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Title: Advances in purification and separation of posttranslationally modified proteins

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Posttranslational modifications (PTMs) of proteins represent fascinating extensions of the dynamic complexity of living cells' proteomes. The results of enzymatically catalyzed or spontaneous chemical reactions, PTMs form a fourth tier in the gene – transcript – protein cascade, and contribute not only to proteins' biological functions, but also to challenges in their analysis. There have been tremendous advances in proteomics during the last decade. Identification and mapping of PTMs in proteins have improved dramatically, mainly due to constant increases in the sensitivity, speed, accuracy and resolution of mass spectrometry (MS). However, it is also becoming increasingly evident that simple gel-free shotgun MS profiling is unlikely to suffice for comprehensive detection and characterization of proteins and/or protein modifications present in low amounts. Here, we review current approaches for enriching and separating posttranslationally modified proteins, and their MS-independent detection. First, we discuss general approaches for proteome separation, fractionation and enrichment. We then consider the commonest forms of PTMs (phosphorylation, glycosylation and glycation, lipidation, methylation, acetylation, deamidation, ubiquitination and various redox modifications), and the best available methods for detecting and purifying proteins carrying these PTMs.

Key words (6):
posttranslational modification; PTM; protein enrichment; PTM detection; proteome; top-down proteomics.

1. Introduction

1.1. So few, and yet so many

Proteomes are expected to be two to three orders of magnitude more complex than would be predicted from numbers of protein-encoding genes present in the respective genomes. There are over 300 known naturally occurring amino acids, but only 64 codons in the genetic code and the actual number of proteinogenic amino acids is even lower. Altogether, 22 amino acids have been proven to be encoded by DNA. However, after protein translation these amino acids may undergo further modifications, which considerably increase the diversity of proteins present in living cells. These modifications can be either transient or permanent, and may result from either targeted, enzymatically catalyzed reactions or spontaneous chemical reactions in the cell. The Unimod Database (http://www.unimod.org; 10/2012) lists almost 1000 different protein modifications that have been detected in mass spectrometric analyses of proteins, but some of them could be artifactual, like methionine and cysteine oxidations during two-dimensional electrophoresis (2-DE), carbamylation by urea on any free amino group in the protein sample (at N-termini, or side chains of arginine or lysine), and modifications introduced by MS analysis (e.g. conversion of phosphoserine to dehydroalanine with neutral loss of phosphoric acid). The RESID database hosted by the European Bioinformatics Institute, EBI (http://www.ebi.ac.uk/RESID/; 10/2012), compiles information on protein posttranslational modifications (PTMs) found in nature and its release 70.01 (08/2012) lists 591 entries. Covalent protein modifications can be classified as a covalent addition of some chemical group, or a covalent cleavage of peptide backbones [1]. Side chain modifications are known for all of the proteinogenic amino acids except Ala and Pyr, and all can undergo crosslink reactions and/or N- or C-modification if they are at the N- or C-terminus, respectively, of their respective proteins (Fig. 1).

<-Figure 1->

2. Methods applied in PTM analyses

2.2. Protein separation or "Thou shalt not use only bottom-up proteomic approaches"

Widely used MS-based approaches in so-called "bottom-up proteomics" (involving digesting samples by selected proteases, MS analysis of the resulting peptides and finally assembly of the identified sequences) are excellent for high-throughput protein identification. However, some information is generally lost when proteins are cleaved into fragments by the chosen proteases prior to MS analysis in typical protocols. Due to the vast ranges of concentrations of proteins in cells and differences in ionization potentials of peptides, it is always challenging to obtain full sequence coverage, and even then it is very difficult to distinguish peptides from partly cleaved protein isoforms. Thus, in bottom-up approaches some of the diversity of isoforms that may result from a single initially translated protein is inevitably lost (Fig. 2). However, a PTM generally changes a protein's chemical and physical characteristics (e.g. molecular weight, shape, charge, p*I*, hydrophobicity and interactions with other proteins or ligands). Thus, proteins carrying PTMs can be separated, and the PTMs can be identified, mapped and characterized by using techniques that exploit these changes.

<-Figure 2->[2]

2.2.1. Size does matter

Protein PTM by a cleavage may result from autocatalytic activity, protease-mediated regulation, or non-enzymatic protein damage [3]. This is theoretically the easiest kind of PTM to detect, because the average molecular weight of an amino acid residue is 110 Da [4], hence losses of one or more can be readily detected by methods separating proteins according to their mass. Apart from mass spectrometry and gradient ultracentrifugation, there are two suitable techniques for estimating the mass of a protein and separating cleaved protein isoforms. The first is size-exclusion chromatography (SEC), also called gel filtration. In SEC samples are introduced in solution to a column containing a solid phase consisting of cross-linked polymer beads with pores or cavities that large proteins cannot enter. Thus, they pass rapidly through the column around the beads. In contrast, smaller proteins are retarded to size-related degrees. This technique can fractionate proteins covering a wide mass range (0.1-100 kDa), depending on the pores' size [5]. The second option is polyacrylamide gel electrophoretic separation in the presence of the denaturant sodium dodecyl sulfate (SDS-PAGE). Approximately one SDS molecule binds per two amino acids, which masks the native charge of a protein and the resulting negative net charge is proportional to the protein's mass. During the electrophoresis, the gel acts as a molecular sieve so the rate of migration of proteins in a sample is inversely related to their mass (high molecular weight proteins are retarded more than smaller proteins). The original setup presented by Laemmli [6] is not suitable for separating low molecular weight proteins (<10 kDa), but there are modifications which overcome this limit [7]. The resolution of both separation techniques allows the estimation of a protein's molecular mass with kDa precision, which means that a loss of ten amino acid residues results in a detectable shift in a protein's mobility. However, while a protein's mobility may be mainly dependent on its mass, shifts may be also due to intramolecular changes. For example, protein phosphorylation can induce calcium ion binding, which reduces SDS-PAGE mobility [8].

2.2.2. Isoelectric focusing

During isoelectric focusing (IEF), charged proteins migrate through a pH gradient. The net charge of a protein is the sum of all the negative or positive charges of the amino acid side chains, which depends on the pH environment and is zero at the protein's isoelectric point (p*I*). Thus, electrophoretic migration of a protein stops at its p*I* and all proteins are focused around their respective p*I*. This is only possible when the three-dimensional protein structure is disrupted by chaotropic agents such as urea, and all charged residues are exposed to the medium. Basic and acidic amino acid residues that contribute to a protein's net charge are often sites of PTMs, and the PTMs often change their charge. For example, acetylation masks positive charge of lysine residues, while phosphorylation or deamidation introduces an additional negative charge to a protein. Thus, PTMs are reflected in changes in a protein's p*I* and can be detected by IEF. In addition to gel-based IEF, which is suitable for analytical studies (see 2-DE), IEF can also be applied in preparative mode. Liquid-phase IEF can be used in preparative analyses, or for separating protein samples into fractions over a specific p*I* range. This technique has lower resolution than gel-based IEF and suffers from pH drifts, but it can separate membrane and hydrophobic proteins, as well as large protein complexes [9]. Off-gel electrophoresis, combining gel and liquid phase modes, provides better performance than liquid-phase IEF, but transferring samples from the gel to the liquid phase can be problematic [10]. Another option is "free flow" electrophoresis, which can also be applied in IEF mode. The method originally developed by Hannig [11] separates protein samples within a thin film of electrolyte that flows laminarly between two parallel cooling plates. After an electric field is applied (perpendicular to the flow), proteins are deflected from the direct flow based on their charge and separated into fractions.

2.2.3. Two-dimensional electrophoresis (2-DE)

Two-dimensional gel electrophoresis has been one of the most successful methods for detecting and analyzing protein PTMs. The method introduced in 1975 [12, 13] combines an IEF separation in the first dimension and an SDS-PAGE separation in the second dimension. A gel with proteins focused by IEF is treated with SDS to cover their native charges, then SDS-PAGE is applied to separate them on the basis of their molecular weights in a plane perpendicular to the plane of the first separation (Fig. 3, A). The reproducibility of 2-DE depends largely on the IEF step and has been significantly improved by using an immobilizing pH gradient [14]. Theoretically, 2-DE offers unsurpassed resolution and a standard large gel (~ 20 × 20 cm) should be able to resolve over 10,000 protein spots. In practice, a protein present in amounts below 0.5 ng is difficult to detect, even with the most sensitive detection stains (silver or fluorescent total-protein stains) and the detection limit of the widely used colloidal Coomassie dye is around 10 ng [7]. Further, PTMs increase the number of spots originating from a single protein, thus quantitative 2-DE analysis is usually restricted to sets of at most a few thousand proteins. The greatest potential of 2-DE is in qualitative analysis. The change in the mass of a protein due to a PTM is often too small to be easily detected by standard SDS-PAGE. However, many PTMs introduce charged groups into the protein, which leads to a detectable change in the position of the protein on a 2D gel. It has been shown that IEF in narrow pH ranges can separate proteins that differ in p*I* values by just 0.001 to 0.01 pH units [15]. In addition, comparisons of theoretical and observed protein p*I* and molecular masses can indicate spliced isoforms as well as proteins with PTMs (Fig. 3, B). Modified proteins can also be visualized in-gel or after Western blotting using specific stains or antibodies, which are available for all major PTMs. However, in addition to the scale limitation mentioned above, 2-DE approaches have several drawbacks. One is that they require roughly 100-fold larger samples than routine LC-MS analysis. A second problem lies in the IEF step. Hydrophobic proteins, large proteins (> 150 kDa), and proteins with extreme p*I* values cannot easily enter the gel and may be lost during this step. Furthermore, very small proteins can be washed away during protein equilibration with SDS. It is also very important to run IEF at an appropriate temperature, generally around 20 °C, otherwise the presence of urea may lead to artificial carbamylation modification of the proteins in the sample. The presence of excessive amounts of salts and buffer ions in the sample can also cause sharp temperature rises, which can promote carbamylation reactions, or even in extreme cases burn the gel strip. Further, IEF is time-consuming and some proteins may become unstable when focused too long, e.g. cysteine residues may be oxidized [16]. The problems associated with separating membrane proteins and proteins with extreme p*I* values can be circumvented by replacing IEF with blue native (BN) PAGE [17], in which proteins in a sample interact with Coomassie brilliant blue G-250 dye before their separation. Binding of the dye results in a negative surface charge, which is essential for electrophoretic separation, but does not denature proteins so their migration (and separation) is influenced not only by their mass (as in SDS-PAGE), but also by their net surface and molecular shape.

2.2.4. Liquid chromatography (LC)

Analysis by 2-DE is time consuming. The lack of automation and the necessity to extract proteins prior to MS analysis led to the development of alternative methods based on liquid chromatography. A two-dimensional column-based liquid chromatographic technique for resolving complex mixtures was initially introduced in 1978

[18], but its first application in proteomics dates to 1990 [19]. Since then the methodology has been further improved and accelerated. For example, while a separation by the original set-up took six hours, the method presented by Stoll *et al.*, (2006) requires only 30 min and provides ca. 100-fold improvement in peak capacity (the number of peaks/protein species that can be separated from one another)[20]. In standard 2D-LC proteomic separation (Fig. 3, C), proteins are separated according to their charge in the first dimension, much like IEF, except that the p*I* axis is in bands instead of continuous p*I* increments. This is done by chromatofocusing, which replaced previously used ion-exchange columns (e.g. strong cation exchange – SCX columns). A pH gradient is created as the eluting buffer, composed of a large number of buffering substances (polybuffer), titrates a weak anion exchanger chromatography medium. The protein with the highest p*I* elutes first and no protein is thus exposed to a higher pH than its own p*I*. In contrast to IEF, this method can separate large proteins and hydrophobic proteins, as well as smaller and hydrophilic proteins [21]. Reversed-phase liquid chromatography is usually used for the second dimension separation, which separates the proteins by hydrophobicity. There are numerous possible combinations of LC techniques and theoretically they could all be combined for multidimensional separation. In practice, mobile phase compatibility restricts the number of on-line modes that are suitable for proteomic analysis. The compatibility restrictions can be lessen by employment of an off-line setup in which collected fractions can be either concentrated by evaporating the buffer or diluted prior to separation in the following dimension [22]. However, only a few successful examples of this approach have been reported. The combination of chromatofocusing and size-exclusion chromatography is, in principle, similar to 2-DE separation, but it only provides comparable resolution over pH intervals of 3 pH units or less. Like 2-DE, multidimensional chromatography is capable of separating proteins carrying PTMs (e.g. [23]). However, it has been more commonly used for peptide fractionation prior to MS analysis, because there is no available technique for visualizing specific PTMs separated by multidimensional chromatography (unless affinity chromatography can be used) that provides comparable sensitivity and resolution to gel staining or Western blotting.

