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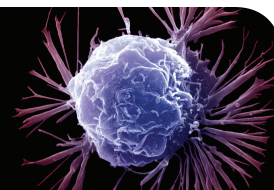

Early Stable Isotope Labeling in Proteomics

Proteomics, the analysis of the proteins expressed by a cell, tissue or organism under a specific set of conditions, continues to see tremendous growth in sample preparation and instrumental technologies. Proteomic studies are typically designed to analyze thousands of proteins in a single analysis and provide a global, dynamic view of changes in protein expression. While proteomics is formally defined as the complete characterization of the protein complement of a cell, including post-translational modifications, much of the effort has been focused on methods to measure changes in relative protein abundances between distinct cell systems.

While changes in protein expression have typically been studied by separating samples of interest using two-dimensional polyacrylamide gels (2D-PAGE) followed by comparing the intensity of the stained spots between gels, this method has many deficiencies related to reproducibility, proteome coverage and quantitation. Fortunately, there have been several recent developments in the use of stable isotope-labeling strategies that allow the comparison of isotopically distinct proteome samples.

While mass spectrometry has not been historically used for measuring relative protein abundances, stable isotope-labeling methods now make this scenario feasible at both the intact protein and peptide level.[1,2] One of the earliest demonstrations of isotopic-labeling strategies for whole proteomes was studying cadmium (Cd^{2+}) stress response in *Escherichia coli. E. coli* was grown in both normal (i.e. natural isotopic abundance) and rare isotope (^{13}C, ^{15}N) depleted media.[1] Relative protein abundances were measured by removing equal aliquots of cells from the unstressed (normal medium) and stressed (depleted medium) cultures at different time intervals after Cd^{2+}-addition. The aliquots were combined and the extracted proteins were analyzed using capillary isoelectric focusing coupled on-line with Fourier transform ion cyclotron resonance (FTICR) MS. Cells have also been cultured in ^{15}N-enriched medium and combined with cells cultured in normal medium and differences in peptide abundances measured by proteolytic digesting the intact proteins.[2] In both of these metabolic-labeling methods, isotopically distinct versions of each protein (or peptide) are observed and their relative abundances are quantified by comparing observed peak intensities of each species in the mass spectra, as shown in Figure 1.

While the metabolic-labeling method described above is limited to cells that can be cultured in specifically formulated media, chemical-labeling methods have been developed that are applicable to proteome samples isolated from any conceivable source. One of the earliest developments in the use of stable isotope labeling to quantify changes in the expression of proteins in proteome studies was the isotope-coded affinity tags (ICAT) method.[3] In ICAT labeling, shown in Figure 2, proteins are modified with a reactive group that covalently modifies Cys residues. The ICAT reagent also contains a biotin tag, allowing the modified Cys-containing peptides to be isolated using immobilized avidin. Changes in the relative abundance of peptides from distinct proteome samples is accomplished by the use of isotopically distinct versions of the ICAT reagent – a light isotopic version and a heavy isotopic version in which eight protons in the linker region between the thiol reactive group and the biotin moiety of the ICAT reagent have been substituted with eight deuterons. ICAT labeling results in both stable isotope-labeled Cys-polypeptides, which can aid identification by providing an additional Cys sequence constraint, and provides a significant reduction in complexity of the mixture being analyzed.

To demonstrate the ICAT strategy, a protein extract from cultured mouse B16 melanoma cells was divided into two equal aliquots. One aliquot was derivatized with the light isotopic version of the ICAT-D0 reagent and the other using the ICAT-D8 reagent. The proteomes were pooled, digested with trypsin and labeled Cys-polypeptides isolated. The peptide mixture was analyzed in a single capillary LC/MS experiment. In this analysis, hundreds of pairs of Cys-polypeptides with the expected integral mass difference of 8.0 Da were observed. A few of these peptides are shown in Figure 3. The average ratio of peak areas for the distinct isotopically labeled versions of each peptide was ~ 1.01. Since identical aliquots of the proteome sample were used in this experiment, average ratio of peak areas for the distinct isotopically labeled versions of each peptide was ~ 1.01, consistent with the expected results.

