REVIEW

Stable isotopic labeling in proteomics

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Labeling of proteins and peptides with stable heavy isotopes (deuterium, carbon-13, nitrogen-15, and oxygen-18) is widely used in quantitative proteomics. These are either incorporated metabolically in cells and small organisms, or postmetabolically in proteins and peptides by chemical or enzymatic reactions. Only upon measurement with mass spectrometers holding sufficient resolution, light, and heavy labeled peptide ions or reporter peptide fragment ions segregate and their intensity values are subsequently used for quantification. Targeted use of these labels or mass tags further leads to specific monitoring of diverse aspects of dynamic proteomes. In this review article, commonly used isotope labeling strategies are described, both for quantitative differential protein profiling and for targeted analysis of protein modifications.

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1 Introduction

Stable isotopes in combination with MS have been used for several decades by clinical chemists and pharmacists as quantification tools to measure concentrations of natural compounds [1]. Today, similar approaches have found broad application in contemporary proteome research. Depending on the introduced isotope spacer and the resolving power of the mass spectrometer, isotope envelopes of labeled peptides can be partly or fully resolved and are used for their quantification. To find proteins or peptides with significantly different concentrations in sampled proteomes, different stable heavy isotope labeling techniques are now fully exploited. Depending on the sample's origin, isotope labeling may be performed on different levels (organism, cell, protein, or peptide) and on different reactive groups. Furthermore, multiplexed labeling increasing sample throughput and allowing temporal and spatial proteomics became possible.

We here discuss commonly used isotope labeling techniques in contemporary proteome research, focus on their potential and shortcomings, and illustrate how isotope labeling can be used for targeted analysis of specific features of dynamic proteomes.

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Abbreviations: COFRADIC, combined fractional diagonal chromatography; **MEF,** mouse embryonic fibroblast; **NHS,** N-hydroxysuccinimide; **PIQS,** parent (precursor) ion quantitation scanning; **QconCAT,** concatamers of quantitative (Q) peptides; **SILAC,** stable isotope labeling by amino acids in cell culture

2 Metabolic labeling techniques for proteomics

2.1 Labeling of cell cultures

Metabolic labeling of cells with stable isotopes for quantitative contemporary proteomics was first described by the lab of Brian Chait in 1999 [2]. His group grew baker's yeast in medium that was for more than 96% enriched in nitrogen-15. The levels of selected, rather high abundant proteins

from two yeast strains – a wild type strain and one over-expressing the G1 cyclin CLN2 – were compared, after growing these in different (^{14}N or ^{15}N) media, by MALDI-MS PMF following protein HPLC and SDS-PAGE protein separation. In the obtained MALDI-MS spectra, peptides were present as couples of light (^{14}N) and heavy (^{15}N) ions. Mass values of the light peptides enable identification by database searching, whereas the ratio of the intensities of the light *versus* the heavy peptides is a read-out for potential concentration changes of the corresponding yeast protein. In the same article, differential phosphorylation of a selected protein, the yeast protein kinase Ste20, was also assessed [2]. Since then, other labs have described similar labeling methods on various small organisms and cell lines (*e.g.*, *Deinococcus radiodurans* and mouse B16 melanoma cells [3], *Arabidopsis thaliana* cells [4], and *Rhodopseudomonas palustris* [5], see also Section 2.2).

This type of metabolic labeling in cell lines or small organisms poses high demands on software algorithms for automated quantification. Amongst others this is because the m/z spacing between light and heavy peptides is not fixed as is the case for stable isotope labeling by amino acids in cell culture (SILAC) labeling (see below) but varies depending on the number of incorporated heavy isotopes. Furthermore, the used media are never fully composed of the heavy isotopic variant implying that the proteins are never fully labeled (although typically nearly complete). Hence, the resulting isotope envelopes of the peptide ions differ from the theoretically calculated and expected ones. This affects recognition of peptide ions as well as integration of their signals. Finally, ^{14}N and ^{15}N labeled peptides never elute perfectly together meaning that during LC-MS/MS analysis, retention time spacing between light and heavy peptides is introduced, further complicating retrieval of peptide couples. RelEx [6] and MSQuant ((http://msquant.sourceforge.net/) and [7]) are two examples of algorithms that were designed to deal with these drawbacks and allow automated quantification. However, from our own experience we learned that such algorithms are often difficult to setup and implement, and always contain little bugs slowing down the whole process and making them often not user-friendly.

The lab of Matthias Mann pioneered the use of stable heavy variants of essential amino acids to metabolically label proteins in cell cultures (SILAC, [8, 9]). SILAC is a straightforward procedure in which natural variants of essential amino acids are left out of culture media and replaced by deuterated, carbon-13, and/or nitrogen-15 variants. These heavy building blocks are readily incorporated into proteins and typically after a few rounds of cell duplication, proteomes are fully labeled and ready for analysis.

By the combined use of heavy variants of lysine and arginine – which on average occur at every tenth position in a protein sequence – and proteome scientist's favorite protease trypsin, not surprisingly SILAC is mainly used for quantitative proteomics [9, 10] as well as for pointing to specific protein binders (*e.g.*, by combining SILAC with RNA interference (RNAi) mediated knock-down of bait proteins [11]). Heavy labeled variants of lysine and arginine that provide ample spacing between isotopic envelopes of light and heavy tryptic peptides (*e.g.*, 10 Da using $^{13}C_6{}^{15}N_4$-Arg and 8 Da using $^{13}C_6{}^{15}N_2$-Lys) are available such that even mass spectrometers with low resolving power (for instance ITs) can be used for quantitative proteomics. Interestingly, since several forms of lysine and arginine are available, they can be "permutated" and thus allow analysis of three different proteomes (*e.g.*, phosphotyrosine proteomes [12]) or more (up to 5-plexing [13]) at the same time.

It should be kept in mind that metabolic conversion of arginine to proline can occur and that the arginine isotope label can thus be "diluted" into heavy proline. Tryptic peptides containing proline will split up into two labeled forms making data interpretation and quantification more complex. Nevertheless, a labeling approach that makes intelligent use of combinations of lysine and arginine and thereby introduces an experimental correction for proline conversion was recently described [14]. Alternatively, algorithm-based corrections for quantitative proteomics are used [15]. Lowering the arginine concentration will also decrease the degree of arginine to proline conversion, but while this is an intuitive way of compensating for isotope dilution, we and other labs observed significant differences in population doubling rates for several cell lines [16] which might also point to synthetically introduced and unwanted alterations in the composition of the labeled proteomes. Thus, ideally, cellular systems that need to be compared are grown in the same SILAC media (containing dialyzed FCS) and only the isotopic nature of the label (the essential amino acid that will be used) and not its concentration differs.

SILAC labeling has been used to study differential protein phosphorylation (*e.g.*, the yeast pheromone signaling pathway [15]) and, recently the dynamic interplay between *O*-GlcNAc modification and glycogen synthase kinase-3 mediated phosphorylation was mapped on a proteome-wide scale [17].

Having several essential amino acids available (*e.g.*, arginine, lysine, tyrosine, and methionine), proteome studies targeting a very specific aspect of dynamic proteomes are now possible. Selected examples of these are discussed in the following paragraphs.