2.2.5. Diagonal separation

In the earliest examples of diagonal separation paper electrophoresis was used to characterize disulfide bonds in peptides[24]. Briefly, mixtures of proteins were separated on a paper strip, treated with performic acid to break disulfide bonds by oxidizing cysteine residues to cysteic acid, and then subjected to a second dimension separation. Peptides that had identical electrophoretic mobility lay along a diagonal line following the two-dimensional separation, while peptides whose disulfide bonds were disrupted lay off the diagonal. This method was later adapted for separating intact proteins in polyacrylamide gels [25], using a similar protocol to 2-DE expect that the first-dimension gels are also SDS-denaturing (Fig. 3, D). After separation, proteins containing no interpolypeptide disulfide bond(s) lie in a diagonal line, whereas disulfide-linked protein complexes are separated into individual components and resolved below the diagonal. The reduction of intrachain disulfide bonds retards migration in the second dimension and the resulting protein spots appear above the diagonal [26]. Diagonal separation has also been extended to LC applications, which are most suitable for analyzing peptides with PTMs, but can also be adapted for proteins with PTMs. In a typical experiment, a sample is fractionated by HPLC, fractions are collected, dried, treated to modify a residue or PTM and again subjected to HPLC analysis. This technique has been used to study modifications including phosphorylation, nitration and *N*-glycosylation [27-30]. The latest development in this field is an automated enzyme-based approach involving capillary electrophoresis, in which the distal end of the first capillary incorporates an enzyme-based microreactor [31]. Obviously, it is currently designed to detect modified peptides, because the immobilized enzyme requires unhindered access to the site of the PTM, but it shows promising potential for further development.

<-Figure 3->

2.2.6. Affinity separation and enrichment

The separation of proteins by means of the electrophoresis or chromatographic methods described in the previous section is based on their mass, charge or hydrophobicity. Thus, protein PTMs can be detected by the shift in net mass/charge/hydrophobicity they introduce to the modified protein. This approach is excellent if a PTM is not known, the proteins of interest are abundant, or if there is no available enrichment technique. In other cases, enrichment is highly advisable, and the main available enrichment techniques are described below.

2.2.6.1. Immunoaffinity

PTM-specific antibodies can be raised against virtually any protein that can be purified. The Human Protein Atlas Project has provided a massive array of antibodies, covering target proteins encoded by over 14,000 human genes (http://www.proteinatlas.org/;10/2012). However, it is difficult to obtain specific antibodies targeting only a PTM group and no other protein epitopes, although this problem can be circumvented (to some degree) by raising antibodies against synthetic peptides bearing PTMs or artificial substances that mimic the structure of a targeted PTM [32]. Well-designed anti-peptide antibodies provide much (but not all, as discussed below) of the specificity required for purifying protein families, e.g. substrates of a specific kinase [33]. Commercially

available antibodies cover several PTMs, including phosphorylation, acetylation, glycosylation, myristoylation, prenylation, sumoylation and ubiquitination. They are generally either cross-linked to a resin or labeled with a tag that facilitates their retrieval after a modified protein is captured and they can be used for either immunoprecipitation or in immunoaffinity columns. For example, proteins with *N*-trimethylated lysine residues can be purified by biotinylated antibodies (e.g. ImmuneChem; http://www.immunechem.com).

2.2.6.2. Ligand affinity enrichment

Some PTMs have an affinity for metal or inorganic ions. For example, immobilized Ga ions can separate sulfated peptides [34] and several combinations of metal ions and metal oxides are widely used for phosphoproteome analysis (see 3.1.2.). Naturally occurring PTMs often participate in cell signaling and protein-protein interactions. Thus, it is also possible to employ innate protein-ligand interaction systems to bind modified proteins, notably lectin-based affinity columns for glycoproteome enrichment, and media incorporating a ubiquitin-binding domain for ubiquitinome analyses (see respective parts of this review).

2.2.6.3. Derivatization

In cases where there is no suitable affinity enrichment technique, a PTM can sometimes be labeled with a tag *in vivo*, using the native cellular machinery to incorporate modified biomolecules such as tagged carbohydrates or lipids [35]. In more elaborate approaches for *in vitro* tagging, purified recombinant enzymes or chemoselective reactions can be used. Introduced labels can serve for detection (e.g. fluorophores) or isolation, usually via biotin-avidin affinity enrichment of biotin-tagged proteins/peptides. The purity of enriched PTM proteins can be further increased by including a cleavable linker in biotin tags, thus allowing bound proteins to be released without risking contamination by natively biotinylated proteins or a need for harsh denaturing conditions that may lead to the elution of non-selectively bound proteins [36]. Chemical tagging procedures are available for all major PTMs, including acetylation, acylation, prenylation, phosphorylation and gylcosylation ([35] and references therein).

2.3. Deeper mining to detect hidden proteins and PTMs

The past decade has seen huge advances in proteomics. There is no doubt that this is mainly thanks to interest in the human proteome and its apparent potential for medical applications, but all sectors of proteomics are rapidly advancing. For example, there were less than 1000 experimentally identified gene products of the model plant *Arabidopsis thaliana* in 2004 [37], but the Arabidopsis proteome pep2pro database now includes proteomic data for over 14 500 proteins (excluding proteins with only single hits) (http://fgcz-pep2pro.uzh.ch; [38]). Ordinary proteomic experiments based on 2-DE and liquid chromatography do not usually cover more than a fifth of this number, and it is estimated that alternative splicing and PTMs increase the number of different proteins to at least 100 000. Thus, the most comprehensive studies, covering several thousands of identified proteins, are only scratching the surface of the whole proteome. This is partly due to the presence of several highly abundant proteins, which interfere with the detection of less abundant proteins. Examples include: collagen, actin, myosin and keratin in many animal tissue samples; albumin, which accounts for up to 60 % of plasma proteomes; and RuBisCO, which may account for more than 40 % of the protein content in some plant tissues [39]. In marked contrast, proteins and many PTMs are frequently below the sensitivity threshold of available analytical methods, and this problem cannot (generally) be solved by crude fractionation, tissue sampling or organelle purification.

2.3.1. Immunoaffinity depletion

Several approaches are used to deplete abundant proteins in samples and increase the proteome coverage. The first option is to use immunoaffinity separation. Commercially available kits offer immunoaffinity partitioning of highly-abundant proteins, for example from plasma/serum proteomes, with antibodies for up to 20 proteins. Further, moderately abundant proteins can be further depleted by antibodies obtained from immunizing chickens with plasma proteins from which the highly-abundant proteins have been removed. This two-step procedure theoretically provides a fraction of low-abundance proteins (in the flow-through) and one of moderately abundant proteins (in the eluted fraction) [40]. Of course, this approach has limits and the price of mixed antibody columns is quite considerable.

2.3.2. Proteome equalization

The second widely used option is based on peptide affinity subtraction. This technique has been in development for over two decades, the first reports dating back to the early 1990s [41]. Its commercialized versions employ a bead-based combinatorial peptide ligand library (CPLL), each unique ligand theoretically binding a unique protein epitope. In theory, when a complex biological sample is applied to the beads, highly abundant proteins saturate their high-affinity ligand or ligands, and excess protein is washed away. In contrast, low-abundance proteins are concentrated on their specific affinity ligand or ligands. The major targets of peptide affinity subtraction are plasma and serum proteomes, but it can be used to reduce the dynamic range of proteomes from

any complex biological samples [39, 42]. It provides a relatively cheap alternative to immunoaffinity separation that has several advantages, including cross-sample/species applications. The decomposition of affinity beads also poses much minimal risks of sample contamination, unlike antibody degradation. Furthermore, samples can be further divided by sequential elution [43]. On the other hand, results of CPLL extractions depend on protein-ligand affinity interactions and are highly unpredictable. Jiang *et al*. (2010) have even presented indications that this method does not improve the quantitative results of proteomic analyses [44], but this may have been due to their use of a gel-free approach, because we have found that 2-DE benefits from a CPLL application (Fig. 4).

<-*Figure 4*->[45]

3. Major protein PTMs found in proteomic studies

Many protein modifications have a significant physiological/biological impact. Both enzymatic and non-enzymatic (chemical) PTMs occur, and with limited knowledge of their origins it is difficult to correctly distinguish their nature. Historically, the most thoroughly studied type of PTM is phosphorylation, largely due to the relative ease of detecting protein *O*-phosphorylation *in vivo* and *in vitro*. While the enzymatic origin of *O*-phosphorylation was soon accepted, *N*-phosphorylation was long considered an experimental artifact or a product of non-enzymatic activity. The existence of *N*-kinases was recognized much later [46, 47], and the possibility that this kind of PTM could occur spontaneously has still not been ruled out. A similar controversy surrounds other PTMs, for example protein *S*-ubiquitination, which is also considered to be non-enzymatic [48]. We are still far from fully understanding functions of individual gene products and it is likely that future discoveries will lead to the reevaluation of enzymatic and non-enzymatic PTMs. Some PTMs are clearly results of protein ageing, especially in proteins that are slowly metabolized, and thus are exposed for longer times to reactive endo- and exogenous compounds. However, following discovery of the role of oxidative damage in cell signaling, it is tempting to speculate that at least some of these modifications could be of enzymatic origin, even if it is through an indirect effect of metabolite production and channeling. In the following text we summarize techniques for detecting and enriching protein PTMs, focusing on their applicability for large-scale proteomic analysis. The selected PTMs (Fig. 5) represent not only the most commonly studied protein modifications, with numbers of entries in the UniProt database [49] reaching up to 10 % of all proteins currently reviewed by its host consortium, but each presents specific problems, and requires an appropriately adjusted proteomics approach for detection and enrichment. We do not strictly separate PTMs usually classified as enzymatic from non-enzymatic PTMs, partly because the methodology for a given type of PTM is easily transferable and partly because a non-enzymatic modification may result in loss of an enzymatic PTM, as believed e.g. for *N*-phosphate dephosphorylation or protein demethylation (see respective parts of the review).

<-*Figure 5*->[50, 51]

3.1 The phosphoproteome

In 1906 Leven and Alsberg discovered phosphorylation of the egg yolk protein vitellin. It took nearly 30 years to track down this phosphorylation to serine residues and a further 20 years to discover that it is actively catalyzed by enzymes [52]. Today, protein phosphorylation is probably the most intensively studied PTM. Numerous studies have demonstrated that reversible phosphorylation is a key posttranslational modification, and is involved in nearly all cell processes. It has been shown to participate in signaling and the regulation of diverse enzyme activities, protein-protein interactions and protein targeting (e.g. [53-58]). Loss of phosphorylation is also known to render some proteins susceptible to degradation, thus regulating their lifespans [59, 60]. The aim of so-called phosphoproteomics is to study protein phosphorylation by identifying phosphoproteins, quantifying phosphorylation, precisely mapping phosphorylation sites, and ultimately revealing their biological functions [61]. Potential phosphorylation sites are estimated to be approximately three times more numerous than genes and a third of expressed proteins are assumed to be phosphorylated at any given time. The largest study of the humans phosphoproteome detected 20 443 phosphosites, and the Arabidopsis phosphoprotein database PhosPhAt (http://phosphat.mpimp-golm.mpg.de; 10/2012; [62]) lists 12 613 unique phosphosites in 5663 phosphoproteins. Regulation of such extensive and dynamic phosphorylation states requires about 3 % of human genes and 5 % of the genes in *Arabidopsis thaliana*, encoding about 1100 protein kinases and 100 to 200 protein phosphatases [63]. Eight amino acids can be phosphorylated (Fig. 5, A), but in eukaryotic cells, protein kinases and phosphatases are believed to act mainly on Ser, Thr and Tyr residues, typically resulting in pSer/pThr/pTyr ratios of ca. 1800/200/1. The stability of phosphorylation at the other amino acids is lower. Phosphoramidates, i.e. phosphohistidine, phosphoarginine and phospholysine, mostly go undetected in conventional studies of protein phosphorylation because of the instability of the phosphate-nitrogen bond in acidic solutions [64, 65]. However, reversible His phosphorylation is estimated to occur in a pHis/pTyr ratio of 10/1-100/1 [63]. The stability of acyl-phosphates is even lower, but they play a role for instance in signaling pathways. While protein phosphorylation is considered to be solely catalyzed by enzymes, protein dephosphorylation may be either enzymatic or non-enzymatic, and the latter may be simply related to protein ageing. However, losses of phosphorylations, especially temperature and pH-labile phosphorylations, is also considered to participate in fast intrinsic responses to stimuli [64].

