peptides [21]. When human transferrin (hTf) was digested in an immobilized trypsin column using an aqueous–organic mobile phase 42 peptides were observed by MALDI-MS in contrast to 12 peptides from a solution digest.

With increasing interest in miniaturization and the movement to micro-total analytical systems (μTAS), immobilized enzymes are being used for on-chip proteolysis. The simplest approach is to place chromatographic supports carrying an immobilized enzyme into either sample wells or channels on the chip [22–24]. Proteolysis is achieved in the same manner as described above. The difference is that liquid is moved through the chip with either a micro-mechanical pump or by electroosmosis. A second approach is to etch pores into channel walls within the chip and immobilize a proteolytic enzyme in these pores [25,26]. Etching substantially increases the surface area of the reactor and concomitantly the enzyme loading capacity.

5. Selection of peptides with specific amino acids

In silico analysis indicates that low abundance amino acids such as histidine and cysteine are expected to occur individually in only 10–20% of the tryptic peptides from a proteome [27]. Methionine-containing peptides are found in even lower abundance, occurring in *E. coli* at an average frequency of 6 per protein. Although any one of these amino acids occur in tryptic peptides at a frequency less than one in six, they are found in more than 90% of the proteins in a proteome. Thus, selecting peptides containing a low abundance amino acid would reduced sample complexity 80% or more while still obtaining at least one peptide from more than 90% of the proteins in a proteome.

5.1. Selection of histidine-containing peptides

Copper loaded immobilized metal affinity chromatography (Cu(II)IMAC) columns have been widely used to select histidine-containing proteins and peptides [28,29]. It has recently been shown that most histidine-containing peptides can be captured from tryptic digests with little non-specific binding through the use of very hydrophilic IMAC columns and imidazole as a displacer [30,31]. Peptides that contain multiple histidine residues can also be selected with Cu(II)IMAC, representing less than 5% of all peptides in a proteome digest [32]. This may be of particular value in the study of histidine rich domains in zinc-finger and metal binding proteins.

Quantification of expression using histidine peptides has been accomplished through differential coding of control and experimental samples with isotopically distinct forms of 4-trimethylammonium butyrate (TMAB) [33]. Following coding, samples were mixed, histidine-containing peptides were selected with Cu(II)IMAC, and the selected peptide fraction transferred to a reversed phase chromatography column where they are further resolved before analysis by mass spectrometry. Changes in protein expression were identified through differences in the isotope ratio of peptide isoforms from the two samples. This method has come to be known as the global internal standard technique (GIST) for quantification [33].

5.2. Selection of cysteine-containing peptides

Cysteine-containing peptides are generally selected by derivatization of cysteine residues with biotin [34]. Proteins are first reduced and then alkylated with a biotinylated alkylating agent followed by proteolysis and an ion exchange step to remove the excess biotinylating agent. After selection of the biotinylated cysteine-containing peptides via avidin affinity chromatography they are desorbed from the affinity column with an acidified mobile phase and transferred to the LC–MS for identification. Because the tetrameric form of avidin binds to the biotin with such great affinity, the much lower affinity monomeric form of avidin is generally used in the affinity column.

Cysteine selection has also been used to study protein expression. Quantification is achieved by labeling sulfhydryl groups in proteins with an isotopically coded affinity tag (ICAT) during the normal reduction and alkylation steps preceding proteolysis [7]. Control and experimental samples are treated with isotopically distinct forms of the biotinylated alkylating agent and then mixed before the proteolysis and ion exchange steps preceding avidin affinity chromatography.

Changes in expression are quantified in the same way as with histidine-containing peptides.

It should be noted that peptides derived from the small number of proteins that naturally carry covalently bound biotin would also be selected in this process. In addition, fragmentation of the rather large affinity tag can further complicate the mass spectrum.

5.3. Selection of methionine-containing peptides

The observation was made in the days of paper chromatography that when a sample was chromatographed in one direction and dried, then rotated 90° and chromatographed in a second direction with the same mobile phase that all the analytes appear on the chromatogram in a diagonal line. Analytes that were chemically modified in some way between these two chromatographic dimensions no longer fell on the diagonal analyte line. This technique for recognizing chemical modifications came to be known as *diagonal chromatography* [35,36] and is also possible with HPLC [37].

Methionine is known to be oxidized by hydrogen peroxide. The retention time of a methionine-containing peptide in reversed phase chromatography generally changes a few minutes following hydrogen peroxide oxidation. When the tryptic digest of a proteome is separated by reversed phase chromatography and each of the chromatographic fractions are treated with hydrogen peroxide, the chromatographic behavior of methionine-containing peptides will be different when the sample is rechromatoghraphed [37]. Generally the retention time of methionine containing peptides is reduced 3–6 min after hydrogen peroxide oxidation. More than 800 proteins from *E. coli* have been identified using this diagonal approach for recognizing methionine-containing peptides.