Our lab introduced the combined fractional diagonal chromatography (COFRADIC) technique to sort amino (N) terminal peptides out of digested proteomes [18, 19]. It is essential here to introduce a difference between protein N-terminal peptides and other (internal) peptides so that both types can be distinguished in a diagonal RP-chromatographic setup. This difference is in fact introduced by *in vitro* acetylating all free primary protein amines (α and ε) prior to digestion with trypsin. Since, trypsin cannot cleave acetylated lysines, it acts as endoproteinase Arg-C and generates peptides ending on arginine. Thus, because of these sample preparation steps, eventually every sorted N-terminal peptide ends on an arginine and hence arginine SILAC labeling is a

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straightforward way to distinguish the same N-terminal peptides in different proteomes. A key application of the N-terminal COFRADIC technique is protease substrate mapping, also known as protease degradomics [20]. Protein cleavage creates a new N-terminal end and the corresponding N-terminal peptide will also be selected by the sorting procedure. To distinguish real protein N-terminal peptides from newly formed ones, differential Arg-SILAC labeling is used. For example, "light" (natural) Arg is incorporated in a proteome that will be affected by activated proteases, while "heavy" Arg is incorporated in a control proteome. After mixing and N-terminal peptide sorting, doublet isotopic envelopes point to peptides present in both samples, while singletons are derived from the protease degraded sample. The latter can then be selected for fragmentation, revealing not only the identity of the cleaved protein, but also the exact position of the cleavage site in this protein.

A recent study on taxol-induced cell death of human nonsmall-cell lung carcinoma A549 cells illustrates our protease degradome approach. The proteome of dying cells ($^{12}C_6$-Arg labeled) was compared with that of nontreated cells ($^{13}C_6$-Arg labeled) using N-terminal peptide sorting [21]. Several *in vivo* protein processing events were characterized pointing to both caspases and calpains as being key proteases activated in this cell death model. As an example, processing of human plectin-1 (Swiss-Prot accession number Q15149) after Asp-2771 in taxol-treated A549 cells is shown in Fig. 1 (the corresponding spectra have been generated with a Waters Q-TOF Premier mass spectrometer (for details see [21])).

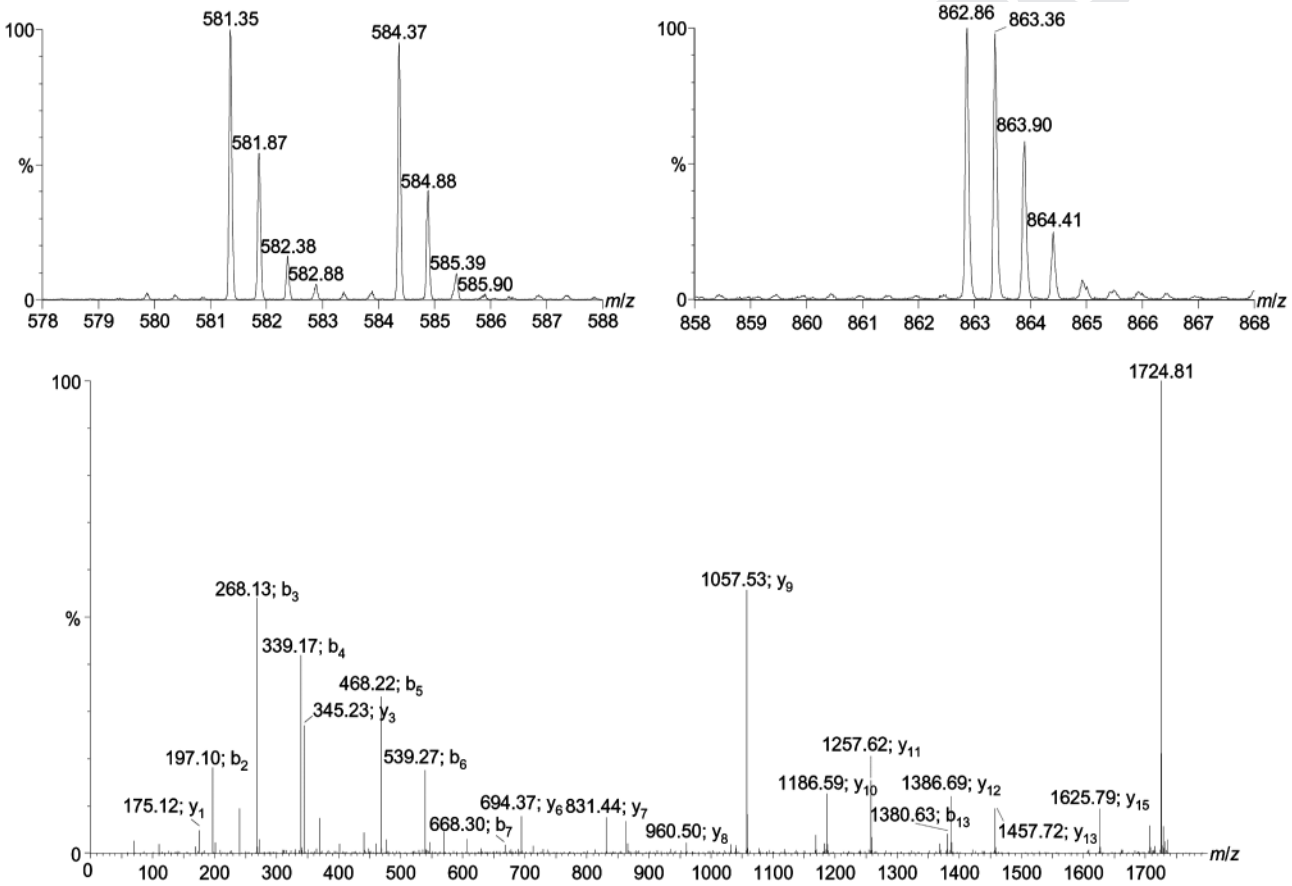

Figure 1. Processing of plectin-1 in taxol-mediated apoptotic A549 cells. Control cells were grown in $^{13}C_6$-Arg SILAC medium while taxol-treated (dying cells) were grown in $^{12}C_6$-Arg SILAC medium. Equal volumes of both cell cultures were mixed and N-terminal peptides were isolated by COFRADIC. LC-MS/MS analyses were performed on a Waters Q-TOF Premier mass spectrometer (for experimental details, see [21]). Two doubly charged peptide ions spaced by 3 Th are shown in the top left mass spectrum. These ions are present in about equal numbers and as expected point to a nonaffected, mature protein N-terminus upon MS/MS analysis; the N-terminus of the vesicle-associated membrane protein 3 (VAMP-3, Q15836; the peptide was identified as STGPTAATGSNR (acetylated at its α-amino group)). Shown in the top right panel is a doubly charged peptide ion that was only present in one sample and was thus expected to originate from *in vivo* protein processing in dying A549 cells. Following MS/MS analysis (the deconvoluted spectrum is shown in the bottom panel; only b and y fragment ions and the precursor ion are indicated) and MASCOT database searching [70], this peptide was identified as GPAAEAE-PEHSFDGLR (α-amino group acetylated and peptide ending on $^{12}C_6$-Arg) starting at position 2772 in plectin-1. Since this peptide is preceded by DALD, this novel N-terminus most probably pointed to hitherto unreported caspase cleavage of plectin-1 at Asp-2771.