3.1.1. Visualization of phosphorylated proteins

Radioisotopes have been used since the beginning of phosphorylation research and experiments with radiolabelled ATP have confirmed that protein phosphorylation is catalyzed by enzymes. This previously widely used method is based on use of [^{32}P] labeled γ-ATP as a substrate for *in vitro* kinase reactions followed by separation of labeled phosphorylated peptides by thin layer chromatography (TLC). Many phosphoproteins can be visualized by autoradiography, but they are not usually present in sufficient amounts to be identified [52, 66]. In addition, radiolabeling *in vivo* is problematic and cannot be used to follow natural phosphoproteome

dynamics. Legal and safety restrictions further limit the use of radiolabeled substances and this methodology is currently in decline, being replaced by immunodetection or fluorescent staining. Increasing numbers of commercially available antibodies can be used for phosphoprotein detection in Western blot analysis (suppliers include Qiagen (http://www.qiagen.com), Santa Cruz Biotechnology (http://www.scbt.com/) and Life Technologies (http://www. lifetechnologies.com). Their use suffers from all disadvantages of antibody-based methods, i.e. they are rather expensive and have questionable specificity. Further, anti-phospo-PTM antibodies are only currently available for pTyr, pSer and pThr. The first fluorescent stain that allowed direct, in-gel detection of phosphate groups attached to tyrosine, serine, or threonine residues (without the need for antibodies or radioisotopes) was Pro-Q Diamond (Life Technologies). It can be used to label proteins in standard SDS-polyacrylamide gels or 2-D gels and the staining procedure has been optimized [67]. The second florescent dye is based on Phos-tag (http://www.phos-tag.com/; [68]), an alkoxide-bridged dinuclear metal (usually Zn^{2+} and Mn^{2+}) complex. These tags preferentially capture phosphomonoester dianions bound to Ser, Thr and Tyr residues. The detection is based either on a mobility shift of phosphorylated proteins in SDS-PAGE (polyacrylamide-bound tags) or tag-reporter conjugates [61, 69]. The fluorescent Phos-tag™ Phosphoprotein Gel Stain produced by Perkin Elmer (www.perkinelmer.com) exploited this technique, but it is no longer available. The only Phos-tag based detection kit currently on the market is a biotin-tagged version for Western blotting, Wako Pure Chemical Industries (http://www.wako-chem.co.jp). These phosphospecific stains have certified ability to detect pThr, pSer and pTyr, but can also detect pAsp and *N*-phosphates [70]. The detection limit of florescent stains is reportedly 1-16 ng of phosphoprotein; 5-20× the sensitivity of techniques based on chemical derivatizations, e.g. by the GelCode Phosphoprotein Staining Kit (Pierce, www.piercenet.com). Phosphospecific stainings share several of the disadvantages of phospho-specific antibodies: their prices are high and their specificity must be usually validated by normalization to a negative control. Furthermore, phosphorylation in proteins detected by phosphostaining is often not detectable by MS analysis, indicating that the stains yield false positives, although this could be at least partly due to their ability to mark labile *N*-phosphates, which escape detection by conventional approaches [65].

3.1.2. Phosphoproteome enrichment by affinity chromatography

The method of choice for identifying phosphorylation site is mass spectrometry, which has replaced Edman degradation. However, the abundance of many signaling proteins is low in cells (even relative to the attomolar sensitivity of modern MS systems), and the proportions of these proteins that are phosphorylated at any given time may also be quite low. Therefore, in a standard LC-MS/MS experiment, the chance of detecting and sequencing many phosphopeptides is quite low [52]. A number of methods to enrich the phosphorylated fraction in samples and thus overcome these problems have been developed. Immobilized metal ion affinity chromatography (IMAC) was originally introduced, in 1975, for purifying native proteins with an intrinsic affinity for metal ions [71] and was focused on isolating His-tagged proteins. The ability of phosphoserine (in phosphorylated ovalbumin) to bind selectively to immobilized iron was demonstrated in 1986 [72]. This is based on the affinity of the negatively charged phosphate group for metal ions (usually Fe^{3+} or Ga^{3+}), but they co-chelate proteins with surface histidines, cysteines or tryptophans. Bound proteins are eluted by introducing excess free ligand (phosphate buffer), or by raising the pH (e.g. by adding NH_4OH), which promotes their dissociation from the IMAC matrix. Non-specific binding of acidic proteins and peptides to the IMAC matrix can be partly circumvented by chemical derivatization of the sample (esterification of carboxyl groups) or adding acetate to the sample. Metal oxide affinity chromatography (MOAC) was introduced for analyzing phosphorylated substances in 1990 [73] and is currently the technique of choice for enriching phosphopeptides resulting from the tryptic digestion of phosphoproteins prior to MS analysis. Two kinds of beads are commonly used: ZrO_2 and TiO_2, which reportedly have specificity (or at least some selectivity) for singly- and multi-phosphorylated peptides, respectively [74]. This methodology has been well optimized (see e.g. [75]), but it is not frequently used for intact proteins. Aluminum hydroxide ($Al(OH)_3$) matrices have also been used for MOAC phosphoprotein enrichment. These matrices are relatively inexpensive and provide comparable yields to those obtained using commercial phosphoprotein purification kits [76]. The numbers of IMAC- and MOAC-based methods are constantly increasing. For example, Ti^{4+}-IMAC and Polymer-based metal ion affinity capture (PolyMAC) techniques have been reported, both of which exploit the titanium atom's ability to bind phosphopeptides [**77**, 78]. Commercial kits with single-use columns and buffers are also now available to explore the broadening avenues of phosphoproteomics. The manufacturers do not describe the composition of the affinity matrices in their kits, except those based on affinity to phosphospecific dyes (Phos-tag agarose, Wako; Pro-Q Diamond Phosphoprotein Enrichment Kit, Life Technologies). However, the similarity of purification results obtained using these kits to those obtained using IMAC/MOAC matrices (e.g. [76]) suggests that they are also probably based on IMAC/MOAC technology. Chromatofocusing and ion exchange chromatography are options that can be used to selectively enrich acidic proteins from a sample. As phosphoproteins are generally acidic, these methods can be used for crude fractionation of proteomes. Even separating differentially phosphorylated protein isoforms is possible, provided the samples are not too complex

[21]. The last phosphoprotein-enrichment method that should be mentioned is hydroxyapatite chromatography; a type of "pseudo-affinity" chromatography or "mixed-mode" ion exchange using a form of calcium phosphate with the formula $Ca_{10}(PO_4)_6(OH)_2$. Several types of hydroxyapatite resins have been designed for protein and DNA separation. Hydroxyapatite has high loading capacity and proteins are eluted by increasing the concentration of phosphate in the elution buffer. This form of chromatography is also not entirely phosphoprotein-specific, as the carboxyl moieties of protein interact with the resin in a similar way (albeit more weakly) to phosphoresidues, but it has been frequently applied for separating proteins that differ only in their phosphorylation (e.g. [54]).

3.1.3. Uses of antibodies and derivatization in phosphoproteome analyses

Antibodies raised against phosphorylated peptide sequences can be used to immunoprecipitate targeted phosphoproteins from a cell lysate. Phosphotyrosine antibodies have been available for several years, and pSer/pThr antibodies are also commercially available, but they reportedly have lower specificity [79]. Producing anti-phosphohistidine or antiphosphoaspartate antibodies is problematic due to instability of these phosphorylations. However, many commercially available anti-phosphotyrosine Ig antibodies also recognize phosphohistidine [64]. Immunoprecipitation is unsuitable for large-scale phosphoproteome studies as several parallel immunoprecipitation reactions are required for each phosphoamino acid, but it can be beneficial if specific phospho-site enrichment is desired [80]. Several derivatization-based phosphoprotein enrichment methods have been developed, but they involve several chemical reactions that are not 100 % efficient. Thus, yields are limited. Further, their specificity is also questionable. For example, replacement of phosphate groups with biotinylated tags [81] does not work with pTyr, but can modify Ser/Thr *O*-glycosylated forms or even non-modified residues [82]. Due to the uncertainty of reaction specificity, the wider spread of derivatization-based techniques for phosphoproteome analyses seems unlikely.

3.1.4. The future of phosphoproteomics

The plethora of methods available for isolating phosphoproteomes does not simplify the task. Many studies indicate that different approaches provide complementary rather than overlapping coverage of captured phosphoproteins/peptides. Further, labile phosphorylations cannot be robustly identified, mapped and characterized using current methodology. Only time will tell which techniques, combinations of approaches or novel procedures will prevail. A promising approach seems to be an automated diagonal capillary electrophoresis technique based on use of immobilized phosphatase, which has demonstrated utility for handling a mixture of phosphopeptides [31]. A similar approach could be theoretically extended to phosphoprotein samples. Of course, the susceptibility of individual phosphoproteins to the phosphatases applied and increased sample complexity would be limiting factors. Today, the best enrichment approach for large-scale phosphoproteome studies seems to be a two-step procedure, starting with phosphoprotein enrichment (using aluminum hydroxide chromatography, for example) followed by digestion and subsequent phosphopeptide enrichment using IMAC or MOAC resins [83].

3.2. Redox proteomics

Numerous posttranslational modifications are caused by chemically reactive species such as reactive oxygen and nitrogen species (ROS/RNS) that are produced via cellular activities (especially, but not solely, under "oxidative stress conditions", when the reductive capacity of the cell's antioxidant systems is exceeded), and are capable of damaging proteins, lipids, carbohydrates and nucleic acids. The reactions involved may be purely non-enzymatic, e.g. in uncontrolled responses to excessive energy absorption during photosynthesis, but reactive species can also be generated to damaging levels in processes linked to physiological cell defense and signaling processes [84, 85]. Proteins are considered to be major targets of the oxidative species, accounting for 68 % of oxidized molecules in the cell [86]. Oxidative protein modifications may be either reversible or irreversible, and may result from either direct oxidation of amino acid residues or the formation of reactive intermediates. Thiol side chains of cysteine residues are particularly susceptible to redox modifications and cysteine residues are attracting increasing research attention. However, protein oxidation processes are very complex and redox proteomics encompassess the full spectrum of PTMs induced by radicals. These can be divided into modifications that alter side chains of amino acids, and modifications altering protein backbones, causing fragmentation and cross-linking [87]. Moreover, non-protein components of cells like nucleic acids, lipids or carbohydrates activated by interaction with reactive species may react with a protein, which further extends the variability of the redox proteome [88, 89]. Here, we focus on cysteine oxidative PTMs, methionine oxidation, protein nitration and carbonylation. Protein glycation and protein deamidation are discussed in separate chapters.

3.2.1. Cysteine oxidative modifications

The amino acid that is the most common site of known PTMs is cysteine (Fig. 1). Its oxidative PTMs are commonly involved in redox signaling and reactive thiol side chains can act as sensors or switches in both signal

transduction and enzyme regulation [90]. Depending on their local concentrations, ROS/RNS can react with cysteine to form several reversible or irreversible modifications (Fig. 5, C). Cysteine oxidative modifications are probably the best characterized redox PTMs to date, and a dedicated database of experimentally verified protein oxidative modifications (RedoxDB) has been established [91].

3.2.1.1. Visualization of modified cysteine residues

The abundance of cysteine in proteins hinders direct detection of modified residues by *in vivo* [^{35}S]cysteine radiolabeling. Several approaches for studying protein glutathionylation involving inhibition of protein biosynthesis and incorporation of radiolabeled cysteine into glutathione [92] have been developed, but they severely disrupt cell homeostasis. However, combining protein extraction with thiol (radio)labeling *in vitro* is feasible. The easiest method available for detecting disulfide bonds is by electrophoretic diagonal separation under first nonreducing and then reducing conditions (see Fig. 3, D). In principle, further disulfide labeling is not essential, but the sensitivity of the procedure is increased. All reversibly oxidized protein thiols can be switched to labeled thiols [93], as follows. First, the background labeling of unmodified cysteine residues is minimized by blocking free thiols using thiol-specific reagents such as maleimides, iodoacetic acid or iodoacetamide. Oxidized thiols are then reduced using modification-appropriate agents (e.g. ascorbate for nitrosylation, arsenite for sulfenylation, hydroxylamine for acylation, tris(2-carboxyethyl)phosphine for disulfides, and oxidoretuctase GRX treatment for glutathionylation), and the newly exposed thiols are simultaneously labeled with a thiol-reactive biotin [92, 94]. The biotin switch method can be used for both enrichment and detection. Once the modified sites have been labeled with the stable biotin group, they can be easily detected by anti-biotin immunoblotting, or enriched by streptavidin affinity chromatography. Alternatively to biotin replacement, reduced cysteine residues can be labeled with a fluorescent dye (redox florescence switching), then readily separated and visualized using 2-DE (particularly the variant DIGE, difference gel electrophoresis, which allows the resolution of several differentially labeled samples in a single 2D-PAGE experiment). Proteins are labeled by Cy3 and Cy5 fluorescent dyes, which have a maleimide group that forms a thioether bond with the free thiols of cysteines in proteins. Comparison of gels stained with these dyes and a total protein stain (e.g. Sypro Ruby) provides exact quantitative and qualitative information about cysteine PTMs in analyzed sample [95]). Some cysteine PTMs can be also detected by specific antibodies. Antibodies have been raised against S-glutathionylated proteins, sulfenic and sulfonic acid PTMs. However, their use has been limited to targeting and assaying the modification status of individual proteins, and they have not yet been used in any published large-scale proteomic analysis [94, 96, 97].