Although not currently explored, it is likely that when coupled with GIST coding it would be possible to quantify relative changes in the concentration of methionine-containing peptides [38].

5.4. Summary and future of specific amino acid selection

Selection of peptides containing specific amino acids will continue to be an important part of proteomics. The great advantage of this strategy is that proteome complexity is substantially reduced and peptide identification is simplified. But there are also disadvantages of this approach. One is that some proteins may not contain selectable peptides. This can be from 1 to 10% of the proteome, depending on the amino acid and the organism. Another disadvantage is that a variety of protein isoforms, be they PTM or multimer variants, may have the same peptide and too few peptides are selected to differentiate between the variants. This is an important issue when ion suppression or co-elution of isobaric peptides causes one or more peptides from a protein to be missed during RPC–MS analyses. If only one or two peptides from a protein were selected and they were missed during the analysis, the protein parent will be missed. A solution to this problem in the future would be to sequentially select different amino acids in multiple RPC–MS analyses of the same samples. This is equivalent to multidimensional chromatography involving cation exchange chromatography followed by RPC–MS [39,40] except that one has knowledge of one or more amino acids in the selected peptide fractions.

6. Selecting N- or C-terminal peptides from a protein

The ultimate in simplification would be to select a single, signature peptide from each protein in the proteome. This has been done in several ways. One is through the use of diagonal chromatography to recognize N-terminal peptides [41]. After acetylation of primary amine groups in proteins, including the amino-terminus, the proteins were digested with trypsin. The critical feature of this process is that proteolysis generates a new set of amino groups on all peptides except those originating from the amino termini of proteins. The tryptic digest was then separated by reversed phase chromatography and the RPC fractions treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS). TBNS peptides display a strong hydrophobic shift and therefore segregate from unaltered N-terminal peptides during a second identical separation step. N-terminal peptides were identified by LC–MS.

Another approach is to select C-terminal peptides from tryptic digests of proteins with an anhydrotrypsin (AHT) column [42]. A unique feature of trypsin digestion is that all peptides in the digest will have a C-terminal lysine or arginine residue except those arising from the C-terminus of the protein. AHT columns form a covalent intermediate with all peptides that contain a lysine or arginine residue at their C-terminus, leaving C-terminal peptides from proteins in the effluent. The exception would be proteins that naturally contain a basic amino acid at their C-terminus. It is important that the trypsin used in proteolysis be free of chymotrypsin because AHT columns do not select chymotryptic peptides and a large number of false positives would be obtained.

These are both elegant methods for sample simplification and will be very useful in many types of studies. But again, methods that depend on very small numbers of peptides for protein identification run the risk of either missing some peptides due to ion suppression during LC–MS, the N-terminal peptide is too small to be captured, or isoforms of proteins can have the same peptide.

7. Selection of enzymatically modified proteins and peptides

Most proteins are modified in some way after leaving the ribosome, be it through derivatization with a unique new functional group, addition of structural units, proteolysis, deglycosylation, or some other form of cleavage. These post-translational modifications are of two major types, alterations

arising from enzymatic reactions and those derived from non-enzymatic modifications.

Proteomics is heavily dependent on DNA databases for protein identification. Through in silico transcription and translation of DNA sequences from databases, protein sequence can be quickly predicted and protein parents identified based on matching either their predicted tryptic peptide mass fingerprint or the sequence of a small number of signature peptides to experimental data. A major problem with this approach in the case of post-translationally modified proteins is that one must know that a tryptic peptide is carrying a PTM and understand the nature of the modification before the mass of these modified peptides can be predicted from DNA databases. This is almost never the case. It is in this context that PTM targeted selection of modified tryptic peptides is of so much interest.

7.1. Glycosylation

Among the many types of PTM, glycosylation occurs most frequently. The glycoproteome plays a key role in cellular regulation and function [43], in addition to being prominently associated with disease. Alzheimer's disease, diabetes, stress, some autoimmune diseases, cystic fibrosis, arthritis, cancer, certain types of heart disease, respiratory illnesses, renal function diseases, and diseases related to cellular adhesion are all associated with aberrations in glycoproteins [44–46]. Proteomics, when coupled with glycobiology, provides a powerful new method to study glycosylation and glycopathologies.