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Methionine is an essential and relatively rare amino acid in mammalian cells (about 2.41% of all amino acids). Proteins can be labeled with heavy variants of Met without risking isotope dilution into other amino acids using cell culture protocols that have been established since many years for ^{35}S-Met protein labeling (*e.g.*, [22]). Only for post-translationally methylated proteins there is a transfer of the ^{13}C-methyl group from methionine *via* the activated methyl pathway (see below). Methionyl peptides can be easily isolated using a simple COFRADIC sorting protocol based on oxidation to the more hydrophilic sulfoxide form [23]. Because methionine appears in nearly all eukaryotic proteins at low frequency, targeted isolation of methionyl peptides strongly reduces the overall peptide complexity, while this selection is just focused on those peptides carrying the differential quantitative information. Figure 2 shows MS spectra of two mouse transgelin tryptic peptides carrying either heavy or light methionine. This actin-associated protein was found highly induced in mouse embryonic fibroblasts (MEF) cell lines in which an actin gene was knocked-out (unpublished results).

A similar sorting protocol can be used for global analysis of *in vivo* methionine oxidation as the result of cellular oxidative stress. This protein modification can be considered as a fundamental sensor of oxygen and oxygen-derived components (such as H_2O_2) in the cell. Like the phosphorylation/dephosphorylation switch, methionine sulfoxide formation can lead to conformational changes in targeted proteins, which may affect their function or downstream signaling, while sulfoxide formation can be reversed by the action of cellular reductases. Global analysis of the methionine oxidation status in cells is difficult because of lack of suitable tools (*e.g.*, modification-specific antibodies). However, if methionines are *in vivo* oxidized, peptides carrying them will no longer be affected by the hydrogen peroxide treatment in our COFRADIC setup and are thus not sorted. We exploited this in an *in vitro* cell model of oxidative burst; human Jurkat T-lymphocytes treated with hydrogen peroxide. These cells were SILAC labeled with either $^{12}C_5{}^{14}N$-methionine or $^{13}C_5{}^{15}N$-methionine and in a typical experiment, one cell culture was treated with 3 mM (final concentration following an initial stimulus with 1 mM) of H_2O_2 for 2 h, whereas the

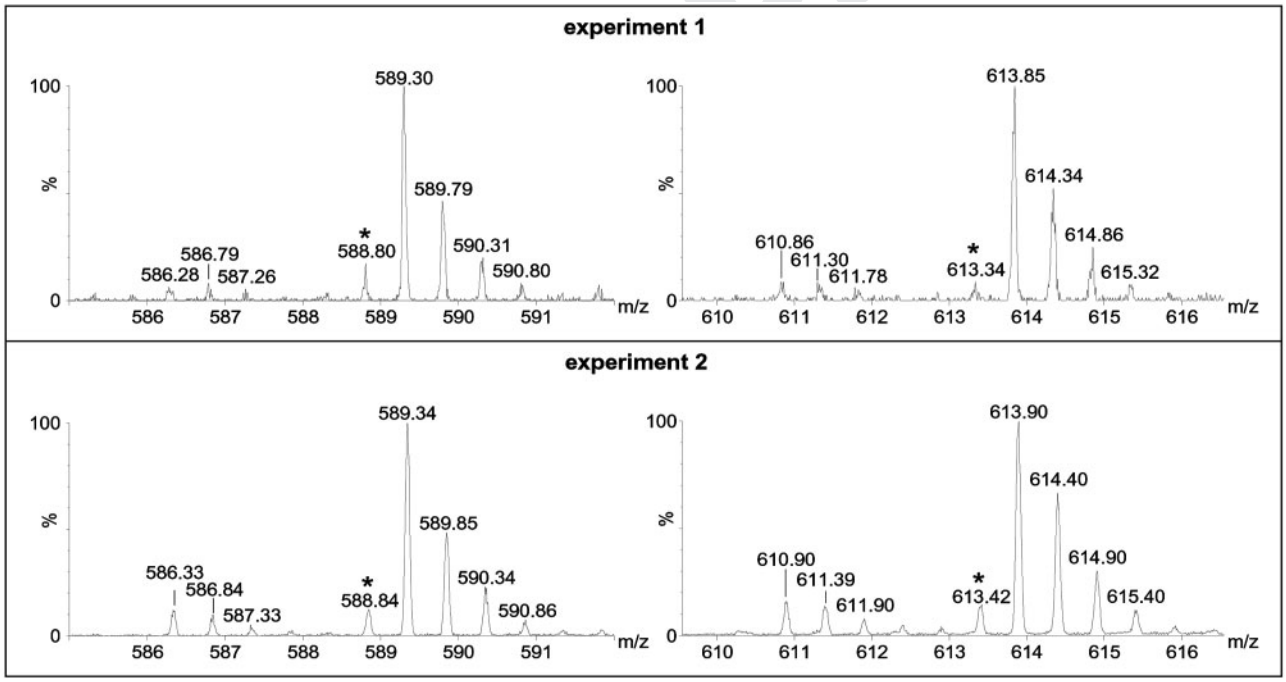

Figure 2. Induction of mouse transgelin expression in β-actin knock-out MEF cells. Proteins were labeled by methionine SILAC (wild type MEFs were grown in $^{12}C_5{}^{14}N$-Met medium, β-actin knock-out MEFs in $^{13}C_5{}^{15}N$-Met medium). MS spectra shown here are from different proteome screens (here denoted experiments 1 and 2) in which methionine COFRADIC was used to compare protein expression levels between wild type MEFs and two β-actin knock-out MEF cell lines (top and bottom spectra, respectively). Following MS/MS analysis, the peptide ion in the left MS spectra was identified as 184-GASQAGM^{ox}TGYGR-195 (M^{ox} = methionine-sulfoxide), whereas that in the right MS spectra was identified as 90-QM^{ox}EQVAQFLK-99 (N-terminal pyroglutamic acid), both belonging to the mouse transgelin protein (Swiss-Prot accession number: P37804). The light/heavy ion intensity ratios point to significant induction of expression of this protein in β-actin knock-out MEF cells (on average more than six-fold). Furthermore, these expression levels differences are very similar for the different peptides identified (compare left to right spectra) and in different β-actin knock-out MEF cell lines (compare top to bottom spectra). Also note that the small peak in front of the monoisotopic ion of the heavy labeled peptide (here labeled with an asterisk), is due to the activated methyl cycle in which the end-standing methyl group of methionine participates. Here, the ^{13} C-methyl group is gradually replaced by ^{12} C-methyl derived from other cellular components. This effect is more pronounced in MEFs compared to other cell lines.

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other cell culture was untreated and served as a control. Methionine oxidation is a limited and specific modification as evident from the majority of sorted methionyl peptides that appeared as couples spaced by 6 Da and showing no altered concentrations. However, several peptides were present at reduced levels in the proteome digest of Jurkat cells under H_2O_2 stress and thus pointed to *in vivo* oxidation, although at this stage increased protein turnover in these cells cannot be ruled out. An example of a sorted peptide holding a potential *in vivo* oxidized methionine in β-filamin is shown in Fig. 3.

