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Review

Current chemical tagging strategies for proteome analysis by mass spectrometry

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Abstract

Proteomics, the analysis of the protein complement of a cell or an organism, has grown rapidly as a subdiscipline of the life sciences. Mass spectrometry (MS) is one of the central detection techniques in proteome analysis, yet it has to rely on prior sample preparation steps that reduce the enormous complexity of the protein mixtures obtained from biological systems. For that reason, a number of so-called tagging (or labeling) strategies have been developed that target specific amino acid residues or post-translational modifications, enabling the enrichment of subfractions via affinity clean-up, resulting in the identification of an ever increasing number of proteins. In addition, the attachment of stable-isotope-labeled tags now allows the relative quantitation of protein levels of two samples, e.g. those representing different cell states, which is of great significance for drug discovery and molecular biology. Finally, tagging schemes also serve to facilitate interpretation of MS/MS spectra, therefore assisting in de novo elucidation of protein sequences and automated database searching. This review summarizes the different application fields for tagging strategies for today's MS-based proteome analysis. Advantages and drawbacks of the numerous strategies that have appeared in the literature in the last years are highlighted, and an outlook on emerging tagging techniques is given.

Keywords: Affinity tags; Isotopic labeling; Mass spectrometry; Proteomics

Contents

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1. Introduction

Since the mid-1990s proteomics, typically defined as the complete analysis of the protein complement of a cell or an organism (the proteome), has rapidly grown in importance as a discipline in the life sciences. Since then, several subdisciplines like "descriptive" proteomics, dealing exclusively with the cataloging of proteins, "functional" proteomics, focusing on the dynamic state of the proteome, or "interaction" proteomics attempting to explore protein interactions (the "interactome"), have emerged [1]. Uniting all the knowledge emerging from the various aspects of proteome analysis will eventually lead to substantial progress in biology, medicine, pharmacy and other life science disciplines over the long-term.

Mass spectrometry (MS) plays a central role in proteomics research, not only since the Nobel Prize in Chemistry has been awarded to John B. Fenn and Koichi Tanaka in 2002, for their role in the development of electrospray ionization (ESI) and laser desorption/ionization (LDI). In the 1980s, these so-called "soft ionization" techniques have laid the groundwork for the modern MS analysis of proteins and peptides. But despite the fact that mass spectrometers have become more powerful, easy to use and affordable in recent years, the successful outcome of a proteomics project relies also on the sample handling and prefractionation steps that come before mass spectrometric detection and identification.

For the analysis of proteins from complex biological mixtures, a number of limitations still remain that cannot be overcome simply by improvements of MS systems alone. In this review, we address some of these current "bottlenecks" in proteomics and highlight the role that chemical tagging strategies play to overcome them. We define "chemical tagging" methods here as those methods that involve the modification of functional groups of amino acid residues, including post-translational modifications, in proteins and peptides.

It is our intention to provide a comprehensive account of the status quo, by including lesser-known methods and stating advantages and limitations of both general concepts and individual strategies. However, due to the ever-growing number of novel tagging techniques reported in the literature, it is difficult to describe every single method in detail, so sometimes concepts are only briefly mentioned. To follow the rapid progress in the field, it was attempted to include the very latest developments at the time of writing.

As this review covers only chemical tagging strategies, other complementary protocols will be mentioned only when appropriate. For a more detailed insight into such related topics as well as for more general articles on proteome analysis by mass spectrometry, the reader is referred to several excellent reviews that have been published recently [2–13].

2. Current limitations in MS-based proteomics

We have chosen three topics to demonstrate the impact of tagging strategies on proteomics projects in combination with mass spectrometric analyses. These are:

- the reduction of sample complexity using affinity tags or related methods, typically used in the course of preseparation concepts;
- the relative quantitation of protein levels by comparative stable-isotope labeling;
- the modification of the fragmentation behaviour of peptides using charge derivatization.

These are the most important fields of application where tagging strategies play a significant role. Of course, apart from that there are numerous other approaches in proteome research that aim to increase sample throughput, sensitivity, confidence of protein identification and many other aspects. This, in turn, leads to the situation that sometimes more data is generated than can be reliably interpreted [14]. Public dissemination of raw data is also not yet common practice [15]. Still, we are currently in a period of rapid progress in this field, and concepts based on chemical modifications are part of the methodological advances that can help to fulfil the great expectations in proteomics.

2.1. Reduction of sample complexity

Even the most advanced MS instrumentation available today is not able to deal with very complex biological

samples like cell extracts as a whole, without prior prefractionation. In the past, reduction of sample complexity has mostly relied on two-dimensional gel electrophoresis (2D-GE) combining isoelectric focusing in the first and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) in the second dimension to efficiently separate protein mixtures. Well-known drawbacks of the technique are limitations in the p*I* and molecular weight of proteins (highly acidic and basic proteins as well as very small and very large proteins cannot be effectively separated), difficult automation and reproducibility problems. Furthermore, low abundant proteins are either not detected at all due to the limited sensitivity achievable with commonly used dyes, or they are masked by higher abundant comigrating proteins. Hydrophobic membrane proteins are known to be difficult to solubilize prior to GE separation and are typically underrepresented in proteomic studies using gel electrophoretic separation.

While 2D-GE still can be considered the most widely used separation technique prior to MS analysis, liquid chromatographic and, to a lesser extent, capillary electrophoretic separations are increasingly used in so-called "gel-free approaches". For this set-up, all proteins present in the sample are usually enzymatically cleaved into smaller peptides to obtain a very complex mixture that is then subjected to further separation steps. This strategy, also called "bottom-up" approach, takes advantage of the higher separation efficiency of chromatographic techniques on the peptide rather than the protein level. Gel-free techniques overcome some of the limitations of gel-based techniques although they lack in resolution of individual sample constituents, even when two-dimensional HPLC – typically consisting of an ion exchange step in the first dimension and reversed-phase separation in the second – is performed.

At this point, so-called affinity tagging (or labeling) procedures can be used to specifically enrich a subpopulation of peptides from the total digest (Fig. 1). For example, only those peptides that contain a certain amino acid will be targeted. Via chemical modification, an affinity tag (e.g. containing a biotin moiety) is attached to the functional group of interest, allowing the sample to be purified by affinity chromatography (in this case, biotin–avidin chromatography). If a relatively rare amino acid, like, e.g. cysteine or tryptophan, is chosen as a target, only a relatively small fraction of peptides will carry this residue, resulting in a significant reduction of sample complexity due to the affinity separation concept. However, in most cases, it is still possible to deduce the parent protein from which the peptide originated in the final data analysis step. With a similar strategy, it is also possible to isolate post-translationally modified peptides from a mixture. Frequently, affinity tagging is also combined with stable-isotope labeling in some form to allow relative quantitation (see also the following section).

Sections 3 and 5 of this review will focus on the application of chemical tagging for the reduction of sample complexity.

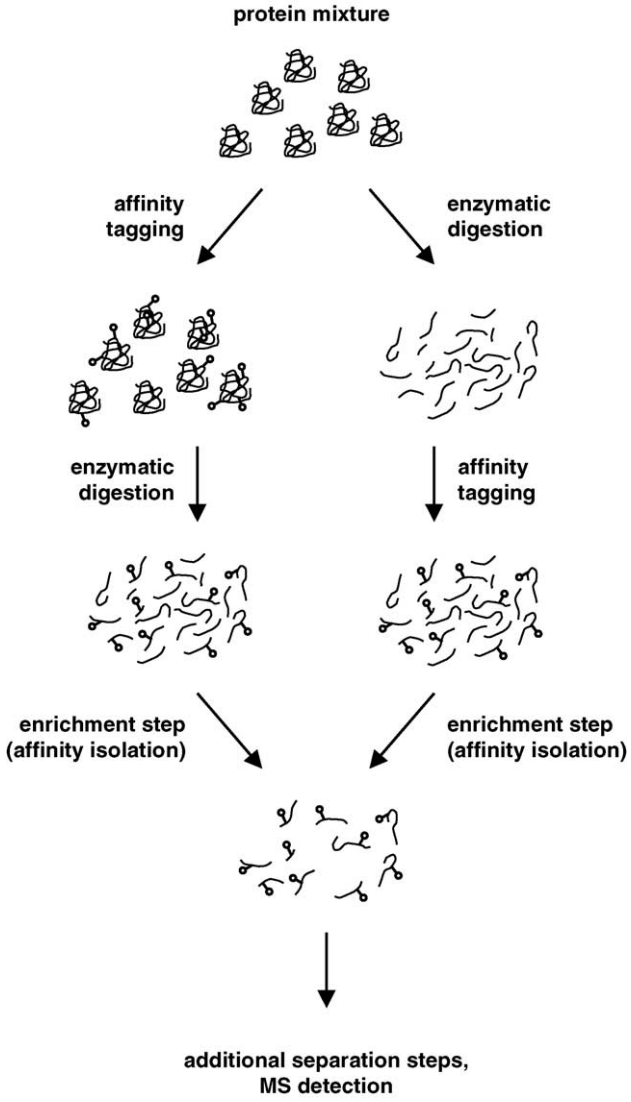

Fig. 1. The use of chemical tagging strategies for sample fractionation. A protein mixture is either first labeled with an affinity tag and then digested (left) or first digested and then labeled (right). In both cases, labeled peptides are subsequently enriched by an affinity chromatography step, so that ideally only the tagged peptides remain.

2.2. Tagging for quantitative proteomics

In large-scale proteomic projects, the *absolute* quantitation of the levels of even the majority of all proteins that are identified is usually not feasible, although it can be performed for selected proteins even in a complex mixture [16–18]. Fortunately, it is very often sufficient to obtain information on the *relative* amounts of peptides (and, subsequently, proteins) in two samples representing two different sample conditions. Examples are cells grown on different culture media, cells from healthy and tumor tissue, etc. In general, the levels of many proteins will be comparable in the two samples, while only a limited number will differ in abundance. These are of high interest, because they can be examined in more detail in further functional studies and their role in disease progression

or other biochemical pathways can be elucidated [1,19]. Frequently, a higher protein level in one sample is directly associated with upregulation of the expression of this protein. As Julka and Regnier [20] pointed out in a recent review, this assumption is not entirely correct since an increase might also be caused by a reduction in the degradation rate of the specific protein in vivo.

Traditionally, relative quantitation was performed by differential staining of 2D gels. While new fluorescent dyes have overcome some of the limitations of gel-based quantification [21–22], problems generally associated with 2D-GE (limited molecular weight and p*I* ranges, etc.) still remain. At the end of the 1990s, techniques began to emerge that did not make use of relative quantitation during the *separation* step (i.e. on the gel), but rather during the (mass spectrometric) *detection* step. This is achieved by introducing stable-isotope labels or "tags" into the intact proteins or – after the digestion step – into the peptides. Fig. 2 illustrates the principle: One sample is labeled with an isotopically "light" tag (containing, for example, ^{1}H, ^{12}C, ^{14}N or ^{16}O), the other sample with the "heavy" tag containing ^{2}H (=deuterium, D), ^{13}C, ^{15}N or ^{18}O. Depending on the experimental set-up, samples are combined at a certain point during the sample preparation stage and are introduced simultaneously into the mass spectrometer, after prior gel electrophoretic or liquid chromatographic separation. Thus, both forms of the peptide (light and heavy form) are similarly affected by variations in the ionization process (e.g. suppression effects caused by coeluting compounds in ESI, inhomogeneous crystallization in MALDI). Because light and heavy forms serve as mutual internal standards, the relative intensities of the two forms should accurately reflect the ratios of the peptides (and therefore the proteins) in the original samples. Some groups have, however, reported that quantification is also possible without resorting to isotope coding, by comparing MS signal intensities in the two samples [23–26] or database identification scores [27–28].

Isotope coding can already be performed during cell culture, either by growth on isotope-labeled media [29–34] or by supplementing growth media with labeled amino acids [35–43]. This strategy has the advantage that all further sample preparation steps can be performed after combining the differentially labeled samples, thereby minimizing effects of parallel sample processing.

However, cell culture labeling is unsuitable for many types of samples (body fluids, human tissue, etc.), and so most of the labeling techniques again use some form of chemical tagging. Often, tags differing in their isotopic composition are attached to functional groups of amino acid residues, especially to the thiol group of cysteine. Isotope coding can also be combined with affinity tagging by using affinity tags that are available in two isotopic forms, so that relative quantitation and reduction of sample complexity can be achieved simultaneously. Alternatively, isotope tagging can be performed on the N- and C-termini of peptides, thereby ensuring that all peptides in the sample are differentially labeled and amenable for quantitation. Sections 3 and 4 present the different concepts for relative quantitation that are in use.

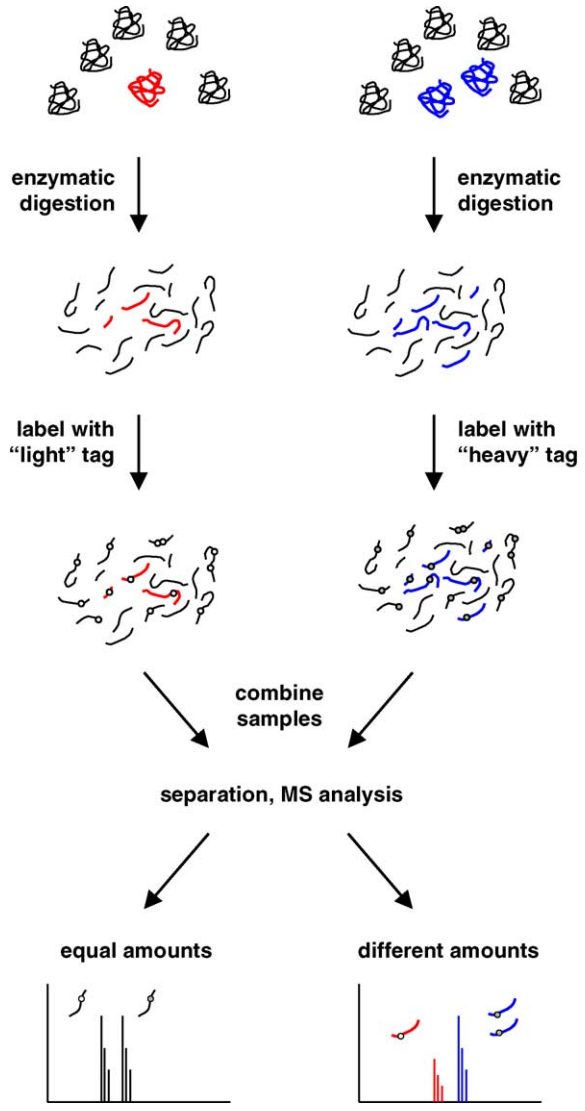

Fig. 2. Concept of stable-isotope labeling for relative protein quantitation. Two samples containing different protein amounts (red and blue, respectively) are digested seperately and the protein mixtures are then individually labeled by an isotope tag in either its light (white circles) or heavy form (grey circles). After combination of the two samples, further analysis is performed on the combined peptide pool. Mass spectra show signal pairs of the same intensity when equal protein amounts were originally present (bottom, left). Differences in abundance are reflected in a ratio other than one, in this case 2:1 (bottom, right). Alternative workflows are also possible.