Glycoproteins occur in two forms. O-linked glycosylation occurs through a post-translational attachment of either mono- or oligosaccharides to the hydroxyl group on serine, threonine, or occasionally hydroxyproline. *O*-Glycosylation occurs independent of protein expression. N-linked glycosylation differs in that a glycan is attached to the amide group of asparagine at –Asn–X–Ser– or –Asn–X–Thr– sequences while the protein is still on the ribosome, i.e. during protein synthesis. Failure to *N*-glycosylate proteins occurs in only very rare cases associated with genetic diseases. This means that the initial *N*-glycosylation is directly proportion to protein synthesis and is not really a post-translation modification. Both O- and N-linked oligosaccharides can undergo extensive post-translational processing involving the deletion or addition of individual sugars [47,48]. Multiple genes regulate this glycan processing through a series of enzymes located at different sites in cells.

Lectin affinity chromatography is by far the most widely used and specific method for selecting glycoproteins and glycopeptides. Lectins are available that target either O- or N-linked oligosaccharides, broad structural features of a glycan, a single sugar residue, or even specific glycopathologies at both the glycoprotein and glycopeptide levels [49–51]. The long history of exploiting lectin specificity as a histological staining agent and in oligosaccharide structure analysis is of great value in proteomics. Based on lectin staining it is well

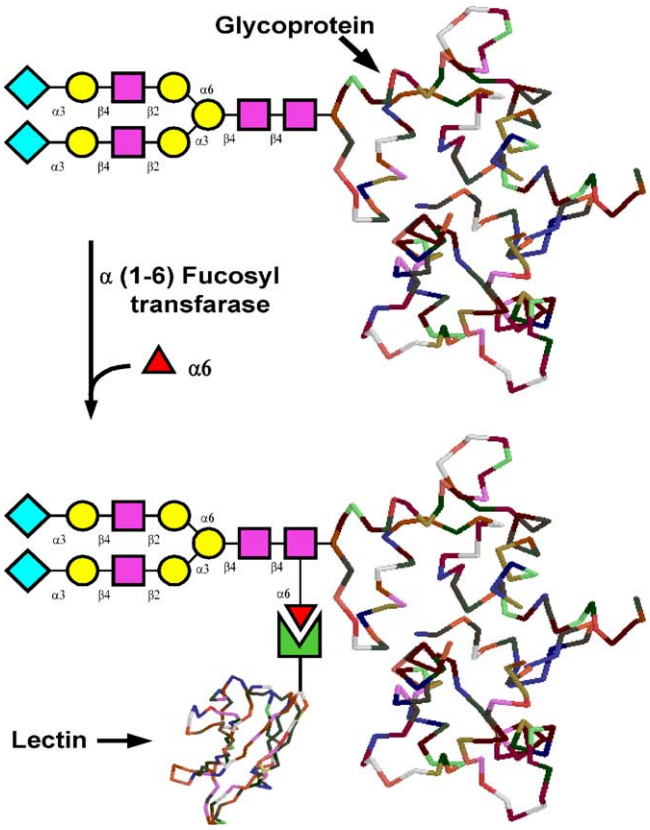

Fig. 2. This is an illustration of how up-regulation in the enzyme α(1–6)fucosyl transferase in cancer patients can cause aberrations in glycosylation associated with disease progression. A lectin that targets fucose in the glycan portion of glycoproteins or glycopeptide has been used to select all glycoforms carrying fucose and show that during chemotherapy, the concentration of some fucose containing glycoproteins associated with metastasis decrease dramatically [52].

known that aberrations in fucosylation and other forms of glycosylation occur in cancer. But few or these aberrations have been connected to specific proteins. One way to address this problem is through the use of a lectin that targets a disease specific aberration in glycosylation, such as fucosylation (Fig. 2). Immobilized *Lotus tetragonolobus* agglutinin (LTA) has been used to select fucose containing tryptic peptides from tryptic digests of blood and show that fucosylation of a large number of proteins was suppressed in lymphosarcoma patients during chemotherapy [52]. One of the complications in glycoproteomics is that glycan heterogeneity in glycopeptides complicates direct MS/MS sequencing and identification of peptides. This is because both the peptide and glycan portions of glycoconjugate are generally unknown and identification through databases is impossible without knowledge of peptide molecular weight. Deglycosylation by either enzymatic or chemical means is necessary before peptide identification is possible, as noted above. The strong point of this approach is that a large number of well characterized lectins are available. The weak point is that there will be types of glycosylation for which there is no specific lectin.