3.2.1.2. Enrichment of proteins containing modified cysteine residues

The instability of redox cysteine PTMs often poses experimental challenges and thiol-disulfide exchange between proteins during isolation/enrichment steps may lead to misinterpretation of results. Hence, it is important to minimize changes in the thioldisulfide status of samples (e.g. alkylation of free cysteine residues) during enrichment steps. The alkylation followed by a "biotin switch" method described above is currently the most commonly used technique for purifying *S*-nitrosylated proteins and can be applied for analyzing any class of cysteine oxidative PTM for which a suitable reducing agent is available. However, the specificity of reducing agents is also a limiting factor here. It has been argued that even the selectivity of the ascorbate method for S-nitrosylation might be compromised by ascorbate-mediated reduction of disulfides [98]. On the other hand, this technique is well established and various useful modifications have been developed. For example, Foresetre *et al*. replaced the biotin tagging with fast simultaneous labeling and capture using dipyridyl disulfide coupled to a resin, which can be followed by direct on-resin digestion and LC-MS/MS analysis [99]. This approach is similar to activated thiol sepharose chromatography, where the pyridyldithiol group is the moiety that reacts with reduced thiol groups and binds them. However, the binding efficiency of thiol sepharose for different proteins may vary, and this method has yet to be further validated [98]. Proteins with reactive disulfides can also be efficiently trapped by thioredoxin (Trx) affinity chromatography. Natural Trx has two cysteine residues in its active center, the first catalyzing formation of a mixed disulfide intermediate between Trx and its substrate, and the second cleavage of this short-lived bond by a nucleophilic attack. If the second cysteine residue is replaced by serine, the mutated Trx can be used to capture the disulfide proteome, and bound proteins can be subsequently released by treatment with dithiothreitol [100]. Some proteins can bind to Trx noncovalently in a protein-protein interaction manner, but this can be circumvented by collecting Trx-protein complexes and resolving them by nonreducing/reducing diagonal electrophoresis [101]. Trx-based enrichment is specific for Trx substrates, but not all disulfides are accessible to Trx and affinities may differ even among those that do bind [98, 100]. Thus, Trx affinity chromatography is not considered suitable for global redox-proteomics experiments.

Glutathionylation results in PTMs that are relatively stable, compared to other oxidative PTMs, and in addition to the "biotin switch" method they can be targeted by specific antibodies, or by supplying a biotin-labeled glutathione ester to investigated cells [102, 103]. Proteins that be potentially glutathionylated can also be

captured with agarose-bound glutathione [104], but this method does not help in monitoring the redox status of a cell. The latest promising strategy is on the border between protein- and peptide-based analyses. It employs two different alkylation agents, the first to protect free thiols prior to SDS-PAGE protein separation, and the second after oxidized cysteine in-gel reduction. Proteins are subsequently digested in-gel and peptides are subjected to $^{16}O/^{18}O$-labeling to differentiate controls and oxidized samples. Peptide-based comparison is then used for simultaneous analysis of dynamic alterations in the redox state of cysteine sites and protein abundance [105]. However, to address low-abundance proteins, this technique has to be combined with further enrichment steps.

3.2.2. Tyrosine and tryptophan nitration

Reactive nitrogen species are generated in various physiological processes, including mammalian inflammatory responses. The most common products of protein nitration are nitrotyrosine, and to a much lesser extent, nitrotryptophan (Fig. 5, B)[106]. Nitrotyrosine can be produced from the reaction between tyrosine and a strong nitrating agent like peroxynitrite and it is considered a general biomarker of disease progression [107]. In low complexity samples, nitrotyrosine can be traced by UV-Vis photometry. Like tyrosine, it has an absorption peak at 280 nm, but nitrotyrosine exhibits additional absorption peaks at ~ 357 and ~ 430 nm in acidic and basic solutions, respectively. Yang *et al*. (2010) exploited this technique to follow the kinetics of protein nitration by coupling HPLC with photo-diode array detection [108]. Similarly, nitrotryptophan formation can be detected by spectrophotometry. Tryptophan exhibits the strongest fluorescence of any proteinaceous amino acid, with an excitation maximum at 280 nm and an emission maximum between 305 nm and 355 nm, and its nitration leads to fluorescence quenching. Both nitrotyrosine and nitrotryptophan can be detected and/or enriched using specific antibodies [109] or derivatized. The derivatization method involves blocking all primary amines, reducing nitro-residues to their amino- counterparts and selectively attaching an affinity handle (e.g. biotin) for enrichment or a reporter group for selective mass spectrometric detection [106, 110].

3.2.3. Methionine oxidation

Like cysteine, the other proteinaceous sulfur-containing amino acid (methionine) is also readily oxidized, usually yielding methionine sulfoxide and methionine sulfone (Fig. 5, D). Sulfoxide formation can be enzymatically reversed by the action of specific reductases and it has been suggested that methionine could serve as an antioxidant that protects other amino acid residues from more deleterious damage [111]. While this possibility cannot be excluded, it has been shown that methionine oxidation can result in loss of a protein function or interference with other PTMs close to the oxidized site [112, 113]. Although methionine oxidation is detected in biological samples relatively often, thorough proteomic methodology for its characterization is still in development. Anti-methionine sulfoxide antibodies have been raised against oxidized methionine-rich zein protein [114], but a recent report indicates that their specificity is questionable [115]. Thus, it seems that the only robust options for analyzing oxidized methionine are peptide-based approaches, which can be based on the 16-Da mass increase upon oxidation [116]. An advanced peptide-based method is diagonal chromatography (see section 2.2.5.), with enzymatic reduction of methionine sulfoxides by methionine sulfoxide reductase [117]. This method has been used to identify 35 *in vivo* methionine oxidations in the mouse serum proteome and could perhaps also be adapted for protein-level analyses.

3.2.4. Protein carbonylation

Carbonylation is considered to be the third most common oxidative PTM, after cysteine and methionine oxidation. Direct protein carbonylation predominantly affects side chains of lysine, arginine, proline and threonine residues [118]. These oxidations are believed to be irreversible and result in glutamic semialdehyde (arginine and proline), 2-amino-3-ketobutyric acid (threonine) or aminoadipic semialdehyde (lysine) (Fig. 5, G) [119-122]. Indirect protein carbonylation is more prevalent and involves a reaction with oxidized AGE (advanced glycation end) products (described in glycoproteomics, section 3.4), or hydroxyl radical-mediated oxidation of lipids. Lipid-derived aldehydes can form Michael adducts with cysteine, histidine, lysine and arginine, and in some cases can further undergo Schiff base formation with amines of adjacent lysines, producing cross-linked amino acids[120, 123, 124]. Analysis of the resulting PTM diversity is hindered by their instability because carbonyl groups readily undergo Schiff base formation with proximate lysine residues, even when stored at -80 °C [122].

3.2.4.1. Isolation and detection of carbonylated proteins

To date, 2-DE analysis has been used in most proteomic studies of protein carbonylation [125]. For detection, reactive carbonyls can be reduced by [^{3}H]sodium borohydride to corresponding tritium labeled alcohols, but most methods rely on covalent labeling with nucleophilic hydrazide- or hydrazine- based probes [126]. Originally a qualitative analytical method used in organic chemistry, based on the formation of nitrogen-carbon bonds (hydrazone) between carbonyls and dinitrophenylhydrazine (DNPH) was adapted to enhance the isolation, identification and quantification of carbonyl-containing proteins. In this approach, derivatized proteins are

detected/captured by DNPH-targeting antibodies [120, 127]. DNPH can also reportedly react with thioaldehyde derived from sulfenic acid [128], but no enrichment of corresponding methionine or tryptophan oxidation products has been observed as yet [125]. Biotin-based hydrazides can be used instead of DNPH and derivatized proteins are then captured by biotin-avidin affinity chromatography (e.g. [125, 129]). However, the binding efficiency of biotin-based tags seems to vary considerably, and the most commonly used (biotin hydrazide) gives the lowest yield [130]. Carbonylated peptides have been successfully enriched by derivatization with Girard's P reagent, which forms hydrazones with peptide carbonyl groups and contains a quaternary amine group that is selectable by SCX chromatography [131]. However, this methodology is unlikely to achieve comparable utility in analysis of intact proteins mainly because of, in general, modest influence of the newly generated positive charge on net charge of the respective protein.

3.2.5. Troublesome path of redox proteomics

Unfortunately, current experimental approaches are not able to deal robustly with the diverse spectrum of PTMs introduced by reactive species. While we are capable of detecting abundant PTMs, using 2-DE and mass spectrometry, for instance, targeted detection and enrichment techniques are available only for specific types of redox PTMs. Further, it might be difficult to distinguish between biologically relevant PTMs and artifacts introduced during an analysis. For example, tryptophan oxidation is considered to be a natural irreversible PTM, but it has been recently found that kynurenine and *N*-formyl kynurenine tryptophan oxidation products can be introduced into a protein during SDS-PAGE separation [132].

3.3. Protein lipidation

Covalent attachment of lipids controls the subcellular localization and activity of diverse proteins. The most common lipidation processes are prenylation, acylation and glycosyl phosphatidylinositol anchor (GPI) attachment (Fig. 5, E) [1, 133-135]. Farnesyl and geranylgeranyl-pyrophosphate molecules can be utilized as electrophilic alkyl donors in posttranslational protein prenylation of conserved cysteine residues at or near the C-terminus of proteins. These PTM types, which were first reported in 1984 [136], are catalyzed by farnesyl transferase or geranylgeranyl transferase at the conserved sequence Cys-a-a-X (where a is an alifatic residue and X any residue), and have been shown to have profound effects on a host of processes involving signal transduction and intracellular trafficking [137-139]. Protein acylation classes of lipidation PTMs include *N*-myristoylation at N-terminal glycine residues and *S*-acylation of cysteine residues by addition of palmitic acid or other long chain fatty acids. The latter is an important mechanism for dynamic targeting of proteins to membranes, and the labile, reversible thioester linkages are enzymatically formed and cleaved by acyltransferases and protein thioesterases, respectively [135, 140]. Non-enzymatic *S*-acylations are also possible, but apart from modifications reported in mitochondria, these reactions probably occur only in vitro [141]. *N*-myristoylation is a cotranslational modification catalyzed by *N*-myristoyltransferases at the conserved sequence Gly-X-X-X-(Ser/Thr/Cys) after cotranslational hydrolysis of the Met_1–Gly_2 bond by methionine aminopeptidase. However, it can occur posttranslationally if an internal glycine residue is exposed upon protease-catalyzed cleavage [1, 135]. Attachment of GPI (which consists of a phosphoethanolamine linker, a glycan core, and phosphatidyl inositol tail) at the C-terminus of a eukaryotic protein results in its plasma membrane localization [142]. Other lipid PTMs, including *O*-acylation and *C*-cholesterylation, have been reported, but they do not seem to be widespread [143, 144].

3.3.1. Analysis of protein lipidation

Attachment of a lipid moiety increases a protein's hydrophobicity, which restricts the methods that can be used to separate lipidated proteins. Unless lipid PTMs are removed, the best methods for resolving lipidated proteomes are multidimensional liquid chromatography or BN-PAGE. However, if the aim of an analysis is solely to identify proteins undergoing lipidation, they can be enriched then released by enzymatic or chemical cleavage. For example, GPI-modified membrane anchored proteins can be enriched from soluble proteins by Triton X-114 partitioning [145], as they separate into the micelles fraction with other membrane-associated proteins. Subsequent cleavage of the GPI anchors by phosphatidylinositol-specific phospholipase C releases these proteins into the soluble fraction, from which they can be separated by repeated Triton partitioning. However, this technique does not provide information about GPI composition, or sites of GPI attachment in the proteins. Moreover, some GPI-anchored proteins are phospholipase-insensitive due to lipid remodeling within the GPI anchor [146]. However, GPI contains glucosamine, which is generally rare in other glycoproteins. Thus, instead of enzymatic cleavage, nitrous acid deamination can be used to break the glycosidic bond between glucosamine and the myo-inositol ring, resulting in cleavage of the lipid portion of the GPI anchor [147].