2.3. Modification of fragmentation patterns

Protein identification by tandem mass spectrometry takes advantage of the fact that peptides typically fragment along their backbone, i.e. at or near the amide bond, when subjected to collision-induced dissociation in the collision cell of a tandem mass spectrometer. Ideally, bond cleavages would occur at every amide bond, however, there are a number of reasons why this is not always the case. Fragmentation

pathways and relative intensities of fragment ions are influenced by the presence of certain amino acid residues, the charge state of the peptide, the size of the precursor molecule and other factors. Incomplete fragmentation is especially problematic when the sequence of a given peptide is not present in a database and has to be elucidated de novo [44]. Also, post-source decay (PSD [45]), a dissociation technique used in combination with matrix-assisted laser desorption/ionization (MALDI), typically generates fragment ion spectra of lower quality, making interpretation more challenging. But even for routine MS/MS analyses in proteomics, a significant number of spectra is of insufficient quality so that database searches do not yield any satisfactory results.

One way to facilitate the analysis of tandem MS spectra is by attaching a permanently charged tag to one of the peptide termini. This way, charge neutralization of ions carrying either the N- or the C-terminus is achieved. Fig. 3 illustrates the principle: When a positively charged peptide ion is fragmented, the charge can remain either on the fragment carrying the N-terminus or on the one carrying the C-terminal end. The relative charge state distribution is dependent on the fragmentation mechanism and on the respective proton affinity of the fragments.

Usually, both N-terminal b-ions and C-terminal y-ions will appear in the spectrum, making assignment of the signals ambiguous. If only b- or y-ions are present, sequence elucidation is much more straightforward. This can be achieved in several ways. For instance, if a negatively charged group is added to the N-terminus of the peptide, this will result in the charge neutralization of all N-terminal fragments carrying the tag, so that only fragments from the C-terminus (y-ions) will be observed in the tandem MS spectrum. A negatively charged group on the C-terminus, on the other hand, would result in the observation of N-terminal ions exclusively.

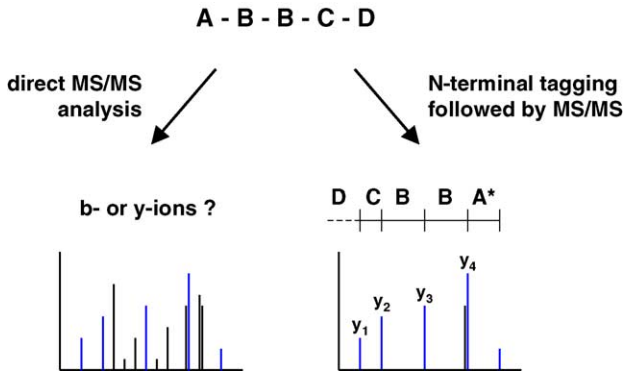

Fig. 3. Simplification of MS/MS spectra by charge neutralization. Left: A model peptide ABBCD (with A, B, C and D representing different amino acids) is subjected to collision-induced dissociation, resulting in a mixture of different fragments, making sequence elucidation de novo difficult. Right: After the attachment of a permanent negative charge on the N-terminus (denoted by an asterisk), the formation of b-ions is suppressed and y-ions constitute most of the product ions, making sequence elucidation straightforward.

Strategies to introduce fragmentation tags will be discussed in more detail in Section 6.

3. Chemical labeling of amino acid residues with isotope and/or affinity tags

As was already noted before, chemical tagging of specific amino acid residues in peptides and proteins can be used to introduce both stable-isotope-coded groups for relative quantitation in complex mixtures and to attach affinity tags to specifically enrich/isolate the peptides containing such a motif.

Considering the nature of the 20 proteinogenic amino acids, the choice of functional groups to be tagged is rather limited. Cysteine is very frequently used because its thiol group can be specifically modified, for example by reagents possessing iodoacetyl or vinyl functionalities. Such reagents have been in use for a long time to alkylate free cysteines after the reduction of disulfide bonds. Due to this fact, many different stable-isotope labeling reagents that modify the thiol group of cysteine have been reported. In addition, Cys is a relatively rare amino acid, with an average relative abundance of only 1.1% across several species [46]. Therefore, it is an attractive target to achieve substantial simplification of peptide mixtures using affinity tags. Cysteine-specific tagging schemes are discussed in Section 3.1.

Specific tagging of lysine (via amidination/guanidination) or tryptophan (modification of the indol system) has also been reported, such methods are among those presented in Section 3.2. In addition, several other specific tagging methods will be presented there.

3.1. Cysteine-specific tagging

Among the amino acid-specific tagging strategies, by far the most are directed towards cysteine residues. Various labeling chemistries specific for thiols are being used that are most often based on the reaction with iodoacetyl groups (for alkylation) or with reagents containing double bonds (for Michael-type addition reactions).

The one that probably had the highest impact of all Cys-specific tags is the isotope-coded affinity tag (ICAT) developed by Aebersold and co-workers [47]. One of the main reasons is that is was the first reagent of its kind that was made commercially available in the form of an analysis kit, by Applied Biosystems (Foster City, CA, USA) in the year 2000. The original version of the ICAT is shown in Fig. 4. The tag consists of a cysteine-reactive iodoacetyl group that allows the specific attachment of the label to the thiol group of the Cys side chain at a pH of about 7–8. A polyether linker serves as the isotope-coded region in the tag: The reagent is available in two forms, the light form containing eight hydrogens and the heavy form containing eight deuteriums. Attached to the polyether linker is a biotin moiety, so that in addition to serving as an isotope-coded label, the ICAT also allows the

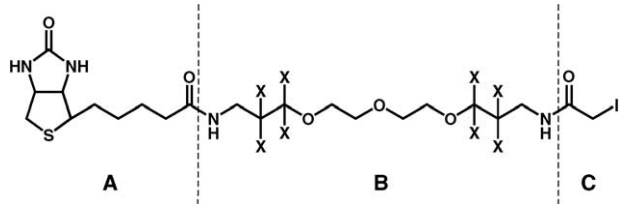

Fig. 4. Structure of the original isotope-coded affinity tag (ICAT) reagent [47]. The tag consists of a biotin moiety that allows enrichment by biotin–avidin affinity chromatography (A), an isotope-coded linker region, using hydrogen or deuterium in the first version (B), and a thiol-reactive iodoacetamide group that allows alkylation of cysteine residues with the ICAT (C).

(more or less, see below) specific isolation of labeled peptides by means of biotin–avidin affinity chromatography.

The affinity step significantly reduces the complexity of the protein or peptide mixture because only a fraction of all peptides resulting from an enzymatic digest will contain cysteine. Even when only Cys-containing peptides are detected by this strategy, this is still theoretically sufficient to receive a high proteome coverage since the majority of all proteins contains at least a single cysteine (approximately 92% in *S. cerevisiae*, generally >80% for all species) and the proteins can be identified from this one single peptide via database searches.

The typical workflow for ICAT-supported proteomic studies is outlined in Fig. 5. It is possible to introduce the affinity label either at the protein stage, e.g. before gel electrophoretic

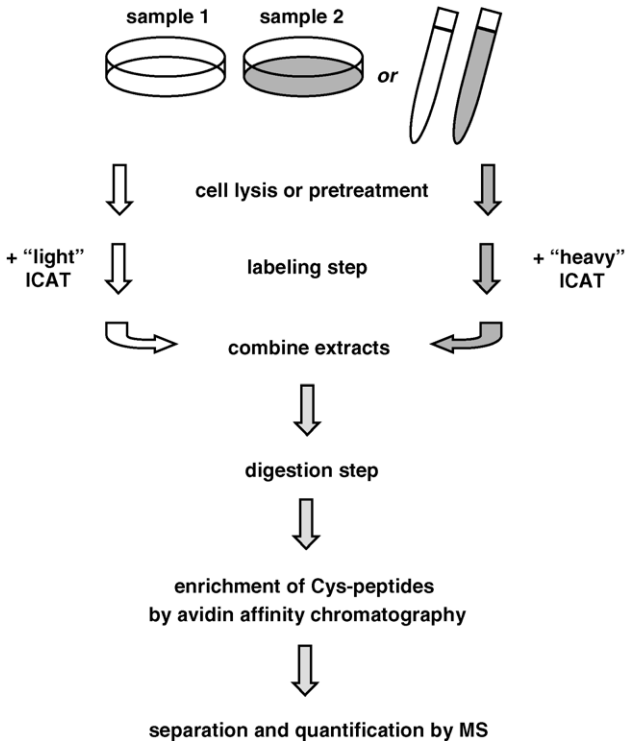

Fig. 5. Schematic representation of the ICAT workflow. ICAT-labeling can also be performed after the digestion step, so that peptides, not intact proteins, are labeled (not shown). For details, see text.

separation or at the peptide level after a digestion step. This is usually the case when gel-free separation techniques are employed. In any case, one sample is labeled with the light (d_0-) form of the ICAT and the other with the heavy (d_8-) form. After this tagging step, the samples are combined and subjected to further fractionation steps that usually also contain the above-mentioned affinity clean-up using immobilized avidin columns.

In contrast to some metabolic labeling techniques, the mass shift that will be observed in the mass spectra is predefined, namely 8 Da. So, a singly charged peptide containing one Cys will appear as a doublet separated by 8 m/z units, its doubly charged form will differ by 4 m/z units and so on.

Since the introduction of the ICAT procedure, a number of applications have appeared in the literature, e.g. [48–58]. Theoretically, the ICAT should be the ideal tag for proteomic applications, especially since software from various instrument manufacturers as well as protein database search engines now routinely allow specifying ICAT labeling as an artificially introduced amino acid modification. Specialized software has been developed that automates the quantification process [59–62]. But some criticism emerged relatively soon after the presentation of the method, possibly boosted by the commercial availability of the kits and its relatively widespread use.

For example, while this approach will cover most of the proteins, there may be some classes of proteins that typically contain very little cysteine. Potentially significant post-translational modifications are rarely observed because they would have to occur on the Cys-peptides because only these are captured during the affinity clean-up step. Of course, this is the same for other amino-acid-specific methods, and it is also possible to further analyze the flow-through from the avidin column. On the other hand, the avidin affinity clean-up step sometimes suffers from significant non-specific binding of Cys-free peptides and/or irreversible adsorption of some Cys-peptides [63].

Regnier and co-workers [64] were the first to point out that liquid chromatographic separation can occur between the d_0- and d_8-forms of ICAT-labeled peptides, in extreme cases even reaching baseline separation. This can severely affect the accuracy of the quantitation, regardless whether mass spectrometric analysis is carried out by ESI or MALDI: For ESI, ionization suppression effects can vary with chromatographic elution time so that the two forms are differently affected. When off-line HPLC prefractionation is performed prior to MALDI-MS detection, e.g. in combination with automated target spotting, the two forms can be present in different fractions that are spotted. Therefore, it is absolutely necessary that the whole chromatographic peak (containing both the d_0- and the d_8-forms) is used for relative quantitation and not just a few selected spectra because they would not accurately reflect the peptide ratios. Further work in the group of Regnier revealed that isotope labels using ^{12}C and ^{13}C as the isotope pair do not exhibit this chromatographic fractionation, as shown for Cys-reactive tags similar to the ICAT

[65–66]. Another disadvantage related to liquid chromatographic separation of ICAT-labeled peptides is that the hydrophobic biotin moiety of the isotope-coded tag influences overall retention behavior of the peptides therefore causing a relatively narrow elution zone of all tagged peptides [67].

Furthermore, the significant mass increase of the label (442 Da for the light tag) causes loss of possibly important low mass fragment ions when ion trap mass spectrometers are used, since they have a lower mass cutoff in MS/MS experiments that is in the range of 1/3 of the *m*/*z* ratio of the precursor ion. As an example, this cutoff is shifted upwards by more than 70 *m*/*z* units for a typical doubly charged peptide containing one Cys. It was also shown that because of its size, the ICAT label itself can yield a number of fragment ions [49,68], therefore complicating product ion spectra or even causing false positive identifications. Smaller labels, including the improved ICAT versions shown below, usually have only little influence on the fragmentation behavior of peptides as peptide bond cleavage is energetically preferred in these cases.

All these possible limitations that have been pointed out by various authors led to the development of modified versions of the ICAT. The first was reported by the Aebersold group in 2001: The "solid-phase ICAT" [69]. This approach used an isotope-coded tag immobilized on glass beads as shown in Fig. 6. The tag is attached to the surface of the beads via an amide bond and includes a photocleavable linker, which makes it possible to detach the tagged peptides by UV irradiation. Simplification of complex mixtures is therefore possible without biotinylation. Isotopic labeling is achieved by including a leucine moiety in either its d_0- or d_7-form and an iodoacetyl group again serves as the Cys-(thiol-) reactive group.