Another recently described route to select glycoproteins is by periodate oxidation and covalent capture of the resulting dialdehydes [53]. Periodate cleaves *cis*-diols in the oligosaccharide portion of glycoproteins to form aldehydes that were captured by a hydrazide resin. Following the capture of oxidized proteins on the resin, bound proteins were trypsin digested and non-glycosylated peptides eluted. Following derivatization with stable isotope coded succinic anhydride that was used later in quantification; the peptide portion of the glycopeptide was removed from the resin by hydrolysis with PNGase F, an enzyme specific for *N*-glycosylation. Released peptides were further fractionated by reversed phase chromatography and identified by mass spectral analysis. All glycoforms that contain one or more diols will be oxidized by periodate. The strong point of this method is that a broad range of *N*-glycosylated isoforms of glycoproteins will be selected. The weak point will be the difficulty in differentiating between them.

Phenylboronates form a cyclic ester with vicinal diols, the rate of which depends on diol stereochemistry. This has led to extensive use of *m*-aminophenylboronate chromatography columns to select carbohydrates and release them under acidic conditions [54,55]. These columns have been used in the capture of ribonucleotides and should be of equal utility in glycoprotein proteomics.

Changes in glycoprotein concentration have also been quantified. Differential coding of tryptic peptides with *N*-acetoxy succinic anhydride [56] or succinic anhydride [57] using the GIST protocol combined with lectin based glycopeptide selection or periodate oxidation and covalent capture along with succinic anhydride coding [40] have been used to recognize and quantify changes in glycoproteins. In the case of cancer, it has been shown that glycoproteins associated with metastasis are reduced in concentration during chemotherapy [58]. Fig. 3 is a reversed phase chromatograph

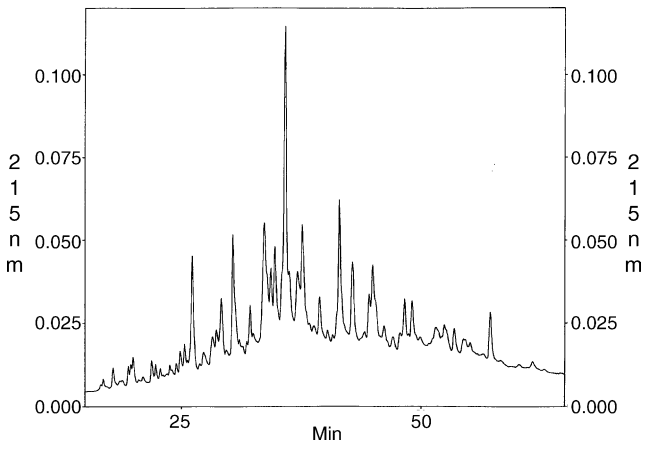

Fig. 3. A reversed phase chromatogram of glycopeptides from human serum. Equal amounts of serum from two samples were tryptic digested and individually coded with acetate d_0 and d_3. After coding the samples were combined and sialylated gycopeptides selected with a Sambucus Nigra Lectin (SNA) affinity column. Following deglycosylation with PNGase, the sample was fractionated with a C_{18} column as seen above.

of sialylated glycopeptides from human serum. Equal amount of serum digests labeled with acetate d_0 and d_3 were combined and selected by Sambucus Nigra Lectin (SNA) affinity column. SNA is specific for glycopeptides carrying a sialic acid attached to terminal galactose in (α-2,6). After deglycosylation with PNGase, the sample was separated with a C_{18} column (Fig. 3).

Although there can be broad oligosaccharide heterogeneity at any one site in a glycoprotein, only a few of these isoforms may be associated with a disease. It is likely that in the future the quantification methods described above will be used to recognize glycoforms associated with regulatory events or disease and study their role in biological processes.

7.2. Phosphorylation

Rapid modulation of signaling is a key component of intracellular regulation and homeostasis. Signaling anomalies can lead to uncontrolled growth or death. In either case, the ability of cells to carry out their genetically determined role is compromised by faulty signaling. It is not surprising that almost 10% of all references in the phosphoprotein literature relate to disease. It is in this context that both phosphorylation and dephosphorylation of proteins in signaling pathways is of such great interest in biology, medicine, and drug discovery. In some respects, monitoring phosphoprotein dynamics is equivalent to “wire tapping” a cell.