A final example of the potential of SILAC is the analysis of *in vivo* methylation by so-called "methyl SILAC" in which advantage is taken from the fact that methionine is metabolically converted into S-adenosyl methionine (SAM), which is the most important biological methyl donor. More specifically, $^{13}CD_3$-methionine was used as labeling reagent which is *in vivo* converted to $^{13}CD_3$-SAM and thus serves as a heavy methyl donor. Quantitative proteomics led to the identification of 59 *in vivo* methylation sites, several of which were hitherto unknown [24].

2.2 Labeling of higher eukaryotes for quantitative proteomics

Some successful proof-of-concept studies in which entire higher eukaryotic organisms were metabolically labeled for quantitative proteomics have been described.

In 2003, the lab of Albert Heck described nitrogen-15 labeling of two small organisms: the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* [25]. *C. elegans* was fed on ^{15}N-labeled *Escherichia coli* and fruit fly larvae were fed on ^{15}N-labeled yeast. For both organisms ^{15}N labeling was reported to be 95% or more complete. A quantitative proteome study was done by comparing the early adult stage proteomes of wild type *versus* glp-4 mutant (^{15}N-labeled) *C. elegans* strains. Since, labeling was done on the level of the organism, equal numbers of both strains were mixed prior to proteome isolation thereby reducing downstream experimental errors. Isolated proteomes were then separated by 2D-PAGE and spots were randomly selected for protein identification and quantification. Several proteins were found in deviating ratios and some were further validated [25].

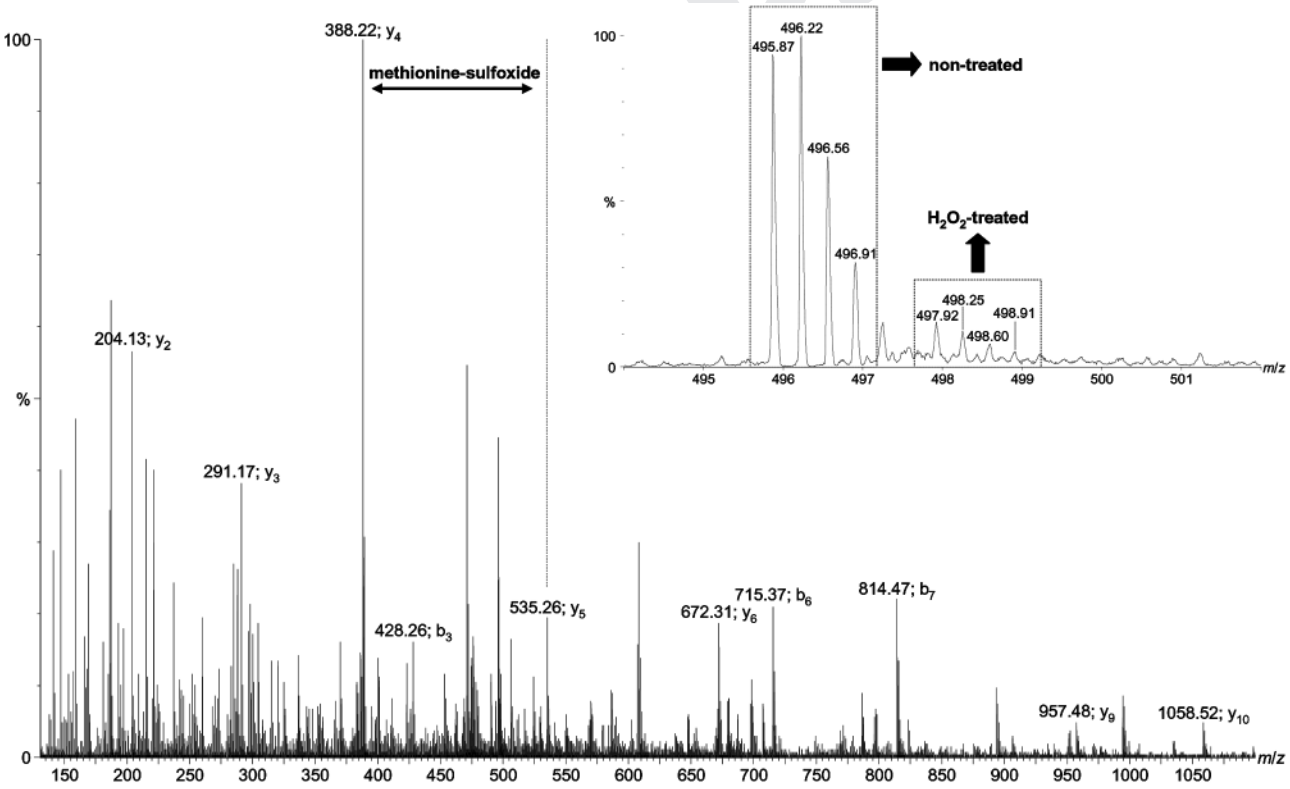

Figure 3. Methionine-1766 of β-filamin is susceptible to hydrogen peroxide stress in human Jurkat cells. Human Jurkat T-cells were cultured in either light $^{12}C_5{}^{14}$N-Met SILAC medium (control cells) or heavy $^{13}C_5{}^{15}$N-Met SILAC medium (H_2O_2 treated). Following induction of oxidative stress (see text), cells were mixed and the extracted proteome was sampled by the methionine COFRADIC technique [23]. An example of a sorted peptide carrying an *in vivo* oxidized methionine is shown. The mass spectrum in the inset shows a triply charged precursor ion which is much more abundant in nontreated cells (more than eight times; compare the signal at 495.87 Th with that at 497.92 Th). Following MS/MS analysis (ESI Q-TOF Premier) of the ion at 495.87 Th (MS/MS spectrum shown; only b and y fragment ions indicated) and MASCOT database searching, this peptide was identified as 1757-KGEITGEVHMPSGK-1770 with Met-1766 oxidized (to its sulfoxide) of human β-filamin (O75369), an actin-binding protein.

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Metabolic labeling of a rat by feeding a protein-free diet supplemented with ^{15}N-enriched algae was described in 2004 by the lab of John Yates [26]. The ^{15}N-labeled liver proteome of this rat was used as an internal standard to which the unlabeled liver proteomes of a control rat and a rat treated with cycloheximide (CHX) were compared by multidimensional protein identification technology (MudPIT), [27]. The levels of about 1000 different proteins were measured and 310 of these were quantified in both liver proteomes. Taking advantage of the ^{15}N-liver proteome as an internal standard, the levels of these 310 proteins in control liver *versus* CHX-treated liver were directly determined and following statistical analysis, 127 proteins with significantly ($p<0.05$) different ratios were identified, most of these functioning in xenobiotic metabolism and protein folding.