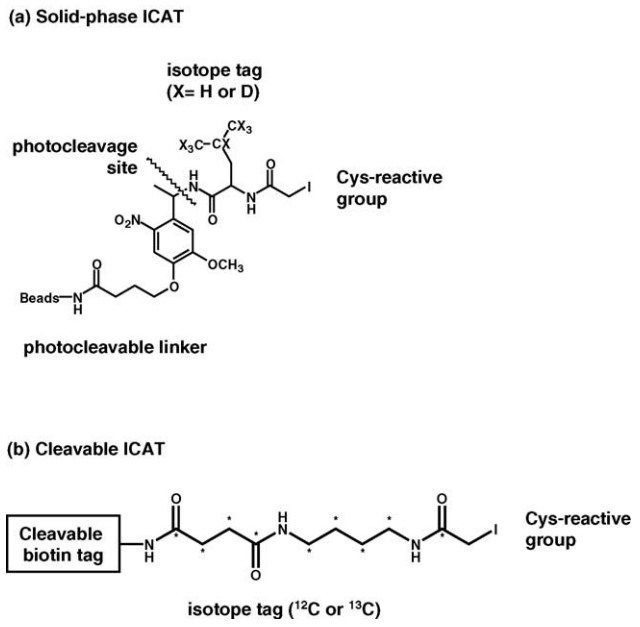

Fig. 6. Improved isotope-coded affinity tags. (a) The solid-phase ICAT [69], including a photocleavable linker region. (b) The cleavable ICAT [67,70–77] with an acid-labile affinity tag region. Asterisks denote differentially ($^{12}C/^{13}C$) labeled carbon atoms.

The whole analytical strategy is similar to the solution-phase ICAT. Two protein mixtures are independently digested and Cys side chains reduced before they are – still separately – applied to the reactive beads. Non-specifically bound peptides are then removed by a thorough washing step and captured peptides are photocleaved off the beads by irradiation in a form directly suitable for subsequent (LC)–MS/MS analysis.

The method has been used for protein profiling of *S. cerevisiae* strains grown under different conditions. The authors state various advantages compared to the classical ICAT approach described above: Because isolation of Cys-peptides and isotopic labeling is performed in one step, this procedure is less labor-intensive; non-specific binding is reduced because more stringent washing steps can be performed on the covalently attached peptides. The smaller isotope label introduced with this protocol also causes less fragmentation of the label itself and less interference with detection on ion traps. However, photochemical reactions are less reliable than other cleavage processes, even more so for on-bead reactions. This may be a cause why, beside the original article, no further applications of the solid-phase ICAT have been reported in the literature so far.

Recently, an improved version of the original ICAT label has been made commercially available by Applied Biosystems. The so-called "cleavable ICAT" (Fig. 6) uses ^{12}C and ^{13}C instead of ^{1}H and ^{2}H and therefore does not cause chromatographic separation of the light and heavy forms. In addition, it contains a linker group that can be cleaved under acidic conditions, resulting in a smaller moiety being attached to the peptide, very similar to the solid-phase-derived tag. First applications have been described [67,70–77]. In particular, Yu et al. [74] addressed important issues such as MS/MS behavior, necessary clean-up steps, completeness of labeling and accuracy of the quantitation. Judging from the available data, the cleavable ICAT seems to represent a significant improvement over the original design. Despite the promising results, the significant cost of the reagent might still be a reason limiting the more widespread use of the ICAT strategy outside of the industry.

Another ICAT variation are the so-called visible isotope-coded affinity tags (VICATs, [78]). The VICAT approach allows the *absolute* quantification of proteins in a complex mixture following a strategy outlined in Fig. 7. In this protocol, three different isotope-coded tags are used: A "normal" tag for labeling the complex sample (VICAT), a tag for labeling the internal standard peptide (^{14}C-VICAT+6), and a third tag which is used as an IEF marker (^{14}C-VICAT-28). The latter two tags are carbon-14 labeled to allow the location of the peptide of interest by scintillation counting. All three tags have in common that they carry a biotin tag for affinity isolation and a cysteine-reactive iodoacetyl group. Similar to the solid-phase ICAT approach described above, a photocleavable linker region is included in the tags as well. Apart

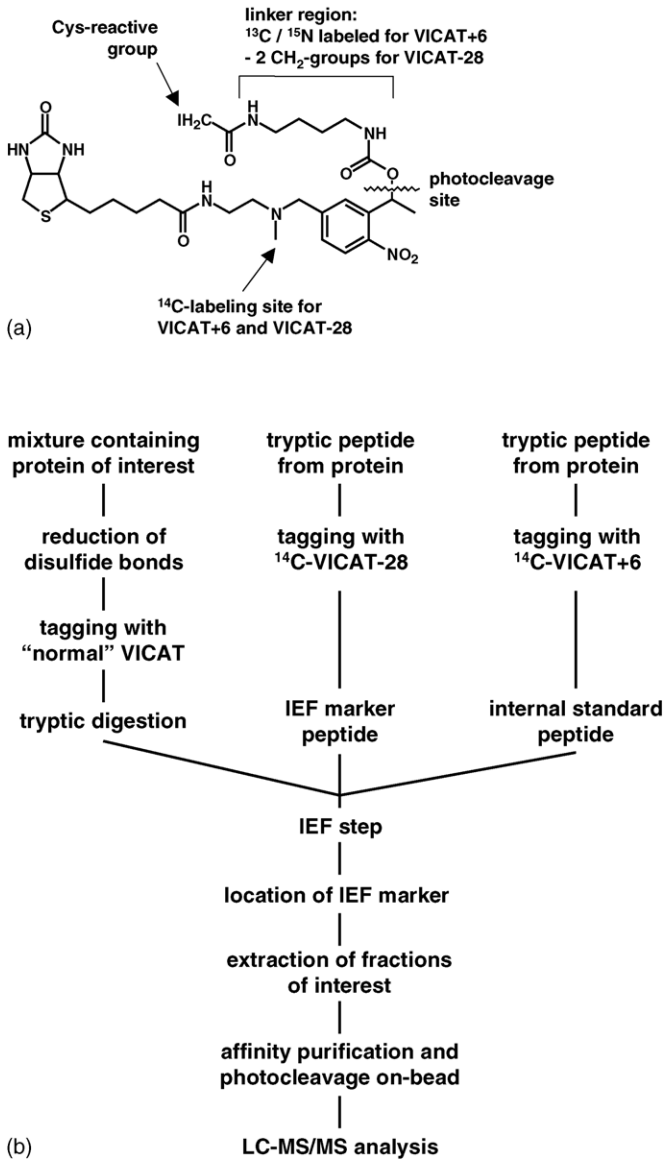

Fig. 7. Concept of the VICAT (visible isotope-coded affinity tag) strategy [78]. (a) Chemical structure of the VICAT reagents. (b) VICAT workflow. For details, see text.

from the *N*-methyl group which carries either ^{12}C or ^{14}C, but which is cleaved off after the enrichment step, the different tags are distinguished by the linker region remaining on the peptides up to the detection by MS: The tag for the internal standard is 6 Da heavier than the normal tag due to the incorporation of ^{13}C and ^{15}N, and the IEF marker tag is 28 Da lighter due to the removal of two methylene groups.

The procedure is performed as follows: The protein mixture which is to be analyzed is denatured and reduced, followed by a labeling step with the "normal" VICAT. After (tryptic) digestion, known amounts of a representative tryptic peptide of the protein of interest are labeled with the internal standard tag and the IEF marker tag, respectively, and added to the digest mixture. The total mixture is now subjected to preparative isoelectric focusing on the peptide level. After

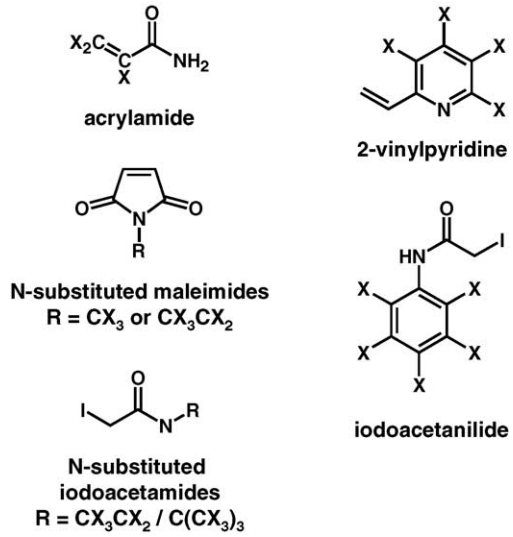

Fig. 8. Thiol-specific reagents for differential isotope coding of cysteine residues (X = hydrogen or deuterium).

the IEF procedure, regions from the IEF gel are sliced and the peptides extracted. Aliquots from the IEF fractions are examined by scintillation counting, allowing the localization of the peptide of interest in the gel via the IEF marker-labeled form. The peptide fractions of interest are captured on streptavidin agarose beads and "eluted" by a photocleavage step (via UV irradiation of the beads). From the purified sample, LC–MS/MS analyses can be performed and absolute quantification is achieved from the comparison of peak areas of the normal-VICAT peptide versus the VICAT+6 internal standard peptide. The shorter VICAT-28-form (the IEF marker) is not used for MS analysis.

The VICAT method was shown to allow quantification of individual proteins even from complex mixtures (cell lysates) and does not need an isotopically labeled peptide internal standard, because the tagging reagent incorporates the isotope tag. However, the use of radiolabeled substances requires appropriately equipped laboratories and will likely limit the broad applicability of the strategy.

A variety of other cysteine-tagging reagents for different purposes have been reported in the literature. For example, commercially available non-isotope-coded biotin affinity tags similar to the ICAT have been used for the isolation of Cys-peptides in a number of studies, e.g. [29,79–83].

Several smaller isotope tags have been proposed for differential quantitation (Fig. 8), including non-deuterated and deuterated acrylamide [84–86], *N*-methyl- and ethylmaleimide [87], *N*-ethyl-iodoacetamide [88], 2-vinylpyridine [89], methyliodide [82], *N-tert*-butyliodoacetamide [90] and iodoacetanilide [90]. These tags mainly differ in the mass shift between light and heavy version ranging from 3 to 9 Da. However, Righetti and co-workers [85,89,91] stated that reagents containing double bonds have advantages over iodoacetamide and related compounds because they allow faster and more quantitative labeling. The issue of chromato-

graphic separation of the two differentially labeled forms seems to be very dependent on the number of heavy isotopes and their location [65–66], so isotope effects are sometimes not as significant as for the ICAT label.

Cysteine tags that contain elements with a characteristic isotope pattern, like chlorine or bromine, can be used to identify Cys-peptides in mass spectra, as demonstrated by Adamczyk et al. [92–93] and Aebersold and co-workers [94]. Alternatively, MS spectra can be acquired before and after alkylation and Cys residues can be identified from the mass shifts that are observed [95–96]. The presence of cysteine in a peptide can be used as a constraint in database searches, increasing confidence in the results and/or reducing search times. The different reactivities of thiol groups in proteins can also be probed by these kinds of tags as shown by Hubalék et al. [97].

An interesting tagging strategy to create enzymatic cleavage sites at cysteines was presented by Loo and co-workers [98]. Conversion of cysteine to *S*-aminoethylcysteine produced a recognition site for trypsin or endoproteinase Lys-C in proteins. When lysine residues are blocked by acetylation before the tagging step, cleavage occurs at the N-terminal side of cysteine and arginine residues (when using trypsin) or at cysteine exclusively (using endoproteinase Lys-C). Specific in-gel labeling and digestion was performed on standard proteins separated by 1D-GE.

Solid-phase capture approaches similar to the solid-phase ICAT strategy outlined above have recently been introduced by Qiu et al. [99] and Shi et al. [100] (see Fig. 9). Qiu et al.'s method (termed ALICE for acid-labile isotope-coded extractants) is based on the immobilization of Cys-peptides onto a polymer resin by the (Michael-type) reaction of the thiol group with a maleimide group at neutral pH. The tag further incorporates an isotope-coded region with a mass difference of 10 between light and heavy form (d_0- and d_{10}-aminocaproic acid was used for synthesis of the two versions, respectively). The coding region is connected to the polymer via an acid-labile anchor group. By using this design, capture is possible at neutral pH and cleavage off the resin is achieved by incubation with 5% trifluoroacetic acid in water. Successful implementation of the ALICE strategy was demonstrated by the isolation and relative quantitation of standard protein mixtures.

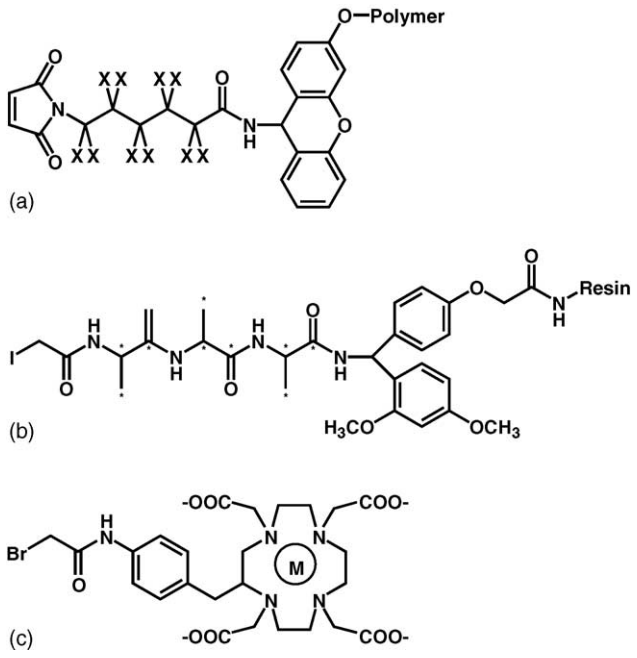

Fig. 9. Various cysteine-specific affinity tags. (a) ALICE solid-phase tag of Qiu et al. [99] (where X = H or D). (b) Solid-phase tag of Shi et al. [100]. Asterisks denote differentially ($^{12}C/^{13}C$) labeled carbon atoms. (c) Element-coded affinity tag of Whetstone et al. [102] (where M is a rare-earth metal).