Since these early developments, there have been a number of improvements in isotope-labeling strategies. Metabolic labeling has advanced to the stage at which isotopically labeled mice can be produced through feeding a specialized diet containing stable isotopes of specific essential amino acids. Chemical labeling methods, such as iTRAQ, enable up to eight samples to be compared in a single LC/MS analysis. If methods such as iTRAQ were combined with metabolic labeling, it may be possible to increase this number to 16 or 24 concurrent comparisons.

While stable isotope-labeling methods have been used primarily to measure relative abundance changes of proteins, other strategies have been developed to quantify changes in the phosphorylation state of proteins. The "phosphoprotein isotope-coded affinity tag"

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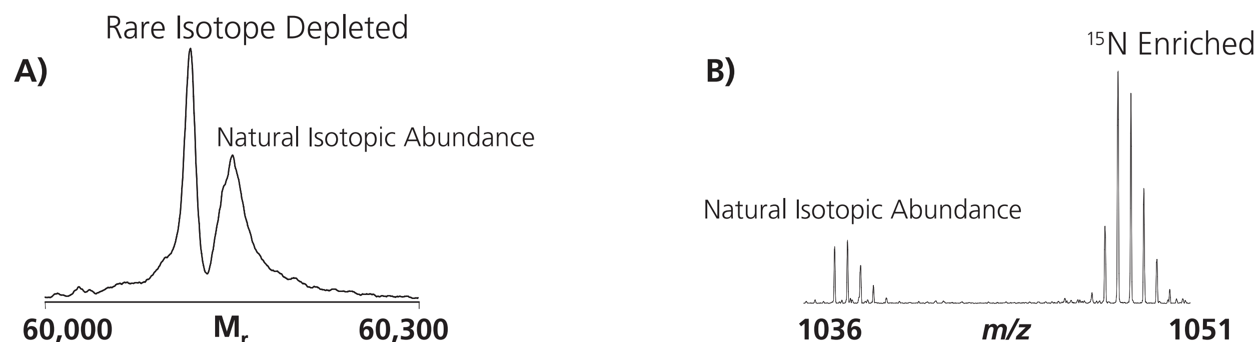

Figure 1. Examples of stable-isotope labeling of an (A) intact protein and (B) peptide observed in the MS analysis of an *E. coli* and *Deinococcus radiodurans* proteome samples, respectively. The two isotopic versions of each were obtained by culturing the cells separately in normal and either isotopically depleted (A) or ^{15}N-enriched (B) media. Combining the two separate cultures provides two isotopic versions for every species present in the samples.

(PhIAT) approach differentially labels phosphoseryl (pSer) and phosphothreonyl (pThr) residues with a stable isotopic and biotinylated tag, as shown in Figure 4.[5] This strategy enriches the phosphoprotein pool and enables a quantitative measurement of phosphorylation between the two distinct protein samples by comparison of the extent of isotopic enrichment. After chemically blocking cysteinyl sulfhydryls, phosphoproteins are selectively modified by removing the phosphate group from pSer and pThr residues via hydroxide ion mediated b-elimination. Michael addition to the newly formed α,β-unsaturated residues is performed using 1,2-ethanedithiol (EDT) containing either four alkyl hydrogens (EDT-D) or deuteriums (EDT-D) to achieve stable isotopic labeling. The sulfhydryl groups present of the labeled proteins are biotinylated using iodoacetyl-PEO-biotin to generate PhIAT-labeled proteins. The PhIAT-labeled proteins are digested with trypsin and isolated using immobilized avidin prior to LC/MS analysis. The resultant spectra show two isotopically distinct versions of the same phosphopeptide allowing changes in the peptide's phosphorylation state to be quantified. Successful PhIAT labeling of a control phosphoprotein, as well as proteins from a yeast extract, was demonstrated.

The above presents only a glimpse into the several different types of stable isotope-labeling techniques that are being utilized in proteomics. As this field continues to develop, a variety of stable isotope-labeling methods are being produced. The use of stable isotope-labeling methods to identify and quantify post-translational modifications will become an area of particular importance and growth.