There are two major classes of phosphoproteins; serine or threonine phosphorylated proteins and those that are phosphorylated on tyrosine. Other types of phosphorylation are seen infrequently or are transitory, as in the case of histidine phophorylation. Tyrosine phosphorylated proteins constitute no more than 0.1% of the phosphoproteome and are thought to be involved in the most crucial signaling [59]. Phosphorylation at multiple sites is very important in regulation [60]. This presents an analytical problem in that phosphopeptides that have been phosphorylated at three to four sites will be much more hydrophilic and may not be retained by reversed phase columns. There is also the prospect that their ionization efficiency will be reduced. Marked differences in the chemical properties of serine/threonine and tyrosine phosphate esters have been exploited in their determination. Phosphate esters of serine and threonine readily β-eliminate in base whereas the tyrosine esters do not. There are also families of phosphatases that differentially hydrolyze these two classes of phosphate esters.

Antibodies specific for either tyrosine phosphorylation or serine/threonine phosphorylation have been used extensively to select and recognize phosphoproteins, either through immunoprecipitation, immunosorbent chromatography, or in western blotting [61,62]. A recent paper describes a protocol for global selection of phosphotyrosine modified proteins using an antiphosphotyrosine immunosorbent column followed by trypsin digestion of the captured proteins, differential coding of control and experimental samples with CH_3OH and CD_3OH, respectively, IMAC selection of the phosphopep-

tides, and reverse phase chromatography–mass spectrometry of the selected phosphopeptides [63]. In general, antibodies targeting phosphorylation seem to be more specific for phosphoproteins than phosphopeptides, as reflected by the literature.

Direct selection of all phosphopeptides from trypsin digests with Al(III), Fe(III), or Ga(III) loaded IMAC columns is another approach [64]. The problem with this method is that peptides with multiple aspartate or glutamate residues are also selected. Half, or more of the selected peptides may not be phosphorylated. Several solutions to this problem have been explored. One is to methylate the carboxyl groups in phosphopeptides [65]. But the tendency of aspartate esters to internally cyclize under acidic conditions through aspartamide formation with the loss of methanol makes them labile during reversed phase chromatography with acidic mobile phases. Another approach is to digest with endoprotease glu-C [66]. This enzyme cleaves proteins at acidic residues and greatly reduces the number of acidic peptides. Ga(III)IMAC selection of endoprotease glu-C digests indicate the roughly 70% of the captured peptides are phosphorylated. Quantification in this method has been achieved with GIST [14] and isotope coding of methyl esters [65].

Another method for selecting all phosphopeptides is through attachment of an affinity tag to phosphate residues [67]. In this process, primary amines in peptides are first protected using the *t*-butyl-dicarbonate (*t*-BOC) chemistry. An aqueous solution of the protected peptides is then treated with ethanolamine and a water-soluble carbodiimide to convert carboxyl groups to amides and phosphate groups to phosphoramidates. Phosphate groups in peptides were subsequently regenerated by treatment with weak acid and again converted to phosphoramidates with carbodiimide catalysis, but with cystamine in the second phosphoramidation. Reduction of cystamine generates a free sulfhydryl group on phosphate groups in peptides. Following removal of non-peptide reactants through reversed phase chromatography, these sulfhydryl-containing peptides are covalently captured by reaction with iodoacetyl groups attached to glass beads. Phosphoramidate bonds in the captured peptides are again cleaved with trifluoroacetic acid, but at a concentration that cleaves the *t*-BOC protecting group as well. Phosphopeptide recovery was roughly 20%.

Specific selection of serine/threonine phosphorylated peptides has also been achieved by replacing the phosphate group with a moiety, such as biotin, which can be affinity selected [68]. Peptides that are *O*-phosphorylated on serine or threonine undergo β-elimination in base with the formation of a conjugated diene –(CH=CHC=O)–. Addition of 1,2-ethanedithiol to this alkene under basic conditions forms a derivative with a free sulfhydryl group. Alkylation of these thiol derivatized peptides with ICAT reagent places an affinity tag on peptides at former phosphorylation sites. Quantification can be achieved with the ICAT method, as has been shown in yeast. But *O*-glycosylated peptides also undergo β-elimination. Prior to application of this method the mixture must first be deglycosylated.

7.3. Nitration

Nitrotyrosine plays a direct role in cellular signaling [69] and is frequently found in proteins. Fluctuation in the concentration of nitrotyrosine has been associated with oxidative stress related diseases [70], diet [71], and even aging [72]. Because nitrophenyl groups are immunogenic, it is possible to prepare antibodies that target nitrotyrosine and use them in the same manner as with phosphotyrosine. There also has been some effort to investigate tyrosine nitration in proteins by mass spectrometry [73].

8. Non-enzymatic post-translational modification

A wide variety of protein modifications occur as a result of the accumulation of low molecular weight species in cells, either from the environment or disease related alterations in metabolism.