The concept of Shi et al. follows a similar strategy. In this case, the solid-phase tag consists of an iodoacetyl group for reacting with cysteines, an isotope-coded region made of three isotopically labeled alanine residues and an acid-labile functionality anchored to the resin. It is noteworthy that ^{12}C and ^{13}C were used for isotope coding in this case, therefore avoiding partial chromatographic separation of the differentially labeled peptides as observed for hydrogen/deuterium-coded pairs (see above). A potential disadvantage is the highly acidic conditions that are required for cleavage off the resin (50% TFA) which might not be suitable for labile post-translational modifications. The application was demonstrated using mixtures of standard proteins. A variation of this tag was recently introduced by Zhang et al. [101] who used d_0- and d_{10}-leucine for isotope coding instead of the Ala_3-form. The use of deuterium, however, lead to the chromatographic separation of differentially labeled peptides.

Whetstone et al. developed a somewhat different Cys-specific mass coding system called element-coded affinity tags [102] (see Fig. 9). The tag contains a chelate binding group that can be loaded with different rare-earth elements (which are mostly monoisotopic). This allows the generation of mass differences between 1 (e.g. for ^{139}La and ^{140}Ce) and 86 (when using ^{89}Y and ^{175}Lu) mass units. Differentially labeled Cys-peptides can be enriched by a special immunoaffinity column containing antibodies that recognize the metal chelate moiety. Proof-of-principle was given for the enrichment of a peptide labeled either with yttrium, terbium or lutetium. It was found that the chelating tag did not interfere with reversed-phase HPLC separation or peptide sequencing by MS/MS. The possibility to differentially label not just two, but multiple samples for one experiment, makes this method highly interesting, however, application to real proteomic samples will have to be shown.

Regnier and co-workers used covalent binding of cysteine to thiopropyl-Sepharose in their prefractionation approach to enrich cysteine-containing peptides [103–104]. This covalent chromatography strategy is based on a disulfide-exchange procedure outlined in Fig. 10. Thiol groups of cysteines (obtained after the reduction of disulfide bonds in proteins) are labeled with 2,2′-dipyridyl disulfide. The modified proteins are subsequently digested with trypsin and the resulting peptide mixture is passed through a column packed with

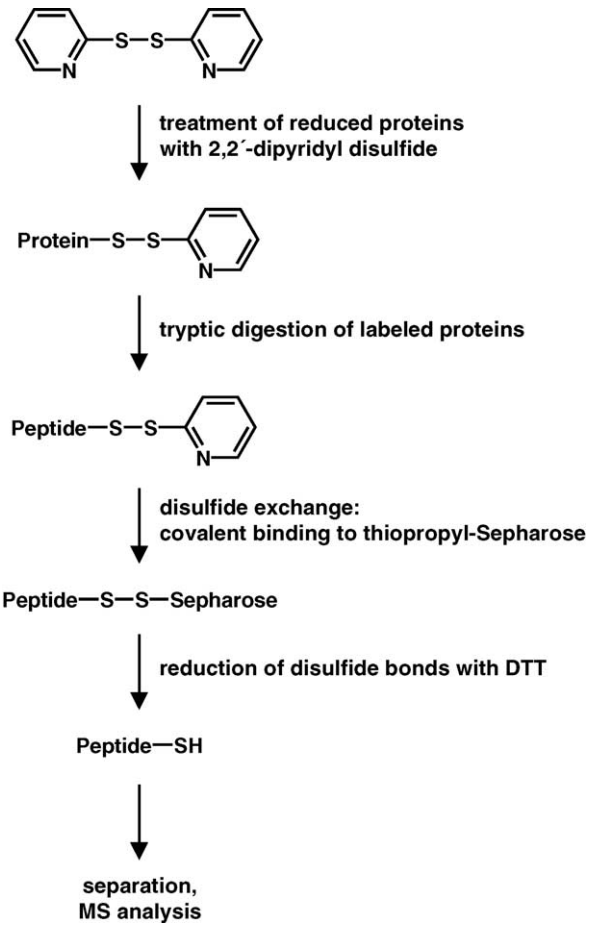

Fig. 10. Principle of disulfide exchange covalent chromatography applied to the isolation of cysteine-containing peptides. For details, see text.

thiopropyl-Sepharose. The large excess of thiol groups on the column causes immobilization of Cys-containing peptides by disulfide exchange with the pyridyl moiety. Elution of the peptides is possible under reducing conditions resulting in peptides with free SH-groups that can then be alkylated prior to their chromatographic separation. Successful application of the method for *Escherichia coli* cell lysates was shown [103–104]. Similarly, Johnson and co-workers [88] used covalent chromatography to enrich Cys-containing peptides prior to differential isotope coding with *N*-ethyl-iodoacetamide as mentioned above, while Smith's group combined it with $^{16}O/^{18}O$ labeling (see below) [105].

Another cysteine-specific tag that can be used to reduce sample complexity was recently introduced by Regnier and co-workers [106]. The new APTA tag ((3-acrylamidopropyl)trimethylammonium chloride, Fig. 11) includes a quaternary amine moiety allowing enrichment by strong cation exchange chromatography (SCX). First, disulfide bonds in the proteins are reduced and a large excess of the tagging reagent is added under alkaline conditions (pH 8.5). Following the removal of the remaining reagent by dialysis, the labeled proteins are digested with trypsin and the APTA-tagged peptides are trapped on a SCX column. After the ion

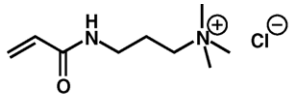

Fig. 11. Structure of the APTA tagging reagent introduced by Regnier and co-workers [106].

exchange step, the Cys-proteins can be analyzed using standard (LC–)MS protocols. The authors state that MS sensitivity for the labeled peptides is generally increased compared to their unlabeled counterparts, which can be attributed to the charged tag. MS/MS experiments revealed that singly or doubly modified cysteine-containing peptides exhibit predominant backbone cleavages, while for very cysteine-rich proteins, cleavage of the tag dominated the MS/MS spectrum. The concept was evaluated using a transferrin digest.

Mann and co-workers [107] combined the concepts of disulfide exchange and immobilized metal affinity chromatography (IMAC) in their "HysTag" approach (see Fig. 12). Cys-peptides are labeled with an isotope-coded peptidic tag containing both a hexa-histidine sequence and a pyridyl disulfide moiety. This way, the tag can be attached to cysteine thiols as a first step. The $(His)_6$ sequence then allows the enrichment of labeled peptides by IMAC (see also Section 5), similar to the protocol used for purifying recombinantly expressed proteins [108]. Alternatively, enrichment is also possible by strong cation exchange chromatography. Because the HysTag also contains a tryptic cleavage site (arginine), a large portion of the tag can be removed in a digestion step after enrichment, leaving only the dipeptide Ala-Cys (with the alanine in either d_0- or d_4-form) attached to the cysteine of the original peptide. The authors reported that mass spectrometric sequencing was not negatively affected since fragmentation of the tagged residue was not observed. In addition, no chromatographic separation of the light and heavy forms was observed, which was attributed to the relatively small number of deuteriums incorporated and the location of the tag. Application of the method was shown by identifying and quantifying a large number of proteins from mouse brain tissue.

Vandekerckhove and co-workers [109] have used a variation of their diagonal chromatography concept (Fig. 13) to specifically isolate cysteine-containing peptides [110] from a complex digest mixture: After a prior reduction step, Cys residues in proteins are first modified by Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)), the protein mixture is then digested and the resulting peptide mixture subjected to fractionation by reversed-phase HPLC (the so-called "primary run"). A following reduction step with tris(2-carboxyethyl)phosphine (TCEP) removes the Cys tag and causes a retention time shift for all cysteine-containing peptides when the isolated fraction is rechromatographed under otherwise identical conditions ("secondary run"). To reduce the analysis time, multiple fractions are collected in a

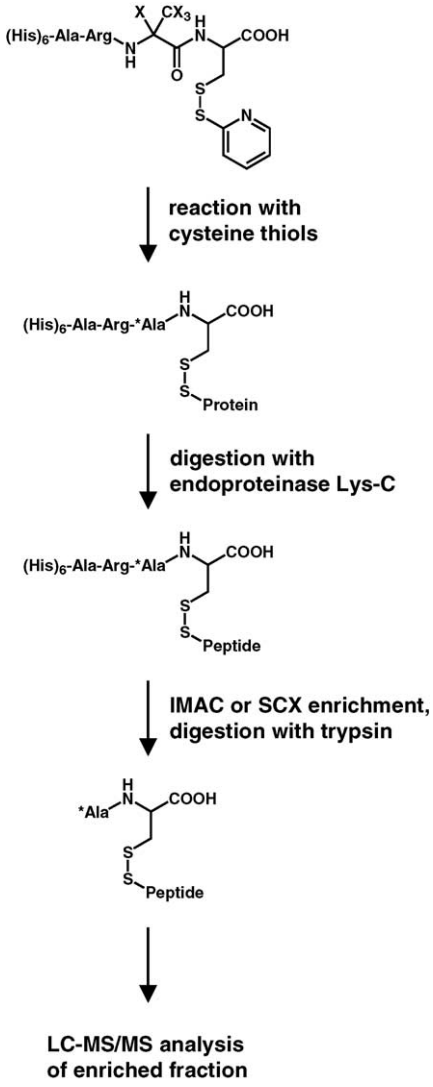

Fig. 12. Principle of the HysTag method [107]. Cysteine residues in proteins are differentially tagged with the peptidic tag (X = H or D) and digested with endoproteinase Lys-C. The $(His)_6$-sequence on the tag allows the enrichment of the tagged peptides by either immobilized metal affinity chromatography (IMAC) or strong cation exchange chromatography (SCX). A second digestion step, this time with trypsin, cleaves the tag C-terminal to arginine, so that only a Cys-Ala*-dipeptide containing the isotope tag (*) remains on the labeled cysteines.

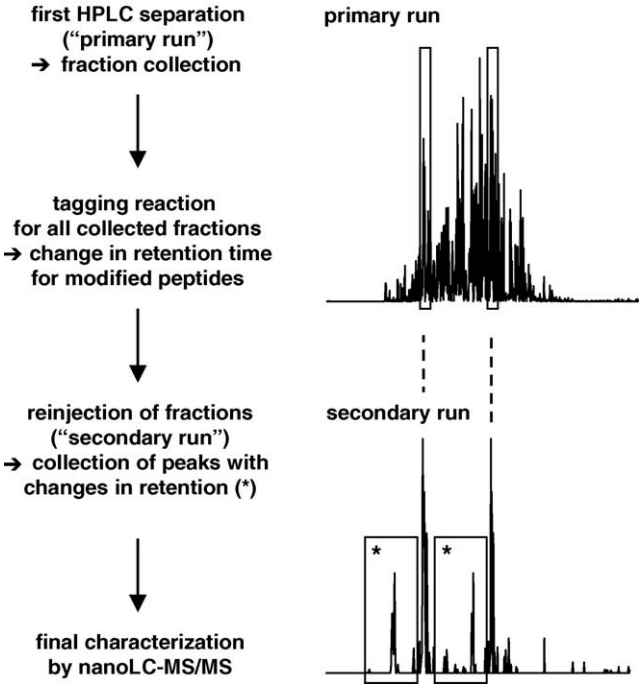

Fig. 13. Concept of diagonal chromatography [109]. The crude sample is fractionated and a subset of the peptides contained in each fraction is tagged in a way so that their chromatographic behavior is altered. Upon reinjection, most of the peptides from the fractions elute at the same position as before, only the tagged peptides show different elution times (boxes marked with an asterisk) and can be collected for further characterization.

way that the elution zones of the modified peptides do not overlap (see Fig. 13). Cys-peptides eluting in these zones can be isolated and identified in a third HPLC analysis step. The method was successfully applied to the identification of human platelet and plasma proteins. A similar method for the isolation of N-terminal peptides has also been described by the same group (see below).

3.2. Tagging methods specific for other amino acids

Apart from targeting the thiol group of cysteine, few other functional groups of amino acids remain that can be modified specifically. However, some methods have been presented that will be discussed below. Fig. 14 illustrates tagging reactions for lysine and tryptophan residues.

Lysine residues can be specifically targeted when the reagent employed does not react with the N-terminus of the peptide, as shown by Peters et al. [111–112] using 2-methoxy-4,5-dihydro-1*H*-imidazole. This tag was reported to improve mass spectrometric response of Lys-peptides in MALDI, and simplified MS/MS spectra were observed when the tag is located on the C-terminus of a peptide, similar to the methods that will be presented in Section 6. This is significant because the two most commonly used proteolytic enzymes, trypsin and endoproteinase Lys-C, cleave C-terminally to lysine residues. Differential quantitation of protein levels was also demonstrated using the deuterated analogue of the tagging reagent. This tagging chemistry has been made commercially available by Agilent Technologies [Palo Alto, USA].

