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Assessment of stable isotope incorporation into recombinant proteins

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ABSTRACT

Stable isotope labeling combined with mass spectrometry has been widely used in a diverse set of applications in the biochemistry and biomedical fields. When stable isotope-labeled proteins are produced via metabolic labeling of cell culture, a comprehensive assessment of the labeling pattern is imperative. In this study, we present a set of mass spectrometry-based bioanalytical tools developed for quantitatively tracing the levels of the stable isotopes incorporated into the recombinant proteins (monoclonal antibodies and Fc fusion proteins expressed in different host systems) that include total mass analysis, peptide mapping analysis, and amino acid analysis. We show that these three mass spectrometry-based analytical methods have distinctive advantages and limitations and that they are mutually complementary in evaluating the quality of stable isotope-labeled proteins. In addition, we show that the analytical techniques developed here are powerful tools to provide valuable insights into studying cell metabolism and performing flux analysis during cell culture.

With the advances in analytical instrumentation and commercially available labeled compounds, stable isotope labeling has been widely used in a variety of biochemistry and biomedical fields [1,2]. Consequently, over the past few decades, a steadily increasing number of quantitative methods employing stable isotope labeling and mass spectrometry (MS)[1] have been reported, including prominent examples of applications in quantitative pharmacokinetic studies [3] and metabolic tracer experiments of drugs or dietary compounds and their respective metabolites [4,5] as well as applications in quantitative and comparative proteomics to determine differential expression of proteins or protein posttranslational modifications (PTMs) [6–19].

Stable isotopes can be incorporated into the protein/peptide of interest via two distinct techniques: in vitro and in vivo labeling. The in vitro labeling usually relies on chemical or enzymatic incorporation of isotopes into the proteins and peptides. The chemical methods frequently target one of the active groups on a peptide with a stable isotope-labeled reagent. In principle, every reactive amino acid side chain can be used for incorporation of an isotope-labeled mass tag by chemical means [20]. In practice, however, Cys and Lys are primarily used as targets for this purpose. These labeling techniques have been commonly known by the acronym isotope-coded affinity tag (ICAT) [21,22] or isobaric tags for relative and absolute quantitation (iTRAQ) [23–25]. Enzymatic labeling is usually achieved through enzymatic protein