8.1. Glycation

Reduction of glucose transport into cells in diabetes results in the elevation of blood glucose. Reaction of protein amino groups with glucose (the Maillard reaction) leads from early stage products such as Schiff bases and Amadori products to advanced glycation end products (AGE), structures implicated in diabetic complications and the aging process [74]. AGE modified proteins have been captured from biological fluids in several affinity chromatography approaches. One is through the use of AGE-targeting antibodies [75]. Another is through the use of natural antibacterial proteins, such as lactoferrin and lysozyme. Both bind specifically to glucose-modified proteins bearing advanced glycation end products [76].

8.2. Protein oxidation

It has been demonstrated that oxidative modification of proteins increases during aging and diseases such as atherosclerosis, arthritis, muscular dystrophy, cataractogenosis, pulmonary dysfunction, various neurological disorders, and possibly even cancer [77]. Oxidative modification can occur in a number of ways. Among the most common are oxidation of advanced glycation end products (AGE), attachment of a lipid fragment from polyunsaturated fatty acid oxidation, oxidation of amino acid side chains, or cleavage of the polypeptide backbone [78]. All of these modifications leave proteins with one or more carbonyl residues. Because the presence of carbonyl groups in a protein is relatively unique to protein oxidation, this can be exploited in their selection. Oxidized proteins and their tryptic fragments have been captured from complex mixtures by first derivatizing

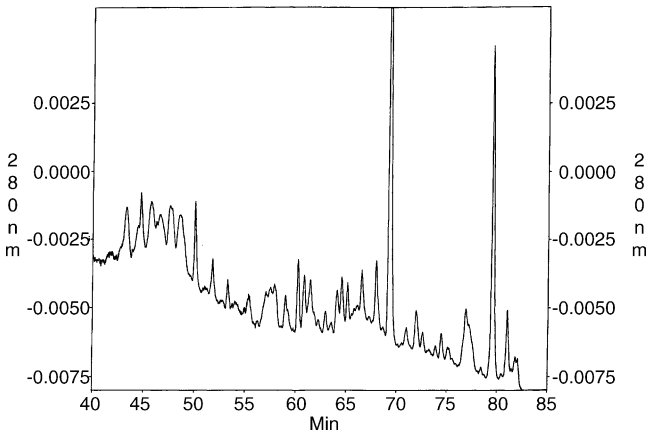

Fig. 4. This figure is a C_8 reversed phase chromatogram of yeast oxidized proteins captured via avidin selection.

their carbonyl groups with 2,4-dinitrophenyl hydrazine [79] or biotin hydrazide [80] and after reduction and removal of excess derivatizing agent the derivatized peptides were selected with either an immunosorbent or avidin, respectively. Oxidized proteins captured in this manner are further separated by RPC then identified from their unmodified tryptic peptides. Fig. 4 is a C_8 reversed phase chromatogram of yeast oxidized proteins captured via avidin selection. The nature of the oxidative modifications was examined by analysis of the tagged peptides. Fig. 5 is a tandem mass spectrum of a peptide (GKFEDMAK$^{\text{biotinylated}}$AGK) from an oxidized protein affinity selected from a liver homogenate of a rat dosed by diquat. The protein was identified to be amphoterin based on the sequence of a selected peptide and the position of oxidation assigned from the MS/MS spectrum. Again the small number of naturally occurring biotinylated proteins and peptides are also selected unless they are removed by an avidin pre-selection.

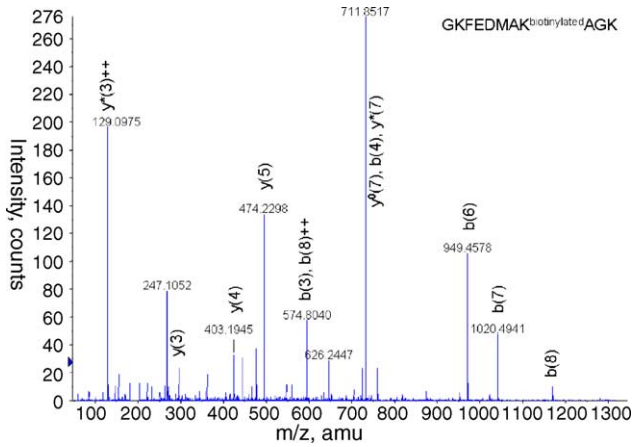

Fig. 5. A tandem mass spectrum of an oxidized peptide affinity selected from rat liver homogenate. The fragmentation pattern of the peptide indicates the presence of a lysine residue oxidized and labeled with biotin hydrazide.