Differential amidination of the amino group of lysine for relative quantitation was proposed by Beardsley and Reilly [113]. In this approach, termed QUEST (for quantitation using enhanced sequence tags), the two samples do not differ in the isotope used in the labeling procedure, but in a methylene group because lysines are derivatized by either *S*-methyl thioacetimidate or *S*-methyl thiopropionimidate. This technique appears to be restricted to MALDI-MS analysis since it can be assumed that chromatographic separation of the two forms would occur. Bergquist and co-workers [114] obtained very large deviations from the expected ratios when using

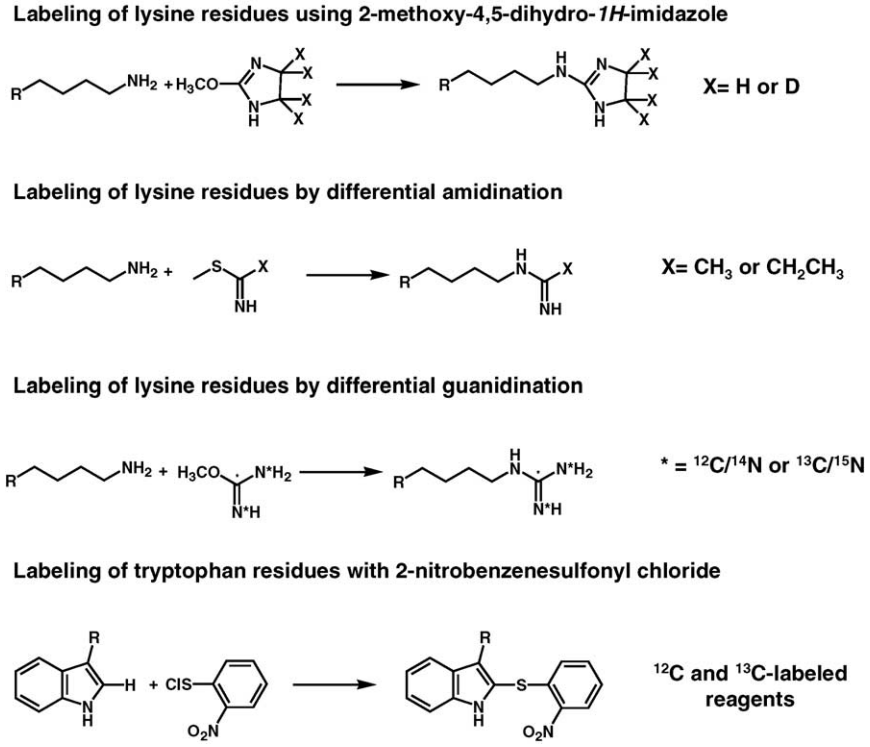

Fig. 14. Various lysine- and tryptophan-specific tags for relative quantification applications.

LC–FTICR-MS. In a recent paper, the effect of this amidination procedure on peptide fragmentation patterns was also examined [115].

A similar approach (called MCAT for mass coded abundance tagging) has been described by Cagney and Emili [116]. Instead of using two different derivatizing reagents, they performed guanidination of lysine residues (i.e. conversion to homoarginine) with *O*-methyl isourea (OMIU) for one sample while the other remained untreated. Despite the fact that significant chromatographic separation was obtained in LC–MS analysis, the authors stated satisfactory accuracy of their approach for relative quantitation.

The first guanidination strategy relying on a stable-isotope-labeled reagent was recently presented by Brancia et al. [117]. In this study, differential tagging was performed using the normal version of OMIU and an in-house synthesized $^{13}C,^{15}N_2$-form, resulting in a difference of three mass units between light and heavy form. Initial application of the method was shown for digests of standard proteins.

Recently, a technique for the differential isotope labeling of tryptophan residues for proteomic applications has been reported by Kuyama et al. [118]. Like cysteine, tryptophan is a less abundant amino acid and can be used for affinity chromatographic prefractionation of complex mixtures. The authors used 2-nitrobenzenesulfonyl chloride (NBS-Cl) for Trp-specific tagging, in addition to the commercially available $^{12}C_6$-form, the $^{13}C_6$-analogue was synthesized and used in this procedure. NBS-labeled peptides are even more hydrophobic than peptides containing unmodified tryptophan and were enriched on Sephadex LH-20 material, where they are strongly retained. Relative quantitation of Trp-peptides from rat sera by LC–MS/MS was demonstrated. Chromatographic coelution of light and heavy forms of the tagged peptides was observed since deuterium-labeling was avoided.

Chelius and Shaler [119] developed an affinity tagging and isolation strategy targeting N-terminal serine and threonine residues. Only when these residues are present on the terminus, a 1,2-amino alcohol structure exists that can be oxidized with periodate, forming an aldehyde group. Subsequently, a biocytin hydrazide tag was attached to this moiety, enabling the isolation by biotin–avidin affinity chromatography. Theoretical calculations have shown that selecting peptides with N-terminal Ser or Thr from a complex digest mixture has approximately the same effect on matrix complexity as Cys-selection. However, suitability of the method was only described for a mixture of four model peptides. Furthermore, acidic cleavage of the biotin tag to release the aldehyde form of the peptides resulted in partial degradation of one peptide and all peptides were observed as free and hydrate forms in mass spectra, complicating data interpretation. It is therefore doubtful whether this technique can be successfully applied to more complicated mixtures.

Similar to their method for isolating Cys-peptides, Vandekerckhove and co-workers [120] have used diagonal chromatography to specifically isolate peptides containing the N-terminus of a protein. In this paper, a two-step tagging procedure was used: First all amino groups of intact proteins

(protein N-termini and ε-amino groups of lysine residues) were acetylated and the protein mixture was then digested with trypsin. After the lysine modification, cleavage at this residue no longer occurs so that arginine residues represent the only cleavage sites. All tryptic peptides with the exception of the ones carrying the protein N-terminus will now carry a free amino group that can be tagged. The authors used 2,4,6-trinitrobenzenesulfonic acid as a tagging reagent to attach a trinitrophenyl moiety to the free amino groups, thereby significantly increasing the hydrophobicity of the tagged peptides, resulting in longer retention in an HPLC run. If the peptide mixture is run two times, once before tagging and once after tagging, only the peptides corresponding to the protein N-termini will have the same retention time in both runs, while all others show a strong shift to longer retention times. The unlabeled fraction was collected off-line and further analyzed in a second chromatographic dimension, resulting in the identification of more than 300 proteins from human thrombocytes. A second paper [121] describes the application of this procedure in combination with ^{18}O-isotopic labeling (see Section 4.2) for relative quantitation.

Hamon and co-workers [122–123] have developed the protein sequence tag (PST) methodology that allows the isolation of N-terminal peptides from cyanogen bromide (CNBr) cleaved proteins. Because it incorporates CNBr cleavage, it was designed particularly with the analysis of hydrophobic membrane proteins in mind. Fig. 15 depicts the strategy: The protein mixture is first solubilized and partially cleaved by CNBr treatment, resulting in cleavages C-terminal to methionine residues. Disulfide bonds in these relatively large fragments are then reduced and free cysteines are alkylated. Then, free primary amino groups (from N-termini and lysines) are protected with a "basic mass tag" (BMT), namely *N*,*N*-dimethylglycine *N*-hydroxysuccinimide ester, followed by a tryptic digestion step. At this stage, trypsin cleaves C-terminal to arginine residues exclusively because lysines have been converted by the BMT and are no longer recognized by the proteolytic enzyme. The peptide mixture now consists of tagged residues – predominantly those with the original N-terminus of the CNBr peptides and arginine on the C-terminus – and a larger number of untagged peptides with a free N-terminus. In the enrichment step, the latter ones are captured on amine-reactive scavenger beads (carrying *N*-hydroxysuccinimide groups). Thus, all untagged peptides are removed from the solution, leaving only the tagged peptides behind which are further fractionated by cation exchange chromatography and analyzed by LC–MS(/MS).

In the initial paper [122], the application of the PST strategy is described using mitochondrial proteins from *S. cerevisiae* and the authors report that a significant increase in the number of membrane proteins identified was possible compared to a standard 2D-GE-based procedure. A second paper [123] describes a refined protocol for the preparation of membrane protein samples resulting in a higher number of low-abundant proteins that were detected.

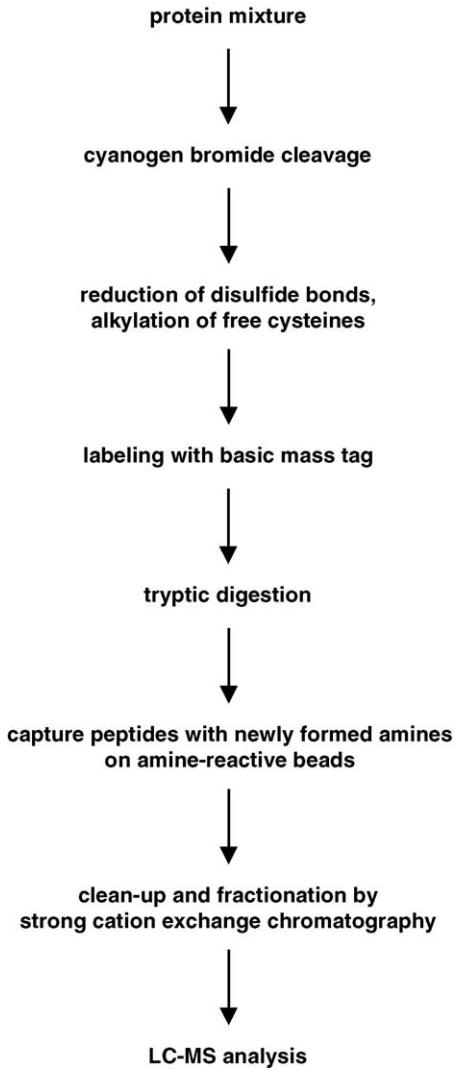

Fig. 15. The protein sequence tag strategy [122–123] for the isolation of N-terminal peptides from cyanogen bromide cleaved proteins. For details, see text.

4. Global stable-isotope labeling strategies

The techniques that were presented in detail in Section 3 rely on the modification of a certain amino acid present in a protein or peptide. Therefore, they are restricted to those analytes possessing such a group. This is advantageous for attachment of affinity tags because of the ensuing simplification of the matrix, additionally, the presence of a certain amino acid can be used as a constraint in database searches. On the other hand, it inevitably leads to reduced sequence and proteome coverage because rare amino acids are targeted.

As complementary labeling approaches for quantitation purposes, other research groups have made use of reactions that modify the N- or C-terminus of peptides. With the exception of possibly modified termini of the intact protein, e.g. due to *N*-acetylation or C-terminal amidation, all peptides resulting from enzymatic digests should be accessible to these

modification procedures. When using these "global labeling" strategies, no simultaneous simplification of the sample matrix via the attachment of affinity labels within the isotope tag is desired. Consequently, they have to rely on more sophisticated separation steps like multidimensional chromatography or high-resolution mass spectrometry to deal with the higher complexity of the mixtures. In addition, tagging reactions that target the N-terminal amino group or the C-terminal carboxyl group generally will also modify amino acid side chains with the same functionalities (Lys; Asp/Glu) so this has to be taken into account. However, some of the methods listed below use more specific reactions.

4.1. N-terminal isotope coding

One such method is the "global internal standard technology" (GIST) pioneered by Chakraborty and Regnier [124]. Stable-isotope labeling of amino groups using either *N*-acetoxysuccinimide [124–131] or succinic anhydride [103–104] (see Fig. 16) and their respective deuterated analogues has been reported. To deal with sample complexity, prefractionation using IMAC for the selection of His-peptides [104,126,131], thiol exchange chromatography for the selection of Cys-peptides [103–104] (see Section 3.1) or lectin affinity chromatography for targeting glycosylated peptides [125–126,130] (see Section 5.2) was sometimes used.

One especially innovative application reported by Liu and Regnier [129] was the identification of single amino acid polymorphisms using GIST. In this study, the proteins of interest, obtained from different species, were digested and the resulting peptides isotope tagged on both N- and C-termini. This was achieved by N-terminal labeling with d_0- or d_3-*N*-acetoxysuccinimide and by C-terminal incorporation of ^{18}O during the enzymatic digestion step (see below). The double labeling approach [128] ensured that all peptides resulting from a protein digest were isotope-coded, even if they originated from a possibly modified N- or C-terminus of the intact protein. All peptides that are identical in both species

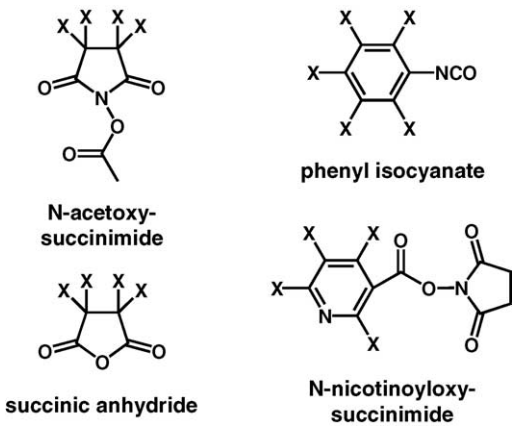

Fig. 16. Isotope-coded tags for labeling of peptide N-termini and lysine amino groups (X = hydrogen or deuterium).

appear as doublets separated in mass by the number of deuterium atoms incorporated, but those appearing as singlets most likely are the result of differences in the amino acid sequences. This way, single amino acid polymorphisms in chicken and turkey lysozyme were observed, as were variants in canine serum albumin among different breeds. So far, one shortcoming of this technique is that the mass spectra have to be manually examined to locate the singlets, therefore this approach is currently limited to less complex mixtures.

Several other groups have also reported methods for N-terminal tagging (Fig. 16). For example, a different isotope coding procedure has been described by Hsu et al. [132], who labeled amino groups of the N-termini and lysine side chains by reductive amination using d_0- and d_2-formaldehyde, resulting in a mass shift of 4 amu. This way, dimethylamino groups are formed, incorporating two isotopically labeled moieties. Application of the method towards digests of standard protein mixtures and cell lysates was shown.

Mason and Liebler [133] tagged peptide N-termini with non-deuterated or pentadeuterated phenyl isocyanate. According to the authors, the attachment is specific at the pH of 8.0 which was used for the reaction, since thiol groups are protected by alkylation beforehand, carboxyl and hydroxyl groups react only at low pH and the ε-amino group of lysine is less reactive (by two orders of magnitude) than the N-terminal amine. Tagging apparently proceeds rapidly and quantitatively in 15 min, as an additional advantage of the method, increased chromatographic retention of small peptides due to the hydrophobic tag was observed.

Münchbach et al. [134] used a two-step tagging strategy to selectively attach a nicotinoyl moiety on the N-terminus. In the first step, intact proteins were treated with succinic anhydride to modify the amino groups of lysine residues. After enzymatic digestion, the nicotinoylation step was performed with either d_0- or d_4-nicotinoyloxysuccinimide and now targeted the N-terminal amines of the resulting peptides exclusively, since lysines were already protected. The authors stated that the N-terminal tag improved mass spectrometric sequencing since the increased basicity led to higher intensities for fragment ions carrying the N-terminus (a- and b-ions, see also Section 6). Relative quantitation of gel-separated proteins was demonstrated using the technique.