3.3.2. Detection and enrichment of lipidated proteins

Like the first methods for detecting most of the long-known protein PTMs, the first methods for detecting protein lipidation were based on radiolabeling, more specifically feeding [^{3}H] and [^{125}I]-labeled fatty acids,

followed by protein separation and autoradiography. This is still one of the methods of choice for confirming the presence of a lipid moiety in a protein since it is not based on indirect detection [135, 138]. In addition, due to the paucity of lipid antigenicity relatively few antibodies that recognize specific lipids have been described [148]. Antibodies for specific GPIs are known (e.g. the immune response to malaria involves the production of anti-GPI antibodies that recognize the unique plasmodium glycan modifications in the *Plasmodium falciparum* GPI anchor), but it remains to be shown if any anti-lipid antibodies could be applied for large-scale protein lipidation analyses. The "Biotin-switch" method described above for cysteine PTMs (see section 3.2.1) has been successfully applied for detecting *S*-acylation (with hydroxylamine as a reducing agent), but there are no reports of a modified version for detecting other types of protein lipidation PTMs. Instead, a new approach has emerged, based on metabolic labeling with lipid analogs containing chemical groups that are not present in biological samples. These chemical groups, e.g. alkyne or azide, can be then targeted either *in vitro* or *in vivo* by "bioorthogonal" chemical reactions, such as Staudinger ligation [149] or copper-catalyzed azide-alkyne cycloaddition (CuAAC) [150, 151]. However, as detection systems these chemical reporters are several orders of magnitude less sensitive than antibody-based systems, and the high concentrations of reactants that would perturb cellular homeostasis severely limits application of this strategy *in vivo* [152]. Nevertheless, azide- and alkyne-functionalized lipid chemical reporters have already allowed tremendous advances in the detection of protein *S*-acylation [153-155], *N*-myristoylation [156] and prenylation [157]. After protein extraction, the chemical reporters are reacted with fluorescent or biotinylated tags for in-gel detection or avidin-biotin enrichment, respectively [135, 158]. The major drawbacks of this technique are the necessity of metabolic labeling, which means that it cannot be applied to characterize standard samples, and the possibility that the unnatural substrates used may be processed by cell enzymes in different ways from natural counterparts. Further, for efficient labeling of prenylated proteins prior to prenylome profiling, the endogenous isoprenoid pool has to be depleted with statins, which precludes comparative studies of different cellular states and analysis of regulatory mechanisms without significant metabolic perturbation of cells [159]. An alternative to bioorthogonal reactions is *in vitro* labeling, which requires use of engineered enzymes with modified substrate specificity, e.g. a protein prenyltransferase with biotin-geranylpyrophosphate specificity for prenylome analysis [160]. Such a modified enzyme can be also introduced into cells, but the technique is better suited for *in vitro* detection of potential protein lipidation targets.

3.4. Glycoproteomics

In 1857 the French physiologist Claude Bernard described "glycogenous matter" in liver as a storage form of glucose. The possible existence of a protein component of glycogen was reported just three decades later, but its presence was only accepted after nearly a century of further research. Nevertheless, we can consider glycogen, with its protein core glycogenin, to be the first studied glycoprotein [161, 162]. Today, we know that proteins can undergo covalent modification by carbohydrates via non-enzymatic glycation or enzymatic *N*-glycosylation, *O*-glycosylation and *C*-glycosylation (Fig. 5, H). These PTMs play important roles in many key biological processes, including protein folding, signaling, enzymatic activity, molecular trafficking, cell adhesion and protein turnover [163, 164]. Glycosylation is believed to be more prevalent than phosphorylation and it is estimated that at least 50 % of all proteins are modified by carbohydrates [163]. *N*-linked glycosylation is by far the most thoroughly studied form of carbohydrate PTM. It predominantly involves cotranslational attachment of a glycosyl moiety to the carboxamide nitrogen atoms of asparagine, almost always in the consensus sequence Asn-X-Ser/Thr (where X is any amino acid residue except proline), by *N*-glycosyltransferases in the lumen of the endoplasmic reticulum and Golgi apparatus [165]. *O*-glycosylation has been frequently documented at serine and threonine residues, less frequently at hydroxyproline and hydroxylysine sites, and very rarely at tyrosine sites. The glycosyl moieties involved are generally shorter and less complex than *N*-glycosyl moieties, which may make it more suitable for reversible regulation by specific transferases and hydrolases. In further contrast to *N*- glycosylation, there is no known conserved sequence motif for *O*-glycosylation [1, 164]. *C*-glycosylation is rare, and has only been found at tryptophan residues, the C2 position of the indole ring being a mannosylation target [163]. Non-enzymatic protein glycation may occur at lysine or arginine amino groups, or the N-terminal amino acid residues of proteins. In the process, the aldehyde group of the open chain of a reducing sugar condenses with an amino group to form a Shiff base, and then an Amadori rearrangement of the double bond to the C2 of the sugar occurs, yielding a stable, covalently linked ketoamine compound (early glycation product This process may slowly and spontaneously revert, thereby regenerating the free sugar and the amine, or the glyco-portion may undergo a further series of reactions leading to the formation of various heterogeneous structures commonly known as advanced glycation end-products (AGE) [166].

The attachment of sugar residues can generate greater structural diversity than any other kind of protein PTM. While three different amino acids can theoretically generate six different peptide-bond trimers, three different hexoses could produce anywhere from 1,056 to 27,648 unique trisaccharides; a massive difference [162]. In nature, the sheer number of combinations is limited by the specificity of glycosyltransferases, but the diversity of

mature glycan chains is still considerable and the remarkable variation in the attached oligosaccharide chains is the major obstacle in glycoproteomics. For this reason, large-scale analyses have typically focused either on characterization of the protein parts and mapping the PTM sites, or characterization of the glycan structures. The latter is beyond the scope of this review and for more information the reader is referred elsewhere, e.g. the excellent tutorial by An and Lebrilla (2011)[167]. Here, we focus on general approaches to detect and enrich glycoproteins from a complex sample.

3.4.1. Visualizing a glycoproteome

Detection of carbohydrate protein PTMs can be facilitated by metabolite labeling with either radioactive or chemically derivatized monosaccharides. *In vivo* treatment with $[^3H]$- or $[^{14}C]$-labeled glycan precursors followed by electrophoretic separation allows autoradiographic detection of any glycoprotein into which they are incorporated [168]. Alternatively, cells can be fed a bioorthogonal chemical tag, usually an azido analog, that allows chemoselective ligation and hence specific enrichment or detection of glycoconjugates in the same manner as described above for lipid conjugates [169]. Both of these approaches can be utilized *in vitro* for detecting potential glycosylation targets using recombinant glucosyltransferases, or for analyzing spontaneous non-enzymatic glycation, because the generation of early glycation products with proteins occurs at a rate proportional to the reducing sugar concentration. Glycoproteins resolved by 2-DE or SDS-PAGE can be visualized by specific staining, most classically by a periodic acid-Schiff stain (PAS) procedure, in which glycans' vicinal diols are oxidized to aldehydes then reacted with Schiff reagent, yielding pink to magenta adducts [170]. The detection limit is reportedly 25-100 ng for free carbohydrates, but the reported sensitivity for glycoproteins is in the range 1-10 μg [171]. Sensitivity has been slightly improved by combining the PAS reaction with alcian blue or dansyl chloride staining, or by tagging periodate-oxidized aldehydes with biotin hydrazide and detecting the biotin (by streptavidin-bound peroxidase for instance), but these labeling procedures are seldom utilized in practice [171-173]. The fluorescent dye Pro-Q Emerald (Life Technologies) also reacts with periodic acid-oxidized carbohydrate groups, but the protocols are more rapid than previously mentioned PAS-based labeling procedures and reportedly at least 50-fold more sensitive [174]. Similar alternatives have been produced, for example by Sigma (Glycoprofile III fluorescent kit; discontinued) and Pierce (Krypton Glycoprotein staining kit) [172]. Specific protein glycosylation PTMs can be detected after electrophoretic separation by Western blotting using glycan-specific antibodies, or overlays with radio-labeled or enzyme-conjugated lectins. Lectins are natural proteins characterized by their ability to selectively bind carbohydrate ligands. Most lectins bind reversibly, do not modify carbohydrate covalent structures, and are non-immune in origin [175]. Hundreds of lectins have been described and more than 60 are commercially available, with specificity ranging from very broad (e.g. Concanavalin A binds *N*-glycoproteins by recognizing a shared trimannosyl motif of the *N*-glycan core structure) to very narrow (e.g. *Sambucus nigra* lectin is specific for sialic acid attached to galactose/*N*-acetylgalactosamine by an (α 2-6) linkage [176](See Tab. 1). Lectin-based detection is also employed in so-called "lectin microarrays", in which discreet fractions of a proteome are usually spotted onto nitrocellulose substrates and the resulting array is subsequently incubated with a specific lectin. An alternative antibody-lectin sandwich version captures specific glycoproteins by antibodies and hybridized arrays are then incubated with fluorescently or biotin-labeled lectins for detection. This approach is primarily used for detecting and comparing differences in glycan structures, but MS can be used to identify the glycoproteins within a sample [177-179]. Glycoproteins may also be detected by mobility shifts, in either the p*I* or MW dimension, in diagonal separations after enzymatic or chemical (e.g. trifluoromethanesulphonic acid-induced) cleavage of glycosylation bonds [29, 180].

<-Table 1->

3.4.2. Enrichment of glycosylated or glycated proteins

Methods described for glycoprotein detection, including metabolite labeling, hydrazide reaction with oxidized carbohydrates, and especially lectin-based procedures, have also been used to enrich glycoproteins. Lectin affinity chromatography can be used with single lectin species, or multiple lectins to capture (for example) all *N*-linked glycoproteins [165, 181, 182]. Depending on the lectin(s) used, binding may require the presence of metal ions or a specific pH. Bound glycoproteins are then desorbed by using a chelating agent or lowering the pH, respectively, but this can of course be facilitated by simply adding a high concentration of the free carbohydrate ligand(s). Hydrazide coupling is the major chemical-based enrichment technique for glycoproteomic analysis. The coupling reagent can be either linked to biotin and captured via biotin-avidin affinity, or immobilized on beads [183, 184]. Similar chemistry has also been used for more specific enrichment. For example, Zeng *et al.* (2009) introduced a method for the selective derivatization of sialic acid by mild periodic oxidation and ligation with an aminooxy tag in the presence of aniline, and the same group has recently described an approach to specifically enrich glycoproteins with terminal *N*-acetylgalactosamine/galactose [185, 186].

Lectin- or hydrazide/aminooxy-bound glycoproteins can be digested on-beads and the remaining glycan-containing peptide(s) can be released and, if desired, various tags can be incorporated into them for quantitative purposes [184, 187, 188]. This is suitable for analysis of *N*-glycans because the known enzyme *N*-glycosidase F cleaves the glycosidic bond between the asparagine residue of a glycosylated protein and the core *N*-acetylgalactosamine, thereby liberating nearly all *N*-linked glycans. This is a particularly attractive reaction, because it results in deamidation of asparagine to aspartate with a mass shift of 1 Da (or 3 Da in [^{18}O] water) which is indicative of a PTM site and can be used for comparative quantitation. However, it should be mentioned that spontaneous deamidation (see section 3.5) has the same effect and may lead to erroneous assignments [189]. A lack of broad-specificity *O*-glycosidases can be circumvented by reductive β-elimination through the use of a strong base [190] or hydrazinolysis [191], both of which release *O*- and *N*-linked glycopeptides. Another method for glycoproteome enrichment is based on the reversible reaction of boronic acid with 1,2-*cis*-diols [192]. These moieties are present in glycan components like mannose, galactose, glucose, and sialic acid, but are also preserved in glycated proteins, and boronate affinity chromatography is currently the only antibody-independent method for enriching glycated proteins [166, 193]. Several derivatives of boronic acid have been introduced for this purpose, including bisboronic compounds, which can also be conjugated with a fluorescence tag and used for detecting protein glycosylation/glycation [194], and both sulfonyl- and sulfonamide-phenylboronic acid derivatives, which can be used at physiological pH. These adjustments have solved the major drawback of the original setup; the need to use alkaline buffers due to the relatively low ionization constants of the commonly used phenylboronic acid ligands [195]. Non-specific boronate affinity has also been successfully combined with lectin affinity in boronic acid-lectin affinity chromatography [196]. In addition, boronate in the form of methacrylamido phenylboronic acid has been copolymerized in acrylamide gel and applied to detect, analyze and separate early and AGE glycation products from unaffected proteins [197]. During the electrophoretic separation, migration of glycated proteins is hindered and gel resolution allows various carbohydrate-protein adducts to be distinguished. A boronate gel was commercialized by Pierce (Glycogel II boronate affinity gel), but it is no longer available. Anti-glycosylated/glycated protein antibodies have also been employed in Western blot analyses, and can be used for specific enrichments. This is especially useful for analyzing protein glycations that are not recognized by lectins and a number of antibodies have been prepared for analyses of AGE protein modifications, notably for detecting ε-*N*-carboxymethyllysine and ε-*N*-carboxyethyllysine [198, 199]. These can be raised, for example, against bovine serum albumin that has been treated with glycolaldehyde, and are commercially available (e.g. Millipore; http://www.millipore.com).