Timothy D. Veenstra, PhD
Laboratory of Proteomics and Analytical Technologies
Frederick National Laboratory for Cancer Research
Frederick, MD

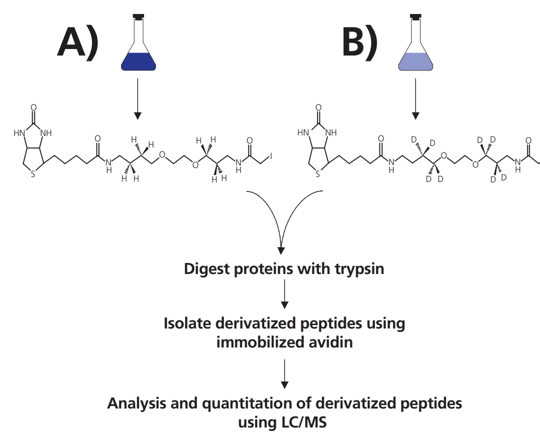

Figure 2. Schematic representation of the isotope-coded affinity tag (ICAT) strategy. Proteins are separately extracted from cells grown under two different conditions (A and B). The proteins for each sample are labeled either with the light (ICAT-D0) or heavy (ICAT-D8) ICAT reagent. After labeling the proteins are pooled and digested with trypsin. The modified peptides are isolated by affinity chromatography and analyzed by capillary LC/MS.

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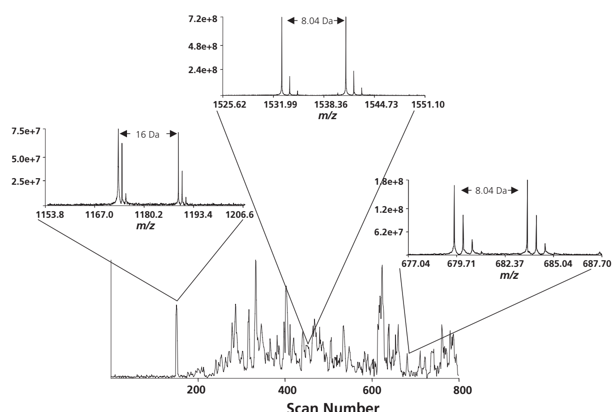

Figure 3. Examples of ICAT-labeled peptides observed in the analysis of mouse B16 melanoma cells. In this analysis, a single proteome sample extracted from the cells was split into two equal aliquots that were then labeled with either ICAT-D0 or ICAT-D8.

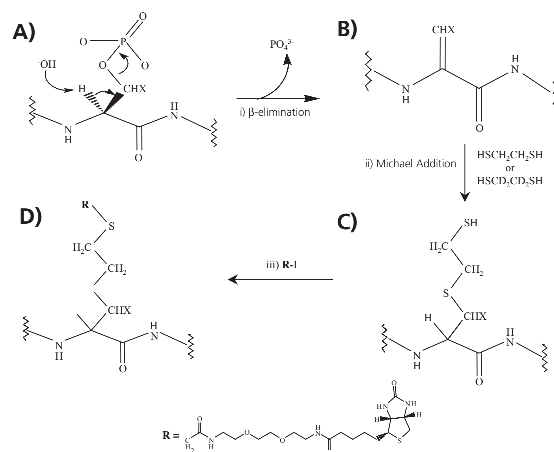

Figure 4. Phosphoprotein isotope-coded affinity tag (PhIAT) labeling method. Proteins containing phosphoseryl (X = H) or phosphothreonyl (X = CH_3) residues are isotopically labeled and biotinylated. After proteolytic digestion, these biotinylated peptides are isolated from non-phosphorylated peptides via avidin affinity chromatography. The ability to quantitate the extent of phosphorylation between two identical peptides extracted from different sources is based on the use of a light ($HSCH_2CH_2SH$, EDT-D_0) and heavy ($HSCD_2CD_2SH$, EDT-D_4) isotopic versions of 1,2-ethanedithiol.

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