Che and Fricker [135] described the relative quantitation of neuropeptide levels by labeling with non-deuterated and deuterated acetic anhydride, respectively; Annan and co-workers [136] used propionic anhydride in combination with enzymatic dephosphorylation to study the stoichiometry of protein phosphorylation (see also Section 5.1).

4.2. C-terminal isotope coding

Isotopic labeling of the C-terminus (along with aspartic acid and glutamic acid residues) can be obtained by converting carboxyl groups to the corresponding methyl esters, as was for example demonstrated by Aebersold and co-workers [61,137]. The reaction is easily performed by resuspending

the dried peptide solutions in methanolic hydrochloric acid prepared from d_0- or d_3-methanol. A possible limitation of this reaction is the possibility that the esters are hydrolyzed during chromatography when using acidic mobile phases. Similarly, Syka et al. [138] used ethyl esterification with d_0- and d_6-ethanol to study differential post-translational modifications of histones in a novel quadrupole ion trap/FT-ICR hybrid mass spectrometer.

Another C-terminal labeling procedure, and one that does not affect Asp or Glu residues is the introduction of ^{18}O into the C-terminal carboxylic group formed during enzymatic digestion of proteins [139–142]. This is achieved by digestion of one protein mixture in $H_2{}^{18}O$, while the control sample is digested in normal $H_2{}^{16}O$. Using trypsin, one or two oxygen atoms from the solvent are built into the C-terminus, which may be dependent on the sequence of individual peptides. Drawbacks of this strategy are the high price of ^{18}O-labeled water and the fact that due to incomplete incorporation of ^{18}O, the mass shift between the two samples is limited and leads to a significant overlap of isotopic distributions of light and heavy forms. Still, because of the straightforward procedure, labeling with ^{18}O is relatively frequently used; for recent examples, see [143–148]. In addition, it has been shown that incubation of an already digested peptide mixture with trypsin also leads to $^{16}O/^{18}O$ exchange. Therefore, it is possible to perform relative quantitation after a prior qualitative identification step, where samples of interest can be identified [145,147–148].

5. Study of post-translational modifications

In addition to the amino acid-specific and the general tagging strategies presented in Sections 3 and 4, methods specifically tailored for the qualitative and sometimes quantitative determination of post-translational modifications (PTMs) have emerged in recent years [149–155]. More than hundred such modifications have been described and they differ substantially in the extent of the modification of proteins, ranging from relatively minor chemical changes like N-terminal acetylation to the attachment of extended carbohydrate chains in the case of glycosylation. For this reason, some PTMs can be targeted more specifically than others, and the tagging concepts currently described in the literature are most often directed towards phosphorylated proteins, although recently methods for the study of glycosylation and tyrosine nitration have appeared.

5.1. Phosphorylation

The (transient) phosphorylation and dephosphorylation of serine, threonine and tyrosine residues are very important regulatory processes involved in metabolic pathways, signal transduction, etc., and therefore there is considerable interest in specifically isolating phosphorylated proteins or peptides. Some currently used techniques for the analysis of phosphorylated proteins are presented in recent reviews [149–150,154–155].

Commonly used enrichment strategies make use of dedicated phosphospecific antibodies or immobilized metal affinity chromatography (IMAC). While antibodies against pTyr are more commonly used, this concept has been less successful for phosphorylation on Ser and Thr. The significant cost and limited availability of the antibodies still prevent a more widespread application. IMAC takes advantage of the affinity of phosphate groups to certain metal ions, e.g. Fe(III) or Ga(III) and has been successfully applied in a number of studies for enrichment of phosphopeptides (see, for example, [131,143,156–164]). However, some authors have noted that non-specific binding of Asp- and Glu-peptides occurs. Ficarro and co-workers [157,159,163,165] have used methyl esterification of carboxyl functionalities in peptides to avoid this unwanted chelation and successfully used the procedure in large-scale phosphoproteomics projects. A similar approach was recently published by He et al. [162]. Still, the significance of non-specific binding of other peptides is unclear since Nühse et al. [161] found in their study that the majority of IMAC-isolated peptides were in fact phosphorylated. In addition, Julka and Regnier [20] point out that aspartate esters could be instable under the acidic conditions during chromatographic separation, causing artifacts.

Both enrichment by antibodies and IMAC can be combined with global isotopic labeling schemes, such as those presented in Section 4, to allow both enrichment and relative quantitation. However, several techniques have now emerged that include the *chemical modification* of the phosphate group in combination with the attachment of an affinity tag and/or an isotope-coded moiety.

The first to report such approaches were Oda et al. [166] and Zhou et al. [167] in 2001, but since then a number of other methods using phosphoamino acid-specific chemical modifications have appeared in the literature [160,166–181]. A short overview of the various techniques is given in Table 1. It has to be noted that the applicability of several techniques has only been proven for phosphorylated standard proteins (e.g. caseins) and not for complex protein mixtures like cell lysates.

The approach of Zhou et al. [167] consists of a six-step reaction (illustrated in Fig. 17): The protein sample is first subjected to proteolysis since the affinity step takes place at the peptide level. Then, after an initial tBoc-protection of amino groups of phosphopeptides to prevent unwanted side-reactions, ethanolamine is attached to phosphate moieties (and, additionally, to carboxyl groups present in the peptides). Treatment with diluted trifluoroacetic acid (TFA) cleaves the ethanolamine from phosphate groups but not from the carboxyl groups, therefore allowing the phosphate-specific addition of cystamine. The reduction of the cystamine disulfide bond sets free a thiol group that is subsequently captured with glass beads containing immobilized iodoacetamide. Cleavage with concentrated TFA then releases the captured phosphopeptides

Table 1
Applications of chemical tagging strategies for the study of protein phosphorylation

Reference	Tagging principle
Strategies based on β-elimination/Michael addition	
Adamczyk et al. [168]	Differential mass tags for MS identification, biotin affinity tag for enrichment
Adamczyk et al. [169]	Isotope-coded tag for relative quantitation
Amoresano et al. [170]	Isotope-coded tag for relative quantitation, affinity tag for enrichment by disulfide exchange
Goshe et al. [171], Goshe et al. [172]	Isotope-coded tag for relative quantitation, biotin affinity tag for enrichment
Knight et al. [173]	Tags for selective cleavage at phosphorylated residues and solid-phase capture
Li et al. [174]	Conversion of pSer and pThr residues to sulfated analogues for stability during MS/MS
McLachlin and Chait [175]	Affinity tag for enrichment by disulfide exchange chromatography
Molloy and Andrews [176]	Differential mass tags for MS identification
Oda et al. [166]	Biotin affinity tag for enrichment
Qian et al. [177]	Tag for solid-phase affinity capture, isotope-coded tag for relative quantitation
Rusnak et al. [178]	Tags for selective cleavage at phosphorylated residues
Steen and Mann [179]	Marker tag for identification for MS detection by precursor ion scanning
Thompson et al. [160]	On-resin elimination in combination with IMAC
Weckwerth et al. [180]	Isotope-coded tag for relative quantitation
Other strategies	
Takeda et al. [181]	Complexation for MS identification
Zhou et al. [167]	Cystamine addition to phosphate groups for solid-phase capture

References are given in alphabetical order.

and simultaneously removes the N-terminal tBoc-protection group.

Despite the multi-step tagging and extraction procedure and the partially extremely harsh reaction conditions, the isolation and successful sequencing of a number of phosphopeptides from a *S. cerevisiae* lysate was reported following this protocol.

Other approaches, by contrast, typically involve the base-catalyzed β-elimination of the phosphate groups of Ser and Thr and a subsequent addition of various tags to the double bond that is created (see also Table 1). Oda et al. were the first to report such a procedure ([166], Fig. 18), consisting of a base-catalyzed dephosphorylation step, followed by a Michael-type addition of ethanedithiol (EDT). The remaining free thiol group then reacts with an activated biotin linker. This way, phosphopeptides can be enriched by biotin–avidin affinity chromatography in the same way as ICAT-peptides. For this step, the same advantages and

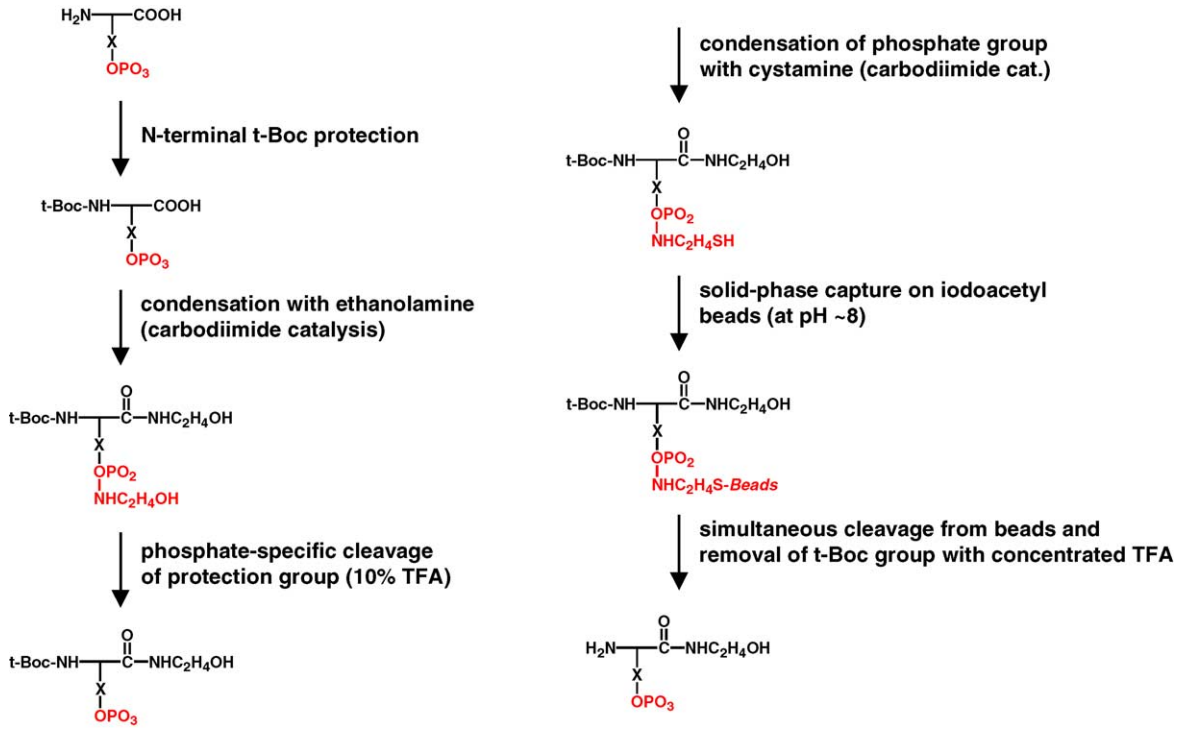

Fig. 17. Reaction scheme of the phosphopeptide tagging approach presented by Zhou et al. [167]. A phosphopeptide is shown in a simplified version highlighting its N- and C-terminus and the phosphate group of a phosphorylated residue within the peptide chain. For details, see text.

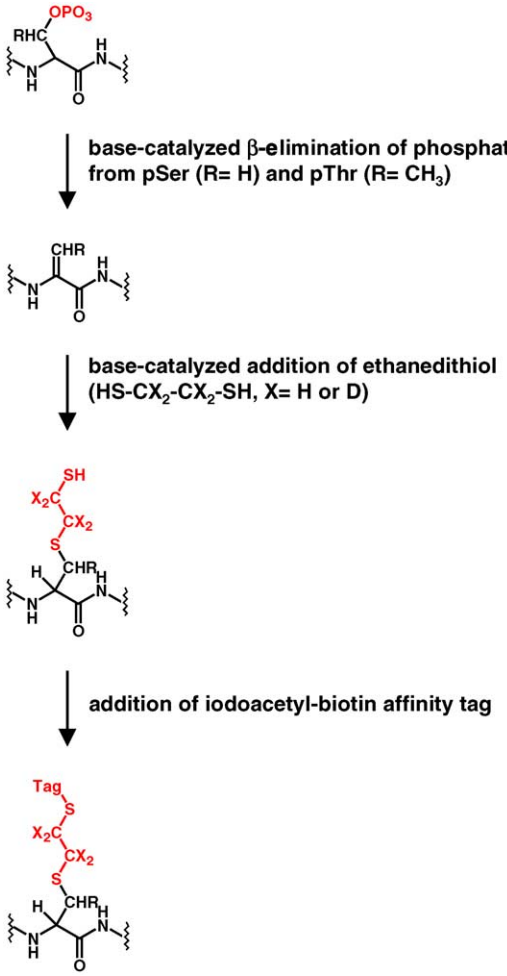

Fig. 18. Conecpt of phosphopeptide tagging using β-elimination of the phosphate group from pSer and pThr and attachment of various affinity tags.

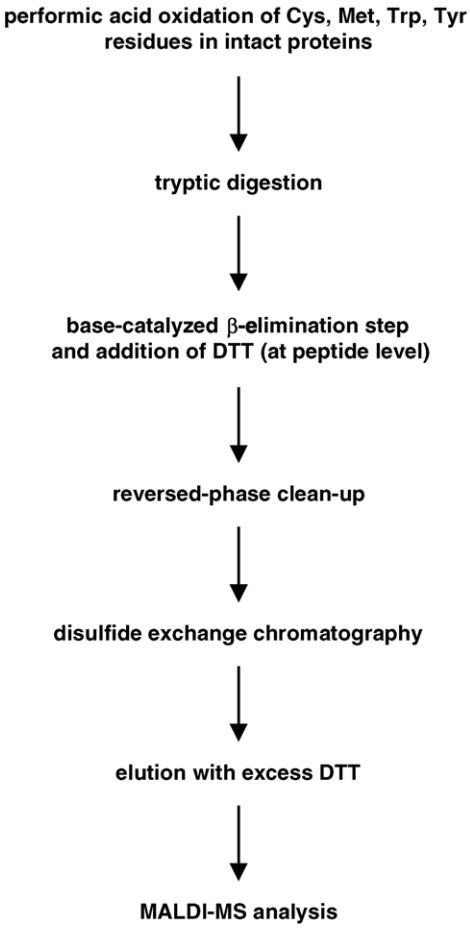

Fig. 19. Phosphopeptide tagging procedure of McLachlin and Chait [175], including a β-elimination step and disulfide exchange chromatography to isolate DTT-labeled peptides. For details, see text.

shortcomings apply as for all other biotin tagging schemes (see Section 3.2). The group of Smith described an almost identical method shortly thereafter, which they called PhIAT, for phosphoprotein isotope-coded affinity tags [171–172]. In addition to Oda's protocol, they also included a stable-isotope labeling step by using two isotopic versions of ethanedithiol, EDT-d_0 and EDT-d_4. Both groups initially demonstrated the isolation of phosphorylated peptides from the model protein β-casein and from complex mixtures.