3.5. Protein deamidation

Asparagine and glutamine residues in both proteins and peptides can be deamidated through spontaneous chemical reactions that may occur either *in vivo* or *in vitro*. The rates of deamidation depend (*inter alia*) on pH, temperature and protein structure. Depending on the surrounding amino acids, half-times of deamidations of specific asparagine and glutamine residues in proteins are within ranges of 1-500 days and 100-1000 days, respectively, at neutral pH and 37 °C [200]. The rates are significantly higher at alkaline pH, which is typical (for instance) in trypsin digestion. Thus, it is difficult to distinguish a natural protein deamidation, which could be a marker of a disease or protein ageing, from artifactual deamidation during proteomic sample preparation [201, 202]. Deamidation proceeds via formation of a cyclic intermediate by condensation of the side-chain amide group with the carbonyl group of the following amino acid, and subsequent opening of this ring upon hydrolysis with water. While this process cannot be completely avoided, even with an improved protocol [202], a potential way to discriminate *in vitro* reactions is to prepare samples in [^{18}O]-water, which increases the molecular weight of deamidation artifacts [203, 204]. The hydrolysis of a ring intermediate can yield two products; aspartic acid and isoaspartic acid in the case of asparagine (Fig. 5, I). The second product is more frequent (yield up to 70 %) and leads to a significant change in the backbone structure of the host protein. Thus it is often accompanied by a loss of biological activity. Deamidation also introduces an additional negative charge in the protein and can be detected by charge-sensitive separation methods (IEF, 2-DE or ion-exchange chromatography) [205, 206]). Isoaspartyl residues can be enzymatically converted to asparagyl residues by protein *L*-isoaspartyl *O*-methyltransferase [207], which can be used to label proteins containing isoaspartyl residues by [^{3}H]-methylation *in vitro*. Proteins can then be separated (e.g. by 2-DE) and converted isoaspartyl residues can be detected by autoradiography [206, 207]. There is no specific enrichment procedure for isolating a deamidation proteome, and large-scale analyses thus depend on advances in mass spectrometry [208, 209].

3.6. Protein acetylation

Acetylation of proteins is a specific form of protein acylation (Fig. 5, E) that was discovered in the early 1960s [210-212]. The most common types are *N*-acetylation of the amino terminus of a protein and *N*-acetylation of lysine residue side chains, but *O*-acetylations of serine and threonine have also been reported [213]. *N*-terminal acetylation rarely occurs in prokaryotes, but it occurs in about 50 % of yeast proteins and more than 80 % of human proteins [214, 215]. It is believed to be irreversible and Hwang *et al*. found that it could act as a signal for

ubiquitin-dependent degradation [216]. *ε*-Lysine acetylation is by far the most extensively studied form of acetylation. It is regulated by *N*-acetyltransferase and deacetylase enzymes, and plays roles in protein stability, localization, enzyme activity, and (in histone acetylation) directly regulates gene expression by changing chromatin structure [217-219]. Analysis of the acetylome is hindered by the high abundance of acetylated histones in the cell and the lack of suitable physical and biochemical properties of the acetyl group that could be exploited for its specific enrichment. However, acetylation masks positive charges of lysine residues (or N-termini) and can thus be detected by changes in proteins' p*I* (IEF, 2-DE) [220]. There has also been some success in the development of anti-acetyllysine antibodies and immunoaffinity enrichment techniques [221-224]. However, Shaw *et al*. clearly demonstrated the limitations of this strategy by showing that sets of acetylated peptides detected by a cocktail of monoclonal antibodies raised against acetylated lysine in different sequence contexts differed from those detected by the polyclonal antibodies used by other groups [225]. Acetylome analyses could benefit from diagonal separation, but the only such technique reported to date is a chemical-based combined fractional diagonal chromatography (COFRADIC) approach to determine N-terminal acetylation [226], and no method employing enzymatic deacetylation has been presented as yet.

3.7. Protein methylation

The first report on the occurrence of a methyl PTM in a protein dates back to 1959, when Ambler and Rees found *ε*-*N*-methyl-lysine in a bacterial flagellar protein [227]. Protein methylation predominantly occurs at lysine and arginine residues, but *N*- or *O*-methylation has also been detected at side chains of other amino acids, N-terminal amino groups and *C*-terminal carboxyl groups. There have even been reports of cysteine *S*-methylation and *C*-methylation at methylene carbons of arginine and glutamine (Fig. 5, F) [1, 228, 229]. Methylation affects proteins' hydrophobicity and can mask a negative charge (by formation of methyl esters). It has demonstrated roles in various processes, e.g. signal transduction, mRNA splicing, transcriptional control, DNA repair, protein-protein interactions, protein stability and protein translocation [229, 230]. Methylation is often catalyzed by methyltransferase enzymes that utilize *S*-adenosylmethionine as a substrate, but *S*-adenosylmethionine can also readily methylate cysteine and histidine residues non-enzymatically *in vitro*, and a similar non-enzymatic mechanism has been proposed for the methylation of some proteins *in vivo*, e.g. human lens crystallins [231]. It was long believed that *N*-methylation is irreversible and that reversibility of *O*-methylation is due to spontaneous non-enzymatic decomposition of the ester bond. However, this view was changed by the discovery of lysine demethylases [232] and the search for arginine demethylases is in progress [233].

3.7.1. Protein methylation detection and enrichment of protein methylomes

The methylation of eukaryotic proteins may be widespread, but the large-scale proteomic techniques that could rigorously test this hypothesis are just emerging. Protein methylation can be detected by metabolite labeling using [^{14}C]- or [^{3}H]-radiolabeled *S*-adenosylmethionine, followed by protein extraction, separation and autoradiography, but to date this procedure has been limited to small-scale analyses and *in vitro* detection of possible substrates of methyltransferases [234, 235]. Anti-methyllysine and anti-methylarginine antibodies have been raised and successfully employed in methylated protein enrichment and Western blot detection [235, 236]. The subtle chemical differences, e.g. between methylated and unmodified arginine, limits the application of antibodies for intact protein enrichment. However, using antibodies in combination with stable isotope labeling by amino acids in cell culture (SILAC) by [$^{13}CD_3$]methionine, Ong *et al*. detected 59 methylation sites in HeLa cell extracts [235]. In addition, Uhlmann *et al*. recently reported a peptide-based method for large-scale identification of protein arginine methylation that seems to outperform antibody-based enrichment [237]. They detected 249 methylation sites in peptides from a SILAC-labeled cell lysate and showed that most arginine methylated peptides were highly basic and hydrophilic, which facilitated their purification. A comparison of the effectiveness of SCX chromatography, IEF and hydrophilic interaction liquid chromatography (HILIC) clearly indicated that HILIC [238] is by far the most effective for this purpose. Surprisingly, however, Uhlmann *et al.* have not found a similar level of enrichment for peptides containing methylated lysine [237].

3.8. Protein ubiquitination

Ubiquitin is a highly conserved protein of about 8.5 kDa, which can be attached in an ATP-dependent manner to a protein. The history of protein ubiquitination research dates back to 1977, when Goldknopf and Busch described an isopeptide bond between a histone 2A lysine residue and the tryptic C-terminal diglycine remnant of a different protein [239]. PTM by ubiquitin and ubiquitin-like proteins represents a major regulatory system. The basic enzymatic cascade couples the C-terminus of ubiquitin via an isopeptide bond to the *ε*-amino group of lysine residues on substrate proteins (Fig. 5, J) or, less frequently, via peptide bonds to the N-terminus, but evidence has also emerged for ubiquitin ester bonds via cysteine, serine or threonine residues [240]. Proteins can be monoubiquitinated, or polyubiquitin chains may form via linkages involving any of the molecule's seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or its N terminus, which have different structures and properties, depending on how the chains are assembled [241]. Protein (poly)ubiquitination is known to regulate

protein turnover by influencing proteasome-mediated degradation, gene transcription, subcellular trafficking and protein localization, enzymatic activity, and to facilitate protein-protein interactions [242-246]. Ubiquitination is catalyzed through the sequential action of three discrete enzymes: the ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligating enzyme (E3). Ubiquitin tags can be removed or edited by deubiquitinating enzymes (DUBs). The number of enzymes involved rivals those involved in phosphoproteome regulation: the human genome encodes approximately 10 E1, 40 E2, over 600 E3 and 80-90 DUBs. Plant signaling is highly dependent on ubiquitin, which is reflected in over 1,000 *Arabidopsis thaliana* genes for E3 [247, 248].

3.8.1. Protein ubiquitination - enrichment

The "ubiquitinome" poses several analytical challenges, including the fact that ubiquitination frequently leads to protein degradation and loss of modified proteins, unless the proteasome pathway is inhibited [249]. Previously, proteomic methods for ubiquitin enrichment have predominantly utilized affinity-tagged labeling and antibody-based capture. Beers and Callis engineered a variant of ubiquitin containing a polyhistidine-tag at its N-terminus for an *in vitro* assay, which allowed IMAC enrichment of proteins with incorporated tagged ubiquitin [250]. A similar approach can also be used for *in vivo* analyses of transgenic lines expressing tagged ubiquitin [251, 252]. Non-specific binding can be circumvented by employing another specific label in addition to ubiquitin (e.g. biotin)[253], but this does not eliminate competition between the tagged exogenous ubiquitin and the endogenous wild-type molecule, which lowers the sensitivity of this method. Further, the possibility that added tags may influence the physiological activity of the molecule cannot be excluded [254]. Some natural proteins are known to associate with ubiquitin and their ubiquitin-binding domains have been used as agarose conjugates for ubiqitinome affinity enrichment [254]. This technique has been further developed by fusing domains into tandem-repeated ubiquitin-binding entities (TUBEs)[255], which reportedly have higher affinity for polyubiquitin chains and are capable of protecting ubiquitinated proteins from proteasome cleavage or enzymatic deubiquitylation, a process difficult to inactivate even in denaturating conditions [256]. Ubiquitinated proteins are routinely detected by Western blotting with anti-ubiquitin antibodies and antibodies have also been used with some success for ubiquitinome enrichment [257]. Antibody-based enrichment has also been applied in peptide-based approaches for ubiquitination detection [258]. Trypsin proteolysis of a ubiquitin-conjugated protein produces a signature peptide for MS-based detection at the ubiquitination site containing a two-residue remnant (glycine-glycine) that is derived from the C terminus of ubiquitin and is still covalently attached to the target lysine residue via an isopeptide bond [251]. Antibodies raised against this sequence are effective tools for profiling ubiquitinated substrates and have significantly increased the number of verified proteins with ubiquitination PTMs [259].

4. Summary

Difficulties associated with MS-based analysis have driven the development of alternative approaches to enrich and detect PTM modified proteins. Advances in MS-based proteomics have greatly facilitated the identification of thousands of PTM sites in eukaryotic cells and revealed novel modifications, but also shifted the focus of protein PTM analyses from a proteome to a peptide level. A key element for the success of these studies is the ability to enrich modified peptides using antibodies or other chemical approaches. This has worked reasonably well for *O*-phosphoproteome analysis, but most other PTMs have been more difficult to address and remain poorly characterized because of the lack of such enrichment methods. However, all known PTMs may be analyzed using the general workflow illustrated in Fig. 6. Indeed, if it was not for the low abundance of modified proteins, 2-DE and/or reversed-phase or HILIC chromatography would be sufficient to detect most known protein PTMs, due to the unsurpassed resolution of the former for p*I*/MW protein separation and the ability of the latter to recognize changes in protein/peptide hydrophobicity. As it stands, these methods only provide satisfactory results for abundant proteins or PTMs tagged with specific (radio)-labels. Sample labeling used to be an essential step for any PTM study and it still remains advantageous. Labels may be introduced by enzymatic or chemical reactions *in vivo* or during/after protein extraction. Labels not only facilitate detection, but can also be used for specific enrichment. The major drawbacks usually lie in reductions in yields when *in vitro* derivatization is applied, and/or the necessity to use enzymes with modified substrate specificity. Analyses of rare protein PTMs may be improved by proteome fractionation, immunodepletion or proteome equalization. While each additional protein isolation step provides indisputable benefits, they all prolong the procedure and usually compromise labile PTMs. Nevertheless, there has been substantial development in PTM enrichment methodology and a number of modifications may be addressed directly by (immuno)affinity enrichment. Enriched samples may be further processed (e.g. by 2-DE) or digested. This is the point where bottom-up and top-down proteomic approaches converge and may proceed through peptide-based enrichment, or directly to MS analysis. In recent years approaches for analyzing protein PTMs have rapidly developed, but large proportions of peaks in MS spectra of protein samples are still typically not assigned, and may originate from hidden PTMs. This hypothesis has been recently corroborated by the detection of a signature fragment of a novel *O*-

acetylglucosamine-6-phosphate PTM in a re-analysis of a large-scale proteomics dataset [260]. Thus, we are still clearly far from an optimal procedure for PTM analyses, and further advances are required not only in protein isolation and MS sensitivity, but also in bioinformatics.