9. Protein–protein interactions

Intermolecular interactions of proteins play an essential role in cellular processes. The association of polypeptide subunits to form a multimeric protein, as in the case of hemoglobin, is a simple case. At a much higher level, large numbers of proteins can interact to form a supramolecular complex as in the case of the ribosome. Roughly 70 proteins are assembled in a highly order structure in ribosomes.

A major problem in proteomics is how to recognize and identify these interacting proteins. Methods have been devised that allow protein complexes to be selected based on the assumption that protein complexes formed in vivo will either remain associated after cell lysis or can be assembled in vitro. One approach adapted from the early affinity chromatography literature is to immobilize one of the components of the complex and let other members reassemble on the surface of a sorbent [81,82]. Three lactate dehydrogenase subunits will assemble around a fourth immobilized subunit [83], for example. This is useful with multimeric proteins but is much less likely to occur with a structure as large as a ribosome. In the case of super-complexes like ribosome, antibody precipitation might be used as the method of affinity selection [84]. Immunological precipitation does not suffer from complex size restrictions as in affinity chromatography.

Molecular biology enables another powerful approach by allowing biosynthetic addition of peptide tags of specified sequence to either the N- or C-terminus of a protein. When the addition of a peptide tag does not interfere with the incorporation of a protein into a complex and the tag appears at the surface of the complex, it is possible to select the whole complex from a lysate. In this manner, the participation of all proteins involved in complex formation can be examined. Obviously it is a tedious process to tag all the proteins in a cell.

One of the major complications associated with recognizing protein–protein interactions is how to differentiate between specific and non-specific binding. This issue has been recently addressed through a method termed tandem affinity purification (TAP) [85] (Fig. 6). A fusion cassette called TAP tag was genetically engineered that codes for a fused polypeptide product containing a calmodulin binding domain, a tobacco etch virus (TEV) protease cleavage site, and an IgG binding domain from protein A. The TAP tag construct was then fused to the target protein gene and introduced into a host cell. Expressed fusion protein was recovered from host cell lysate by selection with an immobilized IgG column that binds to protein A. After removal of unbound proteins, TEV protease was added to cleave the fusion protein complex from the IgG support. The eluate was then added to a calmodulin column in the presence of calcium. This second affinity purification step removes TEV protease and non-specifically bound proteins from the first affinity selection. The final complex of target protein and associated proteins was released from the calmodulin column with EGTA and the associated proteins identified [86,87].

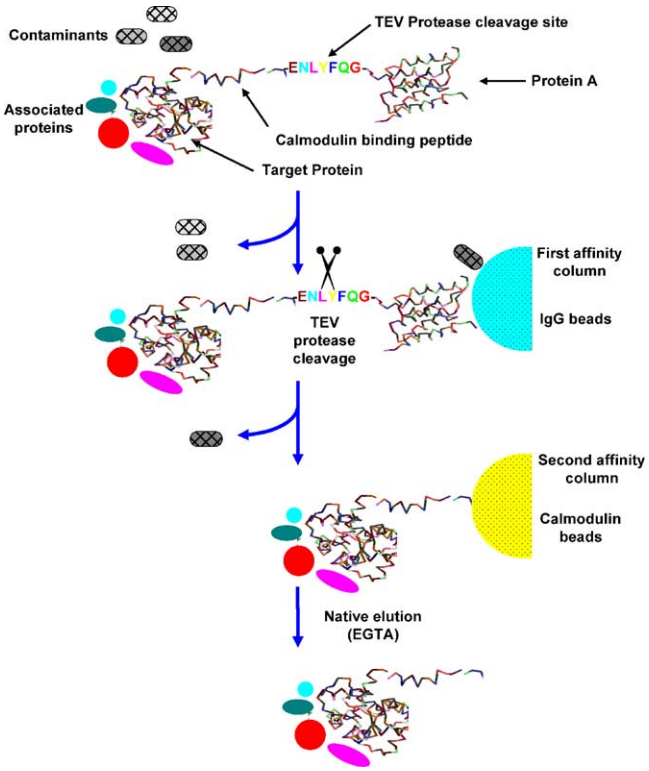

Fig. 6. An illustration of the tandom affinity selection method for the elimination of the non-specific bindings in protein–protein interaction studies. A genetically engineered fusion cassette consisting of a calmodulin-binding domain, a TEV cleavage site, and an IgG binding domain is linked to the protein of interest. After recovery of the protein complex from cell lysate by IgG affinity-selection, it is released from the first affinity column by cleavage of the TEV linker and then captured on calmodulin beads. Subsequent to removal of non-specifically bound proteins, the complex is released by the addition of Ca(II) to the mobile phase and the components characterized by conventional proteomics methods [83].