Recently, Smith and co-workers [177] also reported the development of a solid-phase-based version of PhIAT termed PhIST (for phosphoprotein isotope-coded solid-phase tag). This tagging strategy combines the chemistries of the solid-phase ICAT (see above) and the original PhIAT methodology. β-Elimination of the phosphate group and addition of EDT are performed as previously described, although no stable-isotope-labeled EDT is used. Instead, the isotope tag is introduced during a solid-phase capture step using the same photocleavable linker design as the solid-phase ICAT from the Aebersold group [69]. The only difference are the isotope pairs introduced, because $^{12}C/^{13}C$ and $^{14}N/^{15}N$ are used instead of $^{1}H/^{2}H$, therefore avoiding chromatographic separation of light and heavy forms of the labeled peptides.

The authors claim that their improved approach overcomes the weaknesses of the other techniques presented here. In particular, non-specific binding to avidin columns is avoided because no biotin labeling is performed, the protocol involves less manipulation steps therefore avoiding significant sample loss as reported for Zhou et al.'s method [167] and overall specificity of the enrichment is improved because of the more stringent washing conditions possible on solid-phase.

Another interesting variation based on β-elimination was recently presented by McLachlin and Chait [175] (Fig. 19). Instead of attaching a biotin affinity tag after the addition of a thiol compound, they used covalent chromatography based on disulfide exchange to capture phosphorylated peptides on solid-phase. A sensitive protocol was developed consisting of the oxidation of proteins with performic acid vapor to oxidize cysteine to cysteic acid as a first step, so that immobilization via cysteines does no longer occur (Met and Trp residues are also oxidized at this stage). After that, oxidized proteins are digested and the elimination step is performed in the presence

of dithiothreitol (DTT). DTT is used as the dithiol compound to label the previously phosphorylated site. After a reversed-phase clean-up to remove the reagents, the dephosphorylated peptides are applied to a thiol affinity resin, where covalent linkage via the DTT moiety occurs. After a washing procedure to remove unbound peptides, the immobilized fraction is eluted using an excess of DTT and mass spectrometric characterization is then possible.

In the article, the authors discuss the problem that sensitivity is often limited when such complex schemes are applied (in this case, taking about two days to complete). It is usually necessary to use amounts above 1 μg of starting material to obtain satisfying results. Such an amount might not always be available, especially when taking into account that at any given time, only a small fraction of a protein will be present in its phosphorylated state.

Marino and co-workers [170] describe a similar strategy (cysteine oxidation, β-elimination, addition of DTT and enrichment by covalent chromatography), but introduce differential quantitation by using d_0- and d_6-DTT for isotope coding.

Although possible side-reactions have been mentioned in other articles as well, McLachlin and Chait are the first to give a detailed description about the most likely problem with the elimination step, namely the inadvertent tagging of unmodified serine residues. This, of course, might lead to false positives and can distort the results of a study. Although it was found that the extent of serine labeling is typically below 1% for any given peptide, this still can be significant considering that the relative abundances in a sample can span several orders of magnitude. *O*-Glycosylated serine and threonine residues are also known to be susceptible to β-elimination under the conditions used (see also below). Finally, methods based on phosphate β-elimination are only applicable for the study of phosphorylation on serine and threonine but not tyrosine.

Despite the above-mentioned problems, these novel chemical tagging strategies are emerging as alternatives to the immunoaffinity- or IMAC-based techniques and it can be expected that more such labeling approaches will be presented in the near future.

5.2. Glycosylation

In contrast to phosphorylation, glycosylation as a post-translational modification is much more complex to deal with in terms of the modification itself because of the enormous structural variation of the glycans. While the exact elucidation of the carbohydrate moieties is very demanding (and beyond the scope of this review), it might be sufficient at the initial stage of a "glycoproteomic" study to identify only the glycosylation *sites* in proteins. Two types of carbohydrate attachments are usually dominant, namely N-linked glycosylation on asparagine (in consensus sequences of the –Asn-Xxx-Ser/Thr-type, where Xxx is any amino acid residue) or O-linked glycosylation via serine or threonine. In the last years, several methods have been described that used different methodologies to identify which positions in proteins are involved in glycosylation. Fig. 20 illustrates the various methods.

A classical approach to enrich glycosylated peptides is by using lectin affinity chromatography [182]. Lectins are a group of plant proteins binding to glycan motifs in glycosylated peptides or proteins [183]. A number of different lectins are available that allow the specific selection of glycosylation patterns, but there are also less specific lectins that allow the isolation of a variety of glycoforms, like the widely used concanavalin A (Con A). In combination with global isotope labeling strategies, lectin affinity chromatography allows the enrichment of glycosylated proteins and simultaneously their relative quantitation. Examples are studies from Regnier's group [125–126,130,184–185] and the work of Kaji et al. [186]. Regnier and co-workers used different variations of their N-terminal isotope coding strategy (as outlined in Section 4.1) after specifically selecting glycosylated peptides with different glycan structures (e.g. Con A for broad specificity [125–126,184] or *Lotus tetragolonobu*s agglutinin for fucosylated peptides [185]).

Kaji et al. combined a lectin affinity isolation step with digestion in $H_2{}^{16}O/H_2{}^{18}O$ (see Section 4.2) for relative quantitation in an approach they called isotope-coded glycosylation-site-specific tagging (IGOT). Two batches of protein mixtures from *Caenorhabditis elegans* were prepared. In the first step, glycoproteins with high-mannose and hybrid-type *N*-glycans were isolated by Con A lectin affinity chromatography, and the enriched fraction was subjected to proteolytic digestion by trypsin. The resultant mixture of non-glycosylated and glycosylated peptides was then purified by a second affinity step, again using Con A. After this tandem enrichment step, N-linked glycans were removed by PNGase F (a glucosidase specifically cleaving N-linked glycans) either in $H_2{}^{16}O$ or in $H_2{}^{18}O$. This way, differential labeling was obtained on the former glycosylation position, similar to a method reported earlier by Küster and Mann [187]. After mixing the two isotope-labeled mixtures, peptides were analyzed by multidimensional HPLC and identified by MS; 400 glycosylation sites were identified in this study.

A similar protocol based on tandem lectin affinity chromatography both on the protein and peptide level has been recently described by Bunkenborg et al. [188], although no isotope coding step was involved in this case. Furthermore, Pandey and co-workers [189] recently used a combination of lectin affinity chromatography (for enrichment) and PNGase F digest in $H_2{}^{18}O$ (for labeling *N*-glycosylation sites) to analyze the proteome of human bile, identifying 87 proteins and 33 glycosylation sites.

Apart from their general isotope labeling concept (ICAT, [47]) and their phosphopeptide enrichment strategy [167], Aebersold and co-workers [190] described the isolation of glycoproteins using solid-phase capture: In their approach, *cis*-diol groups in carbohydrate residues are oxidized with periodate and the glycoproteins are coupled to hydrazine beads

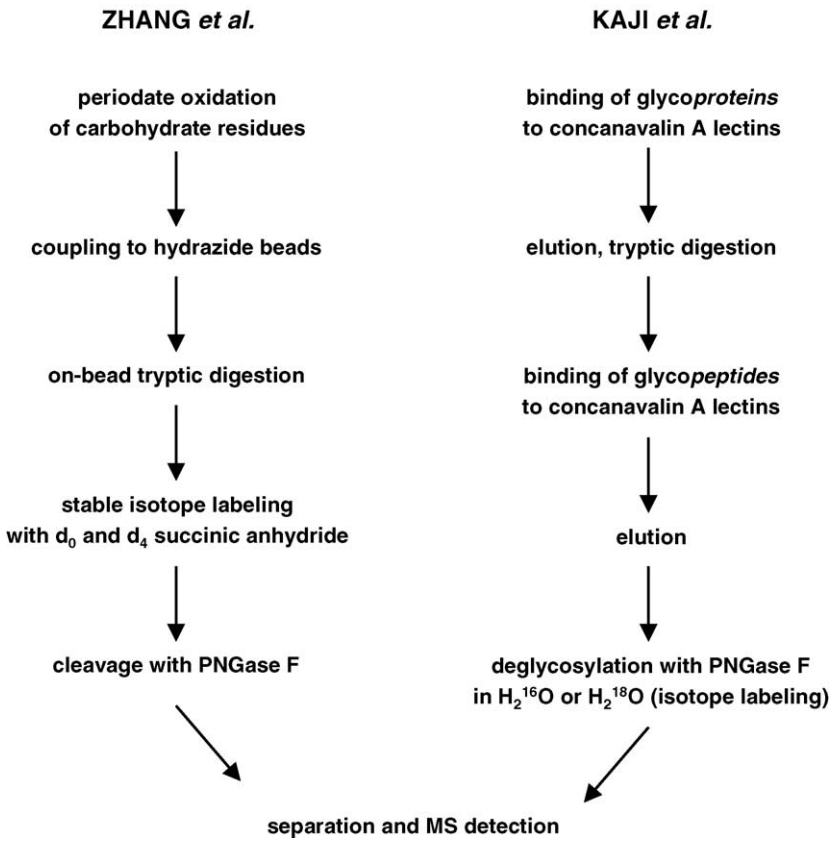

Fig. 20. Identification strategies for N-linked glycoproteins in complex mixtures using solid-phase capture and hydrazide chemistry [190] (left) or tandem lectin affinity chromatography [186] (right), both in combination with differential isotope coding for relative quantitation.

via the aldehyde groups that are formed (Fig. 21). An enzymatic digestion step is then performed so that only the peptides bearing the glycosylation remain while all others are washed away. While still being immobilized, glycopeptides are differentially labeled with d_0- or d_4-succinic anhydride, so that relative quantitation of two populations (captured on two separate batches of beads) is possible. After the isotope coding step, peptides are released in their deglycosylated form after treatment with PNGase F. Analysis was performed by on-line μLC–ESI-MS/MS or off-line μLC–MALDI-MS after combining the two batches. The method allowed the identification of 145 glycosylation sites in human serum proteins.

As already mentioned previously, O-linked glycosides can be removed under basic conditions similar to the dephosphorylation technique used for analysis of protein phosphorylation. Wells et al. used this reaction to identify *O*-GlcNAc-modified sites in various proteins [191]. In their method, O-linked *N*-acetylglucosamine moieties were cleaved off serine and threonine residues by base-catalyzed β-elimination, followed by the addition of DTT or biotin pentylamine. DTT-labeled peptides could be enriched by covalent chromatography on thiol-Sepharose, while the latter reagent allowed the isolation of deglycosylated peptides by biotin–avidin affinity chromatography prior to further mass spectrometric characterization by MALDI-MS or LC–ESI-MS/MS.

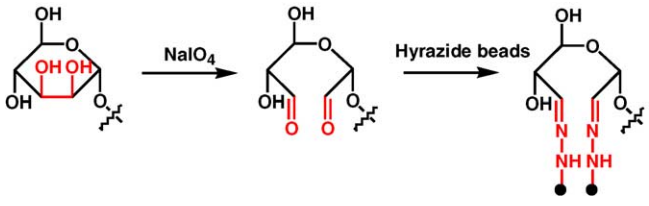

Fig. 21. Periodate oxidation of *cis*-diol moieties in carbohydrates and coupling to hydrazine beads [190].

As was shown in this section, in the case of glycosylation, most methods currently rely on established affinity enrichment using lectins, sometimes in combination with stable-isotope labeling. Nevertheless, chemical tagging concepts like the two examples given here demonstrate that they can be complementary tools.

5.3. Tyrosine nitration

Nitration on tyrosine, a less frequent, but also biologically relevant post-translational modification [192], has been probed by Nikov et al. [193] using another variation of the biotin affinity tag concept. In this approach (illustrated in Fig. 22), the nitro group of 3-nitrotyrosine is first reduced to the amine using sodium hydrosulfite ($Na_2S_2O_4$), followed by the aminotyrosine-specific attachment of a cleavable biotin tag. The lower p*K* value of aminotyrosine (relative to

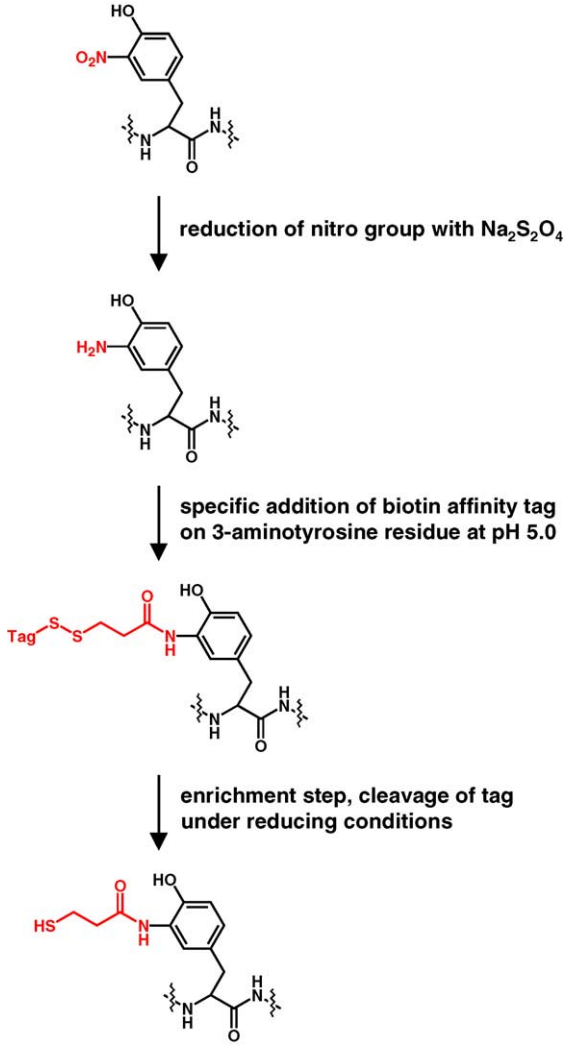

Fig. 22. Strategy for the affinity enrichment of nitrotyrosine-containing peptides [193]. For details, see text.

N-termini or the amino group of lysine) causes the succinimide moiety of the biotin tag to react with this amino group exclusively at a solution pH of 5.0. Following a biotin–avidin affinity chromatography step, the tag is cleaved by reducing the disulfide bond, thereby avoiding interferences during the mass spectrometric detection step. Using this procedure, NO_2Tyr-containing peptides could be successfully isolated from digests of nitrated human serum albumin, although it has not yet been shown how well this procedure can be applied to more complex samples.

6. Tandem MS tagging

As was already pointed out above, tagging strategies for MS-based proteomics are not restricted to affinity labeling for sample prefractionation and stable-isotope labeling for comparative quantitation. Sometimes, it is also desirable to label peptides to improve mass spectrometric sequencing.

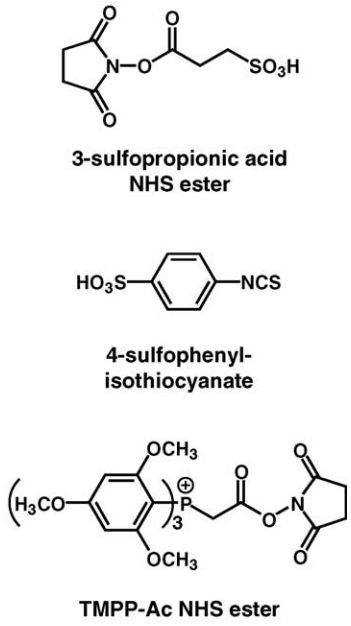

Fig. 23. N-terminal charge derivatization reagents for the modification of peptide fragmentation.

A number of strategies for charge derivatization have been presented in the literature and the developments until the end of the 1990s have been reviewed in detail by Roth et al. [194]. Since then, two techniques have been most prominent in the literature: N-terminal sulfonation and attachment of phosphonium groups to the N-terminus of peptides (see also Fig. 23).

6.1. Addition of a negative charge by sulfonation

In 1999, Keough and co-workers [195] first presented a strategy to introduce a permanently negatively charged sulfonic acid moiety on the N-terminal amino group. The initial method was based on derivatization with either 2-sulfobenzoic acid cyclic anhydride or chlorosulfonyl acetyl chloride. Later, 3-sulfopropionic acid *N*-hydroxysuccinimde ester was used for modification [196]. This reagent was found more suitable because of the possibility of performing the reaction in aqueous solution and even while the peptides are adsorbed to solid-phase extraction tips. A reaction kit based on the latter reagent is now commercially available from Amersham Biosciences under the name Ettan^{TM} CAF^{TM} MALDI sequencing kit [197], where CAF stands for "chemically assisted fragmentation". Various applications have demonstrated that this sulfonation procedure is routinely applicable to derivatize small amounts of peptides such as those recovered from two-dimensional gel electrophoresis [198–200].

The attachment of the negatively charged group effectively suppresses the N-terminal fragment ions, so that only y-ions are observed. Consequently, MALDI-PSD spectra are drastically simplified although the approach is not limited to this

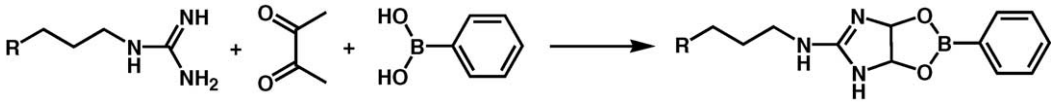

Fig. 24. Modification of the arginine side-chain by the butanedione–phenylboronic acid tag [215].

type of instrument, since the derivative is also reasonably stable during CID as shown in [201].

A different sulfonation protocol is based on the reaction of the N-terminal amino group with 4-sulfophenylisothiocyanate (SPITC). This reaction was first applied to peptide analysis by Gevaert et al. [202] although they reported only limited fragmentation efficiency in MALDI-PSD experiments compared to Keough's original approach. In addition, they stated that the large amounts of sample necessary to perform the reaction were limiting routine application. However, in 2003, Marekov and Steinert [203] presented an improved procedure allowing rapid (20 min) derivatization and PSD sequencing of peptides at a level of approximately 10 pmol. Very recently, two additional articles describing modifications of their procedure by other groups have been published [204–205]. Wang et al. [204] used pure aqueous derivatization conditions and reported a further increase in sensitivity, while Chen et al. [205] developed a protocol to tag peptides while adsorbed on reversed-phase solid-phase extraction microcolumns.

Regardless of the actual reagent or protocol used for sulfonation, it has been necessary to modify lysine residues – typically by guanidination – prior to performing the charge derivatization step. Attachment of a second negatively charged moiety in the peptide would drastically reduce ionization sensitivity, especially in the case of tryptic peptides where lysine is located on the C-terminal end. Lee and coworkers [206], however, noted that under their conditions, lysine residues were not modified by SPITC. In addition, they have used LC–ESI-MS/MS to study the sulfonated peptides. Doubly charged peptides (which are predominantly formed during ESI) mainly showed a loss of the N-terminal amino acid or the tag itself while other y-ions were strongly reduced in their relative abundance. As expected, no b-ions were observed.

6.2. Addition of a positive charge using phosphorous compounds

While sulfonation suppresses fragment ions originating from the N-terminus, the opposite can be achieved by attaching a permanent positive charge on the N-terminus, thereby causing the preferential formation of N-terminal fragments (a- and b-ions). Several strategies have been presented that typically involve derivatization with phosphines or phosphonium salts. Watson and co-workers [207,208] as well as Strahler et al. [209] have used an activated tris(trimethoxyphenyl)phosphonium acetate (TMPP-Ac, see Fig. 23) to attach the positive charge, but other approaches have also been reported [210].

Although applications for peptide sequencing have been shown [211–213], this strategy has not been used widely so far. However, Czeszak et al. [214] have shown that TMPP-modification can be used to analyze *O*-glycosylated peptides by MALDI-PSD. In contrast to fragmentation by collision-induced dissociation, the glycan moiety is not cleaved off under PSD conditions, therefore, this strategy allows the localization of *O*-glycosylation sites. The TMPP-Ac reagent is also commercially available (Fluka, Buchs, Switzerland).

6.3. Significance of charge derivatization reactions

Roth et al. [194] summarized in their review that to be useful, such charge derivatization reactions should be rapid, quantitative and without unwanted side-reactions. Even if these criteria are fulfilled, such methods are not as widespread, in part due to limited availability of reagents or a reduction in detection sensitivity. Still, the CAF reagent has shown that the strategy can be a useful tool for proteomic applications, when these drawbacks are absent [198–200].

When one considers that a large number of tandem MS spectra obtained from LC–MS runs does not yield interpretable information, the development of complementary tagging reagents could be valuable. For example, another, more challenging target for the manipulation of the fragmentation behaviour of peptides is the guanidino group in the side chain of arginine. Especially when multiple arginines are present in a peptide, the number of informative fragment ions can be drastically reduced so that it is not possible to extract enough sequence information for successful database searches.

Our group is dealing with derivatization reactions for the guanidino group to alleviate this problem, and initial results have shown that the fragmentation behaviour of arginine-containing peptides can indeed be altered to some degree. Attachment of the butanedione-phenylboronic acid tag developed in our laboratory (Fig. 24) [215], for example, was found to suppress cleavage C-terminal to acidic residues in arginine-containing peptides [216].

7. Conclusion and outlook

The numerous examples in the previous sections have shown the impact that chemical tagging strategies already have for proteome analysis by mass spectrometry. Various types of tags allow the reduction of sample complexity,

Table 2
Some decision-making points for the choice of chemical tagging strategies

Stage of the introduction of the tag
Isotopic labeling alone or in combination with affinity tagging
Labeling conditions
Influence on chromatographic separation and mass spectrometric detection
Commercial availability and cost of reagents
Software support for data analysis

enable relative quantitation of protein amounts between two samples and aid in de novo sequencing and interpretation of tandem MS spectra. New tagging concepts are emerging rapidly and already established ones are used for more varied applications. To name just a few, isotope-coded affinity tags have been used to study protein interaction networks on a quantitative level [217–218], to identify oxidation-sensitive cysteine residues [219] or to investigate the localization of organelle proteins [220].

However, there are still some general limitations of the various methods published until now. For one, many of the tagging reagents are not commercially available, which limits their widespread use. Amino acid- or PTM-specific tags by far do not cover all interesting targets. There is still a need for methods directed at post-translational modifications other than phosphorylation and glycosylation, and probably for more tags that attach to amino acids other than cysteine as well. The development of new tagging chemistries will therefore be of interest to researchers in proteomics for years to come.

Despite being beyond the scope of a review focusing on chemical tagging strategies, one should not forget that there are also alternative workflows to achieve the same goals like by using tagging schemes. Simplification of complex mixtures is possible by multidimensional fractionation protocols based on chromatography (e.g. by cation exchange plus reversed-phase LC) or gel-based procedures or combinations thereof. High-end mass spectrometers, especially FTICR-MS instruments, can deal with more complex mixtures due to extremely high resolution, dynamic range and sensitivity. We feel that today there is no single strategy that by itself is perfectly suitable for all “proteomic” problems and that approaches based on chemical tagging and those based on sophisticated instrumentation complement each other.

For those researchers who have to choose the most appropriate method to solve their specific problem, the variety of available techniques makes it difficult to find the right one. Many points have to be considered in the process and we have listed some of them in Table 2:

Stage of introduction: This is particularly important for relative quantitation purposes. An early introduction of the isotope tag during the workflow minimizes errors during further preparation steps and it is often argued that in this aspect metabolic labeling is superior to chemical approaches since the incorporation of the tag takes places at the earliest possible time. Still, from the results reported in the literature it can be inferred that instrumental variation during mass spectrometry seems to be another important factor influencing the accuracy of the results. In addition, metabolic labeling is still not possible for all types of samples despite the fact that is has been successfully demonstrated even for mammals now [221].

Use of affinity tags: Whether a specific method for the reduction of sample complexity is necessary or a more general fractionation will suffice very much depends on the question that has to be answered. The use of amino-acid specific affinity tags will inevitably lead to a loss of peptides not containing these particular residues, which is on the one hand desired, but prohibits the detection of most post-translational modifications. Affinity tags are therefore more suitable for general protein profiling, or PTM-specific tags have to be chosen. Non-specific and/or irreversible binding for some techniques has to be also considered.

Labeling conditions: Some methods described here require lengthy sample preparation, sometimes even taking several days. Whether this is acceptable can only be judged on a case-by-case basis. Typically, there is only little information in the original papers on important aspects like specificity or completeness of the tagging reaction, either because it has only be used on samples of limited complexity so that potential limitations were not detected or because such problems were simply not considered during data analysis. A possible instability of various post-translational modifications has also to be taken into account.

Influence on chromatography and mass spectrometry: For stable-isotope labeling, chromatographic separation of light and heavy forms of peptides labeled with hydrogen- and deuterium-containing tags is possible, so this has to be considered during data analysis. Alternatively, labeling with ^{13}C and/or ^{15}N can be performed although usually the reagents are more expensive and the synthesis is more challenging. The attachment of large labels can significantly alter the chromatographic behavior, especially when polar groups are replaced by more hydrophobic ones or vice versa.

As already mentioned above, some tags generate abundant fragment ions in MS/MS experiments thus possibly complicating data analysis. Replacement of polar groups with less polar ones also tends to reduce detection sensitivity, particularly in ESI-MS. Finally, the mass shift for relative quantitation experiments has to be sufficiently high to avoid an overlap of the isotope patterns of light and heavy forms, which is of increasing importance for higher mass peptides where the contribution of naturally abundant ^{13}C is more significant, leading to broader isotope distributions.

Commercial availability and cost: Commercial availability certainly plays a role when laboratories do not have the capacity to perform syntheses themselves, which is less likely for biology-oriented facilities. On the other hand, the cost of commercial kits is sometimes also prohibitive, especially when a large number of complex samples (meaning large amounts of proteins) is being processed.

Data analysis: Data analysis is currently very much the bottleneck for laboratories capable of running high-throughput MS analyses. Commonly used database search programs can usually be adapted to consider artificial modifications, although normally this does not take potentially altered fragmentation into account. Manufacturers of MS instrumentation increasingly provide specialized software for proteomics applications including, for example, capabilities for automated analysis of relative quantitation experiments.

Finally, current tagging strategies almost exclusively rely on chemical reactions prior to any separation or detection steps. Recently, totally new concepts have emerged that might offer complementary approaches in the future. For example, the electrochemical tagging of cysteine residues in proteins directly on an ESI chip has been demonstrated by Girault and co-workers [222–223]. In their approach, free Cys-thiol groups are reacted with *p*-benzoquinone which is formed in situ on the chip via the electrooxidation of *p*-hydroquinone that is added to the sample solution.

Another very interesting approach is gas-phase tagging to identify phosphorylation sites in peptides, as shown recently by Gronert et al. [224]. In this case, trimethyl borate was added to the helium buffer gas in an ion trap instrument, resulting in the gas-phase reaction with phosphate groups.

While these techniques are not yet suitable for routine analysis of complex mixtures, they may give a hint at how creative chemistry can be further introduced and developed to broaden the array of analytical methodologies to reach the goal of unambiguously identifying and quantifying target compounds out of a complex matrix.

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