3 Postmetabolic labeling approaches

3.1 Enzymatic oxygen-18 labeling of peptides

Incorporation of oxygen-18 at the COOH-terminus of peptides during protease mediated-cleavage is known for a long time [28]. It has been used for MS/MS analysis to help distinguishing y-ions from b-ions formed by peptide fragmentation (*e.g.*, when digesting proteins in $H_2{}^{16}O/H_2{}^{18}O$ (1:1) [29, 30]). An interesting aspect is that the ^{18}O-isotopes are not only added during proteolysis, but can also be incorporated postcleavage [31]. Here, proteins are first digested in normal conditions and only after drying the peptide mixture, $H_2{}^{18}O$ is added. Trypsin is here the preferred protease because it efficiently catalyses the exchange of the two C-terminal oxygen atoms of peptides ending on lysine or arginine, with a preference for the latter. Thereby, a mass increase in 4 Da is introduced which is generally sufficient to separate the light and heavy peptide isotope envelopes, opening this method for quantitative analysis.

Although the procedure appears as an ideal postcleavage, stable isotope labeling procedure – it is simple, there is no need to remove by-products and it is quite cheap since only small volumes of $H_2{}^{18}O$ are used – its popularity is limited. There are probably several reasons for this. First, labeling takes place late in the overall analytical process; thus the two samples are processed separately during quite a number of manipulations before being differently labeled and mixed, introducing several sites for technical variation. Second, when trypsin is not fully inactivated or removed after ^{18}O-exchange, trypsin-mediated back-exchange during downstream processing in solvents containing natural water occurs. Importantly, this isotope dilution is also noticed at acidic pH (from pH 4 to 5 and lower) where trypsin-mediated cleavage of the peptide bond is assumed to be completely halted. This was often erroneously interpreted as incomplete or partial labeling since it was assumed that peptide cleavage and oxygen exchange were identical processes, which is not the case. Third, in the absence of trypsin but at lower pH (*e.g.*, 0.1% TFA as used in conventional RP solvents), slow back-exchange also occurs, which can be avoided by working at higher pH (*e.g.*, using ammonium acetate buffering at pH 4.5–5.5). Fourth, we noticed that for highly acidic peptides, ^{16}O-^{18}O exchange tends to be slow and incomplete. Fifth, the 4 Da spacing requires mass spectrometers with high resolving power (*e.g.*, Q-TOF, Orbitrap, and FT-ICR mass spectrometers) to segregate the isotopic envelopes. This also implies that the popular ITs can generally not be used for differential proteomics by ^{18}O-labeling. Although all of this sheds negative light on the technique, trypsin-mediated ^{18}O-tagging has its value, since most issues listed above can be easily controlled and its universal character makes this labeling technique accessible to all types of samples including body fluids. In addition, intelligent software tools have been developed that are able to tackle variable and inefficient ^{18}O-labeling (*e.g.*, [32]).

In order to evaluate the oxygen-18 tagging procedure in relation to the SILAC approach, we compared two sets of differentially labeled N-terminal peptides; one from the human Jurkat T-lymphocyte cell line, the other from the human myelogenous leukaemia K562 cell line. The sorted N-terminal peptides from both cell lines had the same nature: α-N-acetylated and carrying a C-terminal arginine. In both cases, one proteome preparation was labeled with the heavy isotope, whereas the other contained the natural isotope. For the Jurkat proteome, peptides were labeled by ^{18}O-tagging at the peptide level, while for the K562 cells two cell cultures were used, one of which was labeled with $^{13}C_6$-Arg. In both samples the intensity ratios of the light *versus* the heavy forms of the peptides were calculated and we could merely notice differences in the distribution patterns of these ratios (Fig. 4). This shows that oxygen-18 tagging can indeed be used in a differential quantitative set-up, provided that very strict conditions (see above) are created and peptide analysis is done on high-resolution mass spectrometers.

The oxygen-18 labeling "trick" was also used in a COFRADIC-based phosphoproteome approach [33]. Phosphorylated peptides are separated by RP-HPLC in different fractions and in each of these, dephosphorylation by phosphatases is performed. Dephosphorylation produces a hydrophobic shift of affected peptides but since the phosphate group is now lost, it is difficult to ensure that the chromatographic shift is provoked by dephosphorylation and not artificial (*e.g.*, following deamidation). To solve this problem, the sample was split in two and one part was labeled with ^{18}O and fully dephosphorylated. Then, both parts were recombined and subjected to COFRADIC phosphoproteomics. Since now a phosphopeptide and its dephosphorylated counterpart elute in distinct fractions, peptides that shift and appear as ^{16}O-singletons are designated as true "ex-phosphopeptides", while those appearing as a $^{16}O/^{18}O$-doublet are nonphosphorylated peptides.

Oxygen-18 labeling has also been used to more accurately determine sites of N-glycosylation. Here, peptide-N-glycosidase F (PNGaseF) hydrolysis of the N-linked glycan is per-

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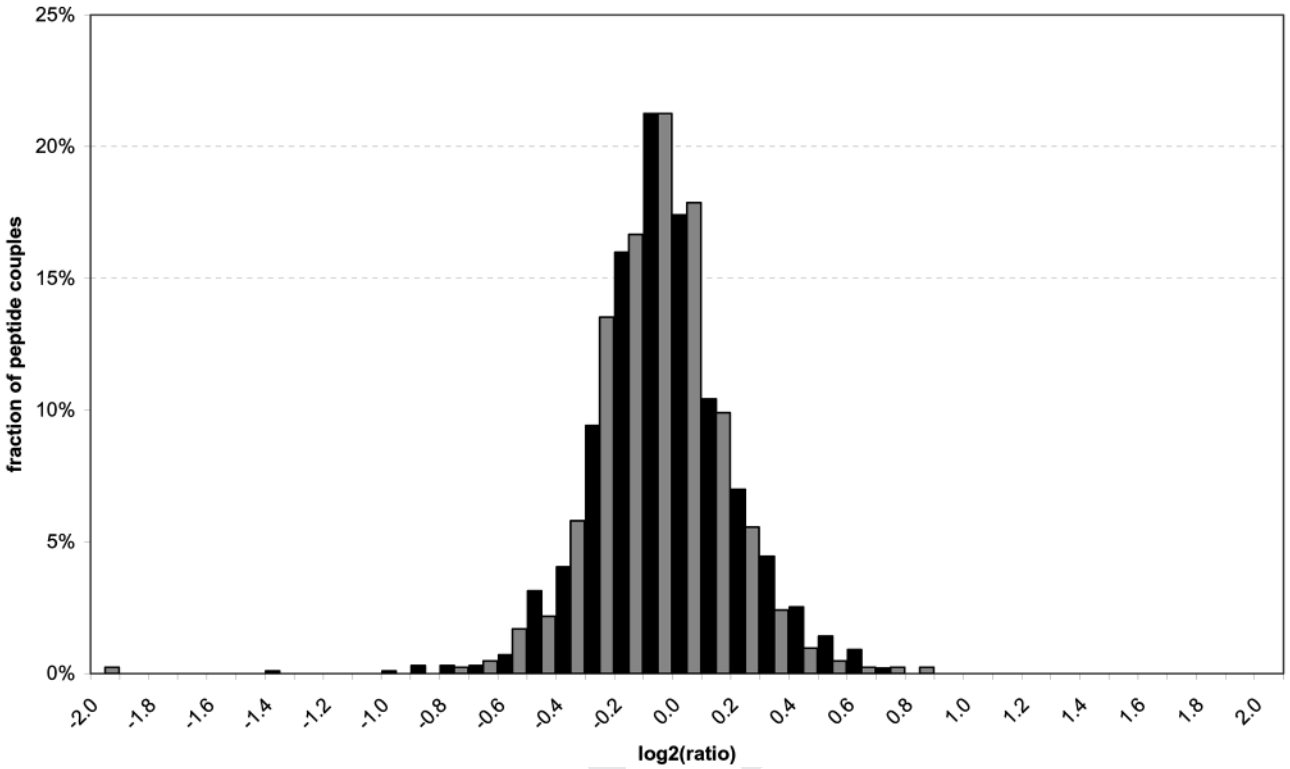