Although affinity methods enable the identification of associating proteins in a complex, they provide little information on their spatial relationship in the complex. Protein cross-linking agents can be useful in this regard. Recent studies indicate that ethanedinitrile readily permeates cells and covalently links associating salt bridges between adjacent proteins with an apparent lack of nonspecific side reactions [88]. Upon trypsin hydrolysis, peptides involved in the cross-linking reaction will have the unique feature of having two C-termini with basic amino acids. When the proteolysis of cross-linked proteins is carried out in $H_2{}^{18}O$, four moles of ^{18}O will be incorporated into all cross-linked peptides instead of the two found in other peptides [89]. (Proteolysis with trypsin is partially reversible and incorporates two moles of ^{18}O.) If the sample is divided into equal parts that are digested in $H_2{}^{16}O$ and $H_2{}^{18}O$, respectively, and then recombined, the digest will be coded in such a manner that cross-linked peptides will appear in mass spectra as doublet clusters of ions separated by 8 amu while none cross-linked peptides will be seen as doublet clusters separated by 4 amu. Cross-linked peptides are then characterized by tandem mass spectrometry to determine the parent proteins from which they were derived.

9.1. Immunoglobulin selection

Protein A from *Staphylococcus* [90] and protein G from *Streptococcus* [91] are among a small group of proteins produced by bacteria that bind to albumin and the constant region of immunoglobulins. Versions of these proteins have now been prepared in which albumin binding domains have been excised through protein engineering. When immobilized, these modified forms of proteins A and G select only immunoglobulins from complex mixtures [92]. This is very useful in the analysis of primary structure analysis and glycosylation in therapeutic antibodies.

9.2. Lectins

Both plants and animals produce proteins referred to as lectins that target oligosaccharide structures. Plant lectins serve a number of roles, including defense against fungal attack. In contrast, animal lectins are more likely to be involved in cellular adhesion or intercellular recognition. It has been noted above that lectins can be used to select classes of glycoproteins from the proteome. The opposite is also true. Immobilized glycoproteins and oligosaccharides may be used to select lectins based on their binding specificity [93].

9.3. Abundant protein removal

One of the problems in proteomics is that a small number of abundant proteins, or peptides in the case of bottom-up proteomics, interfere with the analysis of low abundance analytes. This occurs both by overloading separation systems and suppressing the ionization of low abundance peptides. Preparative immunosorbent columns are frequently used to eliminate interfering, abundant proteins [94]. The concern in abundant protein removal with immunosorbents is that low abundance proteins may bind non-specifically to the immobilized antibody or adhere to abundant proteins being captured.

10. Protein–small molecule interactions

Proteins frequently interact with molecules of a few thousand or less in molecular weight during the course of catalysis and regulation. Many enzymes have been affinity selected through immobilization of a psuedo-substrate, reaction product, inhibitors, cofactor, or allosteric effector. Inhibitors and cofactors will probably be the most useful in proteomics because they bind a broad range of proteins. For example, most enzymes that bind ATP or NAD can be selected with a column that is an AMP mimic. Cibacron blue and AMP columns have been used extensively for this purpose. Covalent inhibitors provide another avenue of selection. For example,

ATP kinases that have been covalently inhibited with fluorosulfonylbenzoyl adenosine can be selected with immunosorbents directed against the inhibitor [95]. A large number of protease inhibitors and dyes have also been used in affinity selection [96].

11. Miscellaneous selection methods

Given the success of molecularly imprinted polymers (MIPs) in structure specific selection [97], it is interesting that they have not been used in proteomics. Apparently, non-specific binding in MIPs becomes dominant in peptides exceeding a few amino acids [98]. Imprinted protein columns have been described [99] but they have not been used in proteomics.

Although not strictly a structure specific selection, proteins can be selected on the basis of bulk properties. Selection by isoelectric focusing in 2D gel electrophoresis has proven to be extremely useful in proteomics. The protein parent of peptides derived from gels can often be determined by comparing the computed isoelectric point of a protein candidate with experimentally determined behavior. The same is true of molecular weight derived from 2D gel electrophoresis.

Combining affinity chromatography with 2D gel electrophoresis is even more powerful in that a three-dimensional selection is being used.

12. Conclusions

Proteomics is dominated by the problem that sample complexity exceeds the analytical capacity of separation systems and mass spectrometers. Structure specific selection of a portion of the proteome affords a rapid method for dealing with this problem while simultaneously simplifying identification. Moreover, structure specific selection frequently allows important biochemical and disease related questions to be probed directly. Taken together these facts provide a powerful impetus for the expansion of structure specific selection in proteomics.

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