<-Figure 6->

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Primary Sugar Specificity	Lectin
Mannose	Concanavalin A (ConA), *Lens culinaris* agglutinin (LCA), Lentil lectin (LCH), Snowdrop lectin (GNA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Pisum sativum* agglutinin (PSA)
Galactose	Jacalin (AIL), Coral Tree (ECL), *Griffonia simplicifolia* lectin I (GSL I), Peanut Agglutinin (PNA)
N-acetylgalactosamine	*Dolichos biflorus* agglutinin (DBA), Soybean agglutinin (SBA), *Sophora japonica* agglutinin (SJA), *Vicia villosa* lectin (VVL)
N-acetylglucosamine	*Datura stramonium* lectin (DSL), *Griffonia simplicifolia* lectin II (GSL II), *Lycopersicon esculentum* lectin (LEL), *Solanum tuberosum* lectin (STL), Wheat germ agglutinin (WGA)
Fucose	*Aleuria aurantia* lectin (AAL), *Ulex europaeus* agglutinin (UEA),
Sialic acid	Elderberry lectin (SNA), *Maackia amurensis* lectin (MAL)

Tab. 1 - Lectins commonly used in glycoproteomic analyses.

Legends:

Fig. 1 - Frequencies of 22 proteinogenic amino acids and their known PTMs. The percentages indicate amino acid usage in complete human (gray), model yeast (*Saccharomyces cerevisiae*, orange) and model plant (*Arabidopsis thaliana*, green) proteomes (i.e. the entire sets of proteins expressed by these organisms, according to information retrieved from http://www.uniprot.org, 10/2012). The bottom part lists numbers of known naturally occurring PTMs at individual residues (RESID database; release 70.01). This plot illustrates the wide variability of protein PTMs, but not their amino acid ratios in the proteomes, which also depend on the relative abundance of the expressed proteins

Fig. 2 - The loss of information in bottom-up proteomic analyses. It is widely accepted that a protein may be identified based on a single unique (proteotypic) peptide with a well-matched mass spectrum. However, while an identified fragment may represent a corresponding theoretical protein (A), alternatively it may be part of a spliced isoform or the result of protein degradation (B), or an indicator of a PTM, especially if the missing sequence fulfills requirements for MS detection [2] (C).

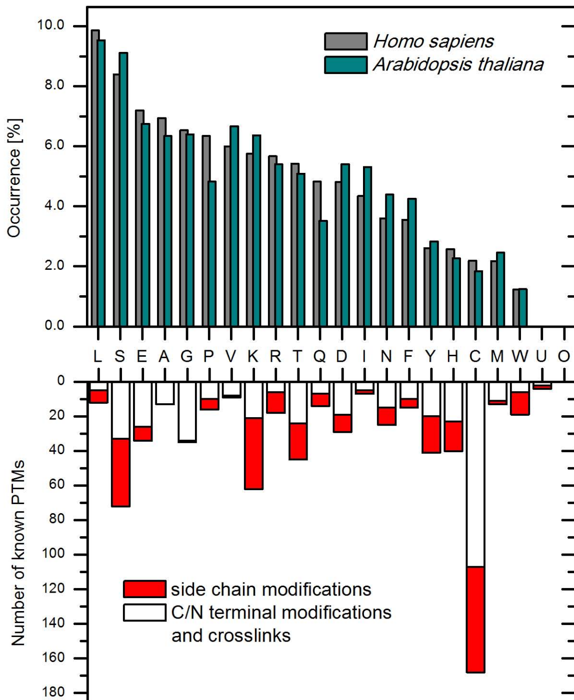
Fig. 3 - Essential methods for protein multi-dimensional separation.
(A) Schematic workflow of 2-DE analysis. Proteins are separated and focused in the first dimension by IEF in a strip with a pH gradient according to their p*I*. The strip with focused proteins is then equilibrated with SDS buffer to form SDS-protein complexes and usually treated with a reducing agent (e.g. dithiotreitol) and iodoacetamide to reduce disulfide bridges and alkylate cysteine residues, respectively. The SDS-protein complexes are then separated in the second dimension by SDS-PAGE. **(B) Detection of a protein PTM by 2-DE.** A subunit of chloroplastic ATP synthase was identified in three neighboring spots. The apparent molecular mass of the protein is 55 kDa, in accordance with the theoretical MW, 55.3 kDa. The comparison of apparent p*I* values indicates that spot (3) represents unmodified protein, while the proteins in spots (2) and (1) bear modification(s) that lower their p*I* to more acidic values. **(C) Analysis by 2D-LC.** Fractions from a first LC separation are collected and analyzed by a second LC with contrasting conditions and/or media. The resulting 2D map is based on UV-VIS profiles and its resolution, especially in the first dimension, is to some extent proportional to the number of collected fractions. **(D) Diagonal separation.** Classical separation and detection of inter- and intra-molecular disulfide bonds, as described in section 2.2.5.

Fig. 4 - Depletion of major abundant proteins can uncover hidden PTMs. Demonstration of immunoaffinity depletion and proteome equalization in the *Arabidopsis thaliana* proteome resolved by 2-DE. A sample of 7-day-old seedlings was divided into three aliquots. One was used for total (acetone/TCA) extraction **(A)**, one was immuno-depleted of RuBisCO using a Seppro IgY RuBisCO depletion column **(B)** and one was equalized by CPLL ProteoMiner **(C)**. Protein spots corresponding to RuBisCO subunits (53 kDa and 20 kDa), show that both B and C successfully depleted RuBisCO. Demonstration of CPLL effectiveness: **Rectangle** – no visible effect of highly abundant protein depletion; **Circle** – seven protein spots identified as RuBisCO activase; the wide ranges of p*I* and MW indicate intensive PTM regulation. Bio-Safe Coomassie stain, 150 µg of protein, 7 cm IPG strip (based on [45]; and our unpublished data).

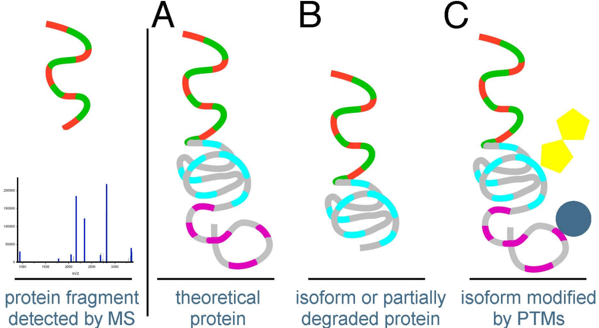
Fig. 5 - Chemical structures of selected PTMs. Protein PTMs described in this review can be separated into 10 distinct but overlapping classes (indicated by colored frames). Individual classes may be further subdivided (dotted lines). **(A) Phosphorylations** found in proteins. Ser, Thr and Tyr are amino acids that can be *O*-phosphorylated; *N*-phosphates (amidates) are formed on residues of Lys, His and Arg; acetylphosphorylation is the least stable protein phosphorylation and has been documented only for Asp (Cys residues are phosphorylated as intermediates during the enzymatic catalysis, but as this bond is transient they are not usually regarded as protein PTMs). **(B) Protein nitration** is documented on Trp and Tyr with 6-nitrotryptophan and 3-nitrotyrosine being the major nitrated products [50]; Cys residues may be modified by S-nitrosylation. **(C) Selected products of cysteine oxidation.** Cysteine is known to undergo a number of oxidative PTMs. The selected PTMs represent only modifications of the Cys side chain, ignoring protein crosslinking and some less common PTMs that may

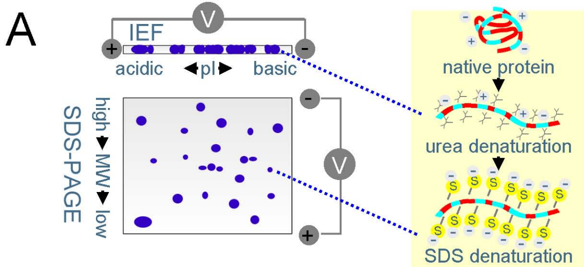
occur, e.g. cystenylation (formation of a disulfide bridge between a protein cysteinyl residue and free cysteine) and thiosulfonate formation (Cys-S-SO_2-Cys). **(D) Products of methionine oxidation. (E) Protein acetylation and lipidation.** Representatives of *S-, O-* and *N*-acylations, an example of a glycosyl phosphatidylinositol anchor, and a Cys/His/Lys/Arg bound product of lipid oxidation (advanced lipidation end-product), 4-hydroxy-2-nonenal (which accounts for > 95% of the total unsaturated aldehydes produced during ***in vitro*** microsomal lipid peroxidation [51]); **(F) Protein methylation.** Representatives of Lys, Arg and His *N*-methylations; *S*-methylation of Cys; *C*-methylation of Glu; and an example of a methyl ester. **(G) Products of protein carbonylation.** The direct oxidation of Arg and Pro (glutamic semialdehyde), Thr (2-amino-3-ketobutyric acid) and Lys (aminoadipic semialdehyde); examples of indirect protein carbonylation products after protein interaction with oxidized lipid or carbohydrate. **(H) Protein glycosylation.** The *N*-glycan core structure transferred to the conserved sequence Asn-X-Ser/Thr (where X represents any amino acid except Pro), and examples of: *O*-glycosylation, *C*-mannosylation of Try, an Amadori product of glucose and Lys interaction, and an AGE-product resulting from further conversion of N^6-fructosamine lysyl and protein crosslinking. **(I) Deamidation and production of an isopeptide bond. (J) Protein ubiquitination.** An example of enzymatic monoubiquitination at Lys, this is essentially a form of protein acylation, but its distinguishing protein-protein nature demands a separate category.

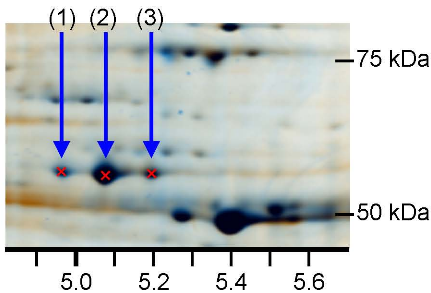
Fig. 6 - Schematic workflow for protein PTM analysis.

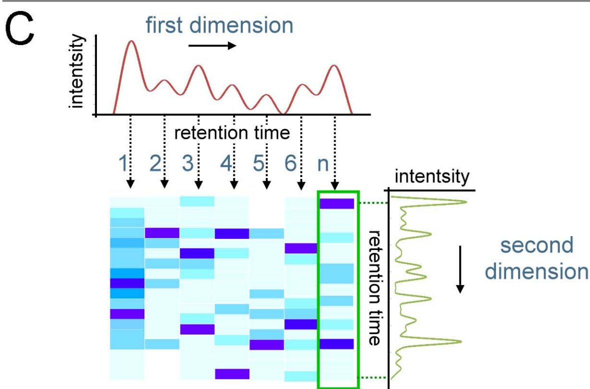


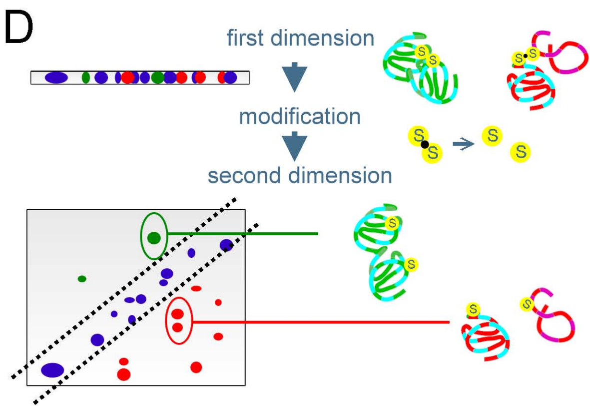
Homo sapiens
Arabidopsis thaliana
Occurrence [%]
10.0
8.0
6.0
4.0
2.0
0.0
L S E A G P V K R T Q D I N F Y H C M W U O
0
20
40
60
80
100
120
140
160
180
Number of known PTMs
side chain modifications
C/N terminal modifications
and crosslinks

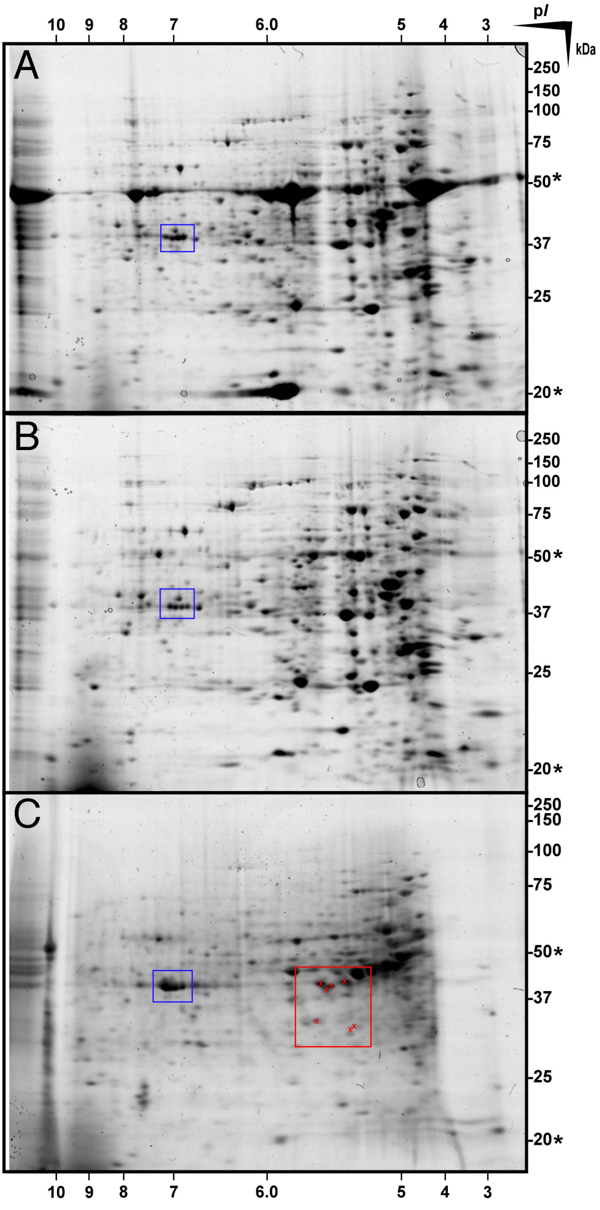

A
B
C
200000
150000
100000
50000
0
1000
1500
2000
2500
3000
m/z
protein fragment detected by MS
theoretical protein
isoform or partially degraded protein
isoform modified by PTMs