Figure 4. Comparison of SILAC arginine labeling and oxygen-18 labeling. Gray bars correspond to couples of $^{12}C_6$-Arg/$^{13}C_6$-Arg N-terminal peptides (414 peptides) isolated from human K562 cells, while black bars correspond to Arg-ending N-terminal peptides (988 peptides) isolated from human Jurkat cells, but now labeled with two ^{16}O or two ^{18}O isotopes (see text). The distribution of the log 2 values of the ratios of light *versus* heavy peptides centers around a value of 0 indicating that, as expected, most peptide ions are present in equal amounts in the sampled proteomes. Furthermore, these distributions are very similar, indicating that ^{18}O-labeling can be as accurate as SILAC labeling for quantitative proteomics.

formed in $H_2{}^{18}O$ by which a mass tag of 3 Da compared to the mass of peptide stored in databases appears following MS (*e.g.*, [34, 35]).

3.2 Nonenzymatic labeling methods

3.2.1 Isotope labeling of amino groups at the peptide level

Primary amines are favorite targets for isotope labeling because of their specific reactivity and the wide variety of available reagents. Rather simple labeling protocols make use of acetylation or acylation of amino groups for quantitative proteomics. For example, mass differences of 3 Da (*e.g.*, by trideutero-acetylation [36] or ^{13}C-propionylation) or 4 Da (*e.g.*, by succinylation [37]) can be introduced. Reductive dimethylation also yields a mass increase in 4 Da when deuterated formaldehyde is used [38]. Here, the tertiary amine created at the peptide's N-terminus is suggested to enhance a1-fragment ion formation, thereby improving database searching [39]. More recently, it was shown that combinations of the used reagents – d0 and d2 formaldehyde and d0 or d3 sodium cyanoborohydride – allow simultaneous analysis of four different proteome samples [40]. Interestingly, the authors reported negligible shifts in RP-column retention times, which is generally a problem when deuterium is used for the creation of heavy mass tags (see the original isotope-coded affinity tag (ICAT) molecules). Because these reagents generally do not distinguish between α- and ε-amino groups, mass tagging depends on the number of lysines present in the modified peptide. This can create problems at two levels: nonuniform mass tagging and risk of variable chromatographic effects. However, this can be controlled when digesting proteins with endoproteinase-Lys-C yielding peptides which have in general only one C-terminal lysine.

3.2.2 Isotope labeling of amino groups at the protein level

Amino mass tagging can also be performed at the protein level. This creates the flexibility to either preprotect ε-NH_2-lysine groups (*e.g.*, by guanidination) or not, thereby steering the tagging either exclusively to protein N-termini or to both α-and ε-amino groups.

N-terminal nicotinylation was introduced by the lab of Peter James in 2000 [41] and became later commercialized as

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ICPL (*i*sotope-*c*oded *p*rotein *l*abeling [42]). Using combinations of d0, d4, and $^{13}C_5$ nicotinic acid N-hydroxysuccinimide (NHS)-esters, multiplexed differential analysis became possible. When only present at the α-amino group of modified peptides, the fixed positive charge of the label at acidic pH directs peptide fragmentation and, by increasing the signals of b-type of fragment ions, can be used for *de novo* sequence analysis. In a follow-up of this chemistry, James's lab introduced the parent (precursor) ion quantitation scanning (PIQS) technology [43]. The general idea is here to only sample peptides that show significant deviating levels for MS/MS analysis. In brief, this is achieved as follows. All protein lysines are blocked by succinylation to prevent them from further reaction. Following digestion, peptides are reacted with iodoacetic anhydride making their N-termini reactive for the PIQS reagents (*e.g.*, different isotopic forms of mercaptobenzimidazoles). In LC-MS/MS mode, mass spectrometers alternatively scan for the light and heavy signature ions from the PIQS reagents (parent ion scans), track such pairs and calculate their ratios. Subsequently, only peptides with a significantly altered ratio are analyzed in full MS/MS mode and identified. This approach seemingly holds great potential for future, more rational, and intelligent differential proteome studies.

We recently showed that our N-terminal COFRADIC technique can be used to assess the *in vivo* acetylation status of protein α-N-termini [18] and thus characterize the general substrate specificities of the α-N-acetyltransferase complexes that cotranslationally acetylate nascent polypeptide chains. In higher eukaryotes, the majority of cytosolic proteins are expected to be acetylated at their α-N-terminus however by lack of analytical tools no real systematic analysis has been performed. Prior to the actual COFRADIC steps, all free amino groups of proteins need to be blocked to allow the largest recovery of N-terminal peptides. This is most conveniently done with an NHS-ester of acetate [19]. Upon replacing this reagent with an NHS-ester of trideutero-acetate, a mass tag is incorporated at all *in vivo* free α-amino groups and all lysines. Hence, following MS/MS analysis of COFRADIC-sorted N-terminal peptides, *in vivo* acetylated protein N-termini are readily distinguished from *in vivo* free N-termini which hold an *in vitro* trideutero-acetylated α-N-terminus. Examples of *in vivo* fully, partially and non-acetylated protein N-termini are shown in Fig. 5. Besides for reporting the *in vivo* modification status of protein N-termini, we use trideutero-acetyl groups to locate protein processing by proteases [44]. As discussed above, when used in a differential modus (*e.g.*, control *vs.* apoptotic cells), N-terminal COFRADIC sorts neo-N-termini that report protein processing events (see also Fig. 1). Since proteases create protein breaks, new α-N-termini arise. When these are now chemically trideutero-acetylated, an additional mass tag is present in the COFRADIC-sorted peptides that is used to directly point out potential protein processing events in result lists of database search engines. Indeed, such reporter peptides start typically many positions away from the initiator methionine and are readily distinguished from *in vivo* acetylated peptides also starting at such positions but pointing to alternative translation starts or gene prediction errors.

An interesting alternative for multiplexed quantitative peptide labeling is offered by the iTRAQ chemistry introduced in 2004 [45]. These amino-reactive reagents are composed of two distinct groups: a balancing group and a reporter group. While the individual moieties hold a gradual change in mass, they are assembled such that the sum of the masses of these two groups is always the same and the reagents are therefore termed isobaric. During PSD or MS/MS, the bond linking the balancing group and the reporter group is broken and the reporter ions are detected in peptide fragmentation spectra and used to quantify the peptide levels in each proteome. Besides for protein quantification, iTRAQ has been used for discovering protease substrates [46, 47]. While the first version of iTRAQ allowed 4-plexed proteome studies [45], the newest version now allows 8-plexing [48] and is expected to speed up the throughput of proteomics significantly. A similar reagent designed to label proteins rather than peptides (protein iTRAQ) appears to have a few problems related to detection of the reporter ions and peptide identification. One interesting observation was that for multiple charged precursor ions, high collision energies were needed for efficient liberation of the iTRAQ reporter ions from modified lysine side-chains, and this resulted in poor generation of b and y fragment ions hindering peptide identification [49].

3.2.3 Labeling of thiol groups

An early example of combining shotgun proteomics and quantification is the ICAT method developed by the group of Aebersold [50]. Originally, a modified thiol-reactive iodoacetamide derivative harboring an affinity tag (biotin) and a linker region was used to modify cysteines such that following protein digestion, cysteinyl peptides could be affinity-isolated for further analysis. Since cysteine is rare amino acid (making up 1.42% of all amino acids), the ICAT approach significantly reduces the sample complexity. The linker region could carry up to 8 deuteriums (d8 ICAT) thus providing sufficient m/z spacing in mass spectra and allowing quantitative proteomics. It was however reported that the d0 and d8 ICAT tagged peptides had different retention on RP columns: d8 ICAT tagged peptides are less well retained and elute in front of d0 ICAT tagged peptides [51, 52]. If this is not postanalytically corrected, erroneous peptide ratios will be determined. Furthermore, the original ICAT tag is very bulky and was reported to pose problems when interpreting MS/MS spectra of tagged peptides since many fragment ions resulted from the tag itself rather than from the actual peptide backbone [53]. More recent, MS/MS fragmentation hindrances were compensated by the introduction of solid-phase tags with cleavable linkers [54] that were further labeled with carbon-13 instead of deuterium by which coelution between light and heavy peptides

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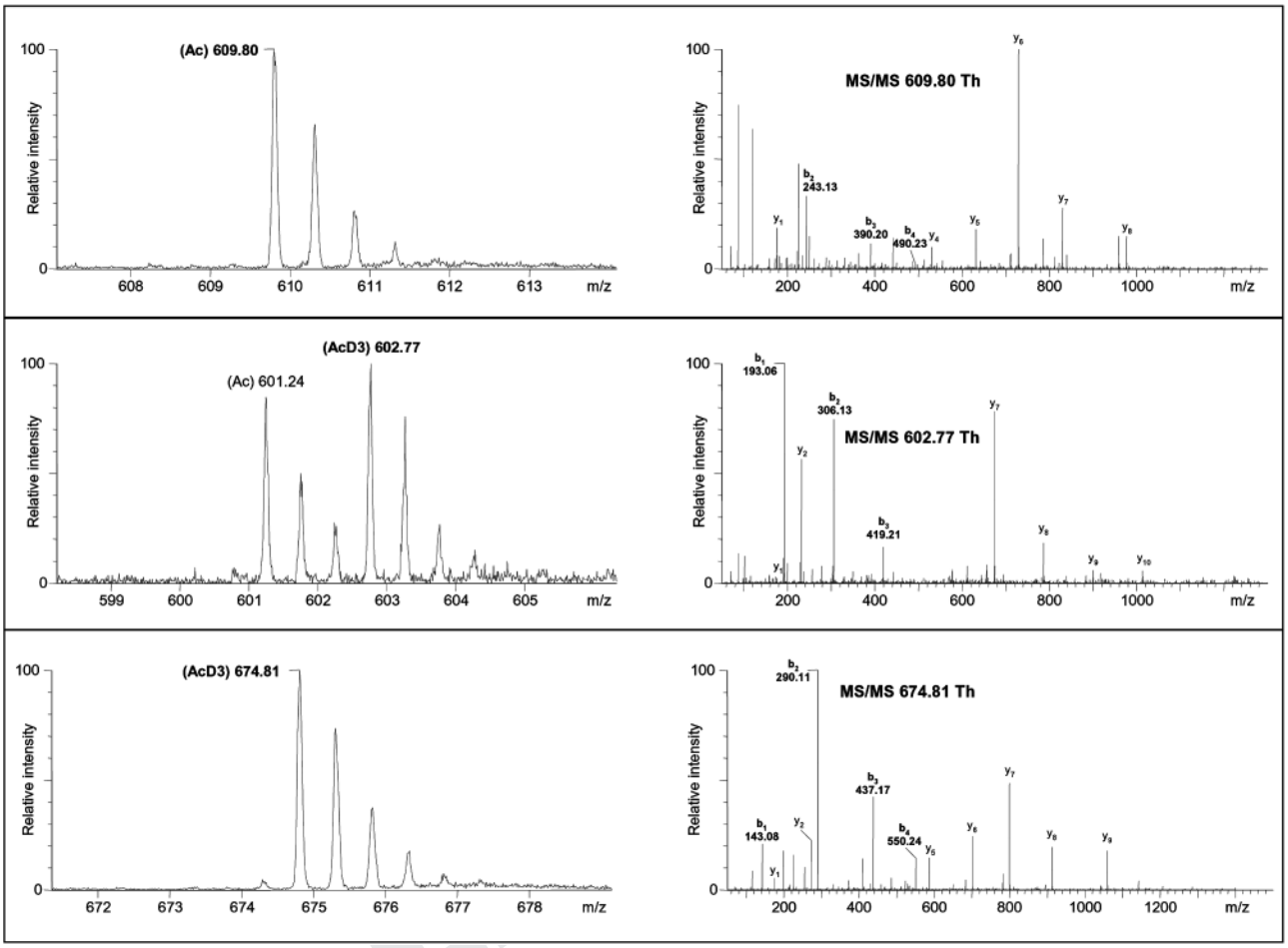

Figure 5. MS and MS/MS spectra pointing to *in vivo* free and acetylated protein α-N-termini. MS and MS/MS spectra of doubly charged peptide ions are shown at the left and right sides, respectively. Spectra shown in the upper panel originate from an *in vivo* acetylated (Ac) α-N-terminus from the Shwachman-Bodian-Diamond syndrome protein (Q9Y3A5). The peptide was identified as 2-SIFTPTNQIR-12, with its N-terminus acetylated. Spectra in the middle panel point to the presence of two variants (*i.e.*, the α-*N*-acetylated and free (AcD3) forms) of the N-terminus of the zuotin-related factor 1 (Q99543). Here, the trideutero-acetylated peptide ion was sampled by MS/MS and identified as 2-MLLLPSAADGR-12 (its methionine was oxidized to a sulfoxide). Spectra shown in the lower panel point to an *in vivo* free α-N-terminus (peptide identified as 2-PMFIVNTNVPR-12 with its N-terminus *in vitro* trideutero-acetylated and the methionine oxidized) from the macrophage migration inhibitory factor (P14174). In all MS/MS spectra, the *m*/*z*-values of the b-type of fragment ions are indicated as are the observed y-fragment ions.

was accomplished [55]. An interesting application of the ICAT technology is the characterization of oxidation-sensitive, protein-bound cysteines (redox proteomics) [56]. The assumption here is that in conditions of oxidative stress some cysteine thiol groups get modified such that these are no longer open to ICAT modification. Upon comparing a control cell population with one under oxidative stress, ICAT-tagged peptide couples showing altered ratios may point to *in vivo* modified cysteines [56]. ICAT labeling has also been used to study protein processing by matrix metalloproteases (*e.g.*, [57, 58]). Here, the levels of secreted and shed extracellular proteins are quantified by ICAT using cell lines that typically over-express the metalloprotease of interest.