A
V
IEF
acidic
pI
basic
SDS-PAGE
high
MW
low
V
native protein
urea denaturation
SDS denaturation

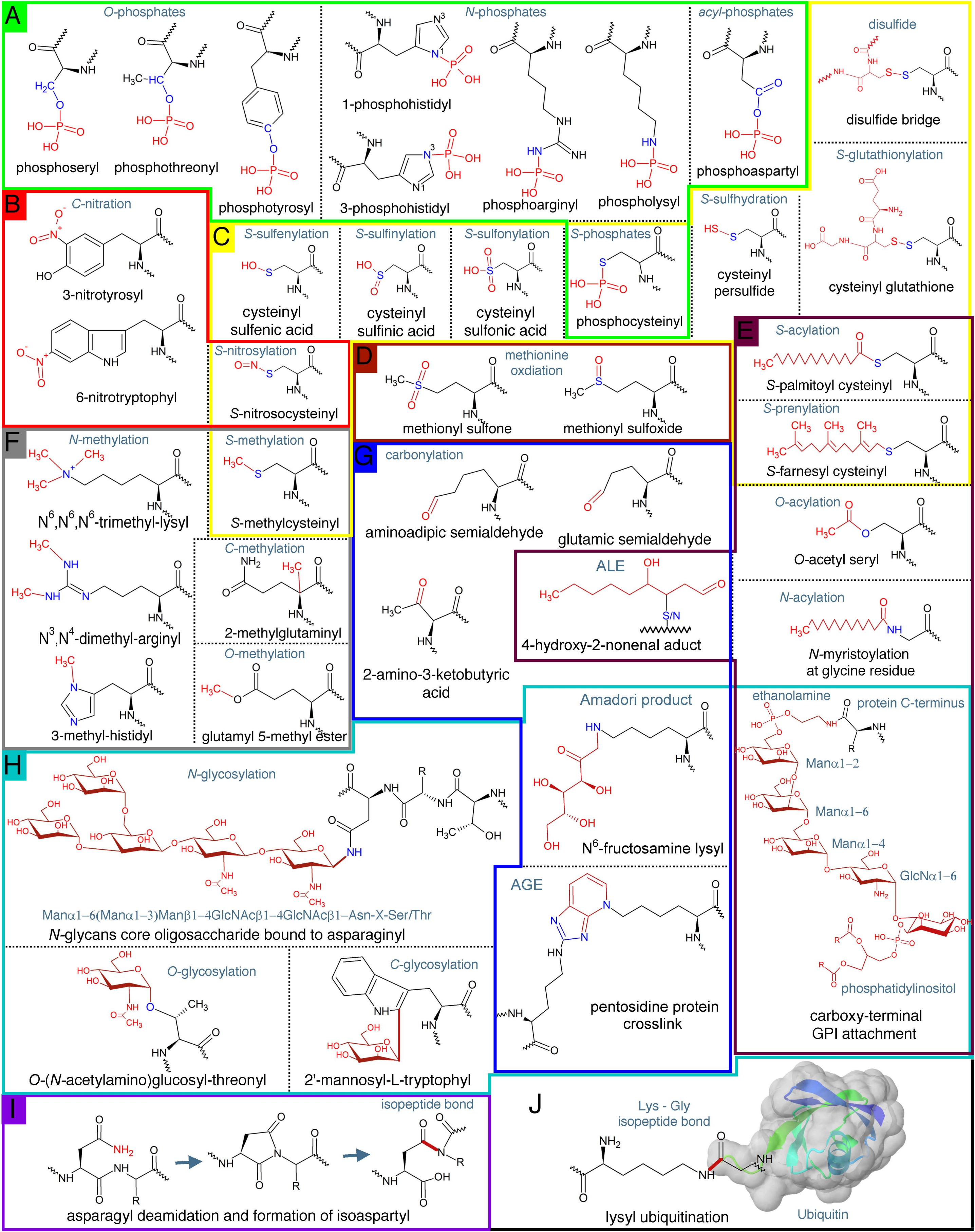

B
ATP synthase subunit alpha, chloroplastic
theoretical MW: 55.3 kDa
theoretical pI: 5.19
(1)
(2)
(3)
75 kDa
50 kDa
5.0
5.2
5.4
5.6


C
first dimension
intentsity
retention time
1
2
3
4
5
6
n
intentsity
retention time
second dimension

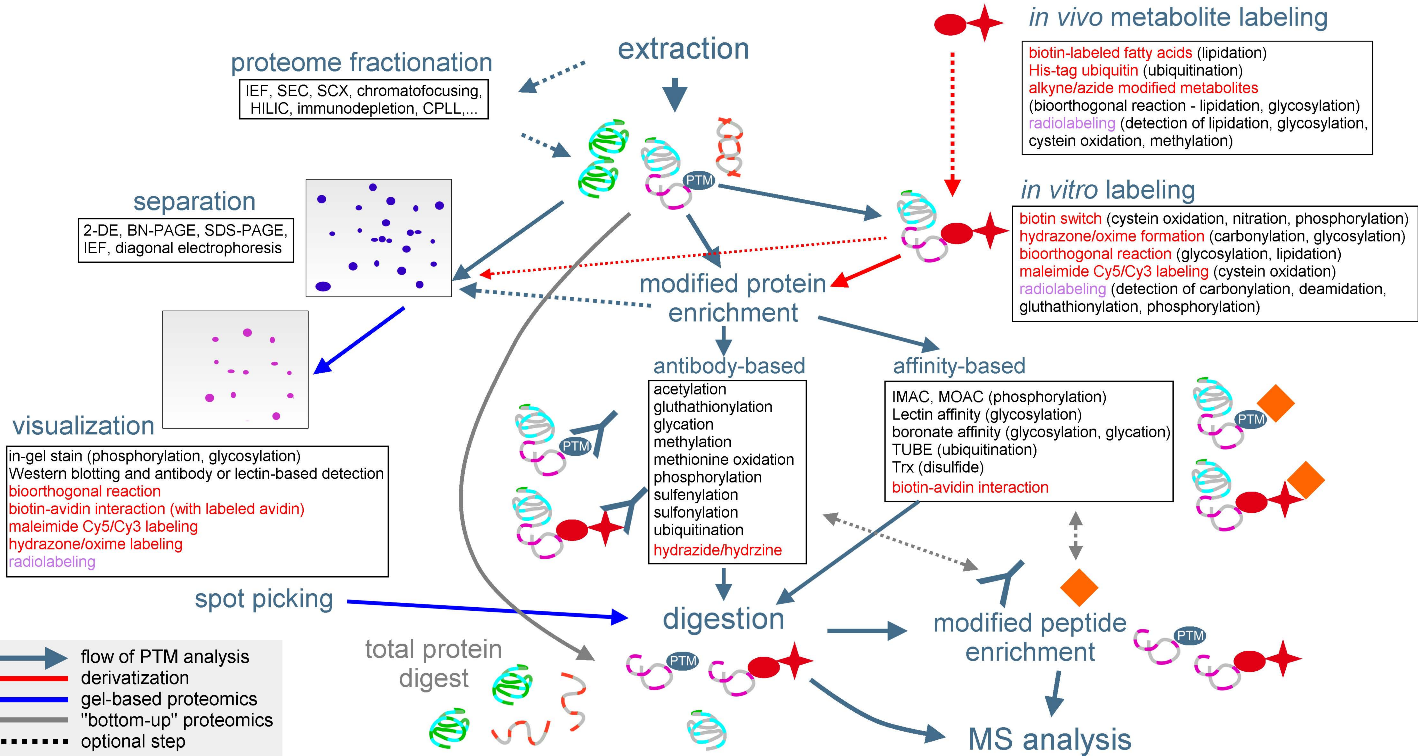

D
first dimension
modification
second dimension



p*I*
kDa
10
9
8
7
6.0
5
4
3
A
B
C
-250
-150
-100
-75
-50*
-37
-25
-20*



A
O-phosphates
phosphoseryl
phosphothreonyl
phosphotyrosyl
N-phosphates
1-phosphohistidyl
3-phosphohistidyl
phosphoarginyl
phospholysyl
acyl-phosphates
phosphoaspartyl
disulfide
disulfide bridge
S-glutathionylation
cysteinyl glutathione
S-sulfhydration
cysteinyl persulfide
B
C-nitration
3-nitrotyrosyl
6-nitrotryptophyl
C
S-sulfenylation
cysteinyl sulfenic acid
S-sulfinylation
cysteinyl sulfinic acid
S-sulfonylation
cysteinyl sulfonic acid
S-phosphates
phosphocysteinyl
S-nitrosylation
S-nitrosocysteinyl
D
methionine oxdiation
methionyl sulfone
methionyl sulfoxide
E
S-acylation
S-palmitoyl cysteinyl
S-prenylation
S-farnesyl cysteinyl
O-acylation
O-acetyl seryl
N-acylation
N-myristoylation at glycine residue
F
N-methylation
N6,N6,N6-trimethyl-lysyl
N3,N4-dimethyl-arginyl
3-methyl-histidyl
S-methylation
S-methylcysteinyl
C-methylation
2-methylglutaminyl
O-methylation
glutamyl 5-methyl ester
G
carbonylation
aminoadipic semialdehyde
glutamic semialdehyde
2-amino-3-ketobutyric acid
ALE
S/N
4-hydroxy-2-nonenal aduct
Amadori product
N6-fructosamine lysyl
AGE
pentosidine protein crosslink
ethanolamine
protein C-terminus
Manα1–2
Manα1–6
Manα1–4
GlcNα1–6
phosphatidylinositol
carboxy-terminal GPI attachment
H
N-glycosylation
Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1–Asn-X-Ser/Thr
N-glycans core oligosaccharide bound to asparaginyl
O-glycosylation
O-(N-acetylamino)glucosyl-threonyl
C-glycosylation
2'-mannosyl-L-tryptophyl
I
isopeptide bond
asparagyl deamidation and formation of isoaspartyl
J
Lys - Gly isopeptide bond
lysyl ubiquitination
Ubiquitin


in vivo metabolite labeling
biotin-labeled fatty acids (lipidation)
His-tag ubiquitin (ubiquitination)
alkyne/azide modified metabolites
(bioorthogonal reaction - lipidation, glycosylation)
radiolabeling (detection of lipidation, glycosylation, cystein oxidation, methylation)
extraction
proteome fractionation
IEF, SEC, SCX, chromatofocusing, HILIC, immunodepletion, CPLL,...
PTM
separation
2-DE, BN-PAGE, SDS-PAGE, IEF, diagonal electrophoresis
in vitro labeling
biotin switch (cystein oxidation, nitration, phosphorylation)
hydrazone/oxime formation (carbonylation, glycosylation)
bioorthogonal reaction (glycosylation, lipidation)
maleimide Cy5/Cy3 labeling (cystein oxidation)
radiolabeling (detection of carbonylation, deamidation, gluthathionylation, phosphorylation)
modified protein enrichment
antibody-based
acetylation
gluthathionylation
glycation
methylation
methionine oxidation
phosphorylation
sulfenylation
sulfonylation
ubiquitination
hydrazide/hydrzine
affinity-based
IMAC, MOAC (phosphorylation)
Lectin affinity (glycosylation)
boronate affinity (glycosylation, glycation)
TUBE (ubiquitination)
Trx (disulfide)
biotin-avidin interaction
visualization
in-gel stain (phosphorylation, glycosylation)
Western blotting and antibody or lectin-based detection
bioorthogonal reaction
biotin-avidin interaction (with labeled avidin)
maleimide Cy5/Cy3 labeling
hydrazone/oxime labeling
radiolabeling
spot picking
digestion
modified peptide enrichment
total protein digest
MS analysis
flow of PTM analysis
derivatization
gel-based proteomics
"bottom-up" proteomics
optional step