4 Methods for absolute quantification

Rather simple absolute quantification was introduced by the group of Steven Gygi in 2003 [59]. The absolute quantification of proteins (AQUA) technology makes uses of heavy isotope labeled peptides as internal standards added preferentially as early as possible in the analytical process. More multiplexed absolute quantification was done by first constructing a nonnatural gene in which the different tryptic peptides one wants to quantify are concatenated. This patchwork protein is expressed in a host cell system for producing recombinant proteins such as *E. coli* grown in nitrogen-15 media (thus labeling the recombinant protein). By use of affinity tags, the protein is isolated and, following

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digestion with trypsin, a set of heavy labeled signature peptides – typically, these are peptides known to ionize well and their sequences are derived from the proteins one wants to quantify – that can be used as internal standards for proteomics is in that way created [60]. In follow-up studies, these so-called QconCAT peptides (or concatamers of quantitative (Q) peptides) were produced in $^{13}C_6$-Lys and $^{13}C_6$-Arg media instead of ^{15}N media [61, 62]. Now, all QconCAT peptides hold the same mass difference (6 Da) compared to their natural counterparts, making quantification more straightforward.

A very important issue relates to the actual peptide that will be selected as an internal standard. When starting from predictions only, these peptides should be well soluble allowing their isolation following production, easily ionized and only point to a single protein (or protein isoform) in the complex background of a complete proteome. Production problems of QconCAT peptides were recently reported [63] and illustrate the necessity of selecting peptides based on experimental data next to plain predictions. Ideally, proteotypic peptides [64, 65] – peptides that are observable by mass spectrometers (thus ionize and fragment well and with m/z-values well inside the practical m/z-range of the spectrometer used) and can be unambiguously associated to a single protein – should be selected and analyzed by targeted multiple reaction monitoring (MRM) (*e.g.*, [66]).

Despite the different solutions proposed for absolute protein quantification, the challenge in this field remains considerable and there are several important issues requiring reflection. In a direct differential comparison (relative quantification), the variables in the two samples are in a physical and chemical identical state, the only differences being their concentrations and their masses. By for instance combining two proteome samples early in a SILAC proteomics schema, all consecutive losses encountered in the following steps will be identical for the two protein (and later on peptide) isotopic variants. Thus, one may logically expect that the peptide ratios will correctly reflect the ratios of their parent proteins. The same reasoning will hold for an absolute quantification strategy if the protein to be measured is compared to the same protein generated as an isotope variant added at the beginning of the entire process. Unfortunately, while this might be possible for one or a few proteins (for which each time an isotopic variant has to be generated, purified, and exactly quantified) such strategy cannot be followed for screens of tens or hundreds of proteins simultaneously. Therefore, peptides have been suggested as preferred "standard compounds" to measure the concentrations of their naturally occurring parent proteins. Peptides are readily synthesized, purified, and quantified in large quantities and further tagged with a large variety of ^{13}C- or ^{15}N-labeled derivatives, not only restricted anymore to the essential amino acids.

This is fine when the standard is added in an appropriate amount to the biological sample immediately before (as a concatenated artificial protein) or after (as individual peptides) the proteolytic cleavage process and when the generated peptide mixture can be directly injected in the mass spectrometer for analysis. Unfortunately, the complexity of biological samples is generally too high such that preprocessing is necessary. This can for instance be depletion of the most abundant serum/plasma proteins, protein precipitation, prefractionation, protein modification such cysteine side-chain stabilization, *etc.*. It is clear that reference peptides can only be added at later stages, which means that the quantification will not include the variable and unpredictable individual protein losses that may be encountered in all preprocessing steps. Taking these limitations into account, the peptide reference approach would still remain valuable when the preprocessing steps are standardized and carried out in a highly reproducible manner.

5 Conclusions

Tagging proteins or peptides with stable heavy isotopes has found broad applications in contemporary proteomics, which is relying on MS as the analytical instrument. Generally, heavy isotopes of hydrogen (D), carbon (^{13}C), nitrogen (^{15}N), and oxygen (^{18}O) are used. Although the choice of isotopes is often connected to the proteomic question and sample to be analyzed, major selection criteria are determined by the mass difference produced upon isotope labeling and the extent of the potential shift that is produced during chromatographic separation of the normal and heavy peptide variants. As a general rule, mass differences should preferably not be less than 4 Da, since too much overlap between the isotopic envelopes of the light and heavy variants will occur. When the two variants are at less than 4 Da mathematical corrections are necessary taking into account natural isotope distribution, mass and/or sequence of the peptides [32]. It is now generally accepted that natural peptides and their heavy isotope variants may elute differently during RP-HPLC (depending on the elution system). Deuterated components are frequently reported to be more sensitive to such chromatographic effects in particular when the deuterium is bound to a sp^3 carbon (*e.g.*, as in the original d8 ICAT molecule, see above). Other isotopes – ^{13}C, ^{15}N, and ^{18}O that are amongst others present in different SILAC amino acids as well as in iTRAQ reagents – appear much less sensitive and are therefore preferred above deuterium.

In this paper, we illustrated different uses of stable heavy isotope labeling. The most direct applications are differential quantitative analyses, where two or more samples are differently tagged, mixed in equal amounts, and simultaneously analyzed. While all isotopic variants are assumed to behave in an identical manner during consecutive purification steps, the resolution and quantification of the different isotopic variants is left to the mass spectrometer. In this situation, it is of high importance to incorporate mass tags as high upstream as possible in the entire process. This is most conveniently done using a SILAC-type of approach. If impos-

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sible, tagging either at the protein or even lower, at the peptide level, is performed. Here, it is realized that introducing the isotope tags downstream the process may increase the risk for uncontrollable variations in the different samples. In most cases, two different proteome samples are compared, however more complex labeling strategies allow multiplexing as described above for SILAC and iTRAQ.

As described herein, heavy isotopes can also be used to direct proteomics to the peptides of interest. Indeed, peptide-centric proteomics generally deals with an extremely high number of different compounds that cannot be fully covered even with the best mass spectrometers [67]. Therefore, strategies have been developed to either reduce the complexity or to target the peptides of interest. Very often, one can use isotope tagging to discriminate targeted peptides. From our own work we have shown examples where peptides reporting a proteolytic cleavage site (*e.g.*, due to activity of apoptotic proteases) can be distinguished from normal protein N-termini (*e.g.*, [21, 68, 69]). Similarly, we have used the doublet-*versus*-singleton approach to identify and confirm phosphopeptides [33]. Differential acetylation/trideutero-acetylation allows distinguishing *in vivo* α-*N*-acetylated from nonblocked proteins [18]. Finally, we have been able to characterize *in vivo* methionine oxidation during oxidative stress. In each of these examples a different isotope tagging method was used: ^{18}O-^{16}O exchange, arginine SILAC, trideutero-acetylation and methionine SILAC, illustrating the possibilities of a tool that makes proteomics more versatile and more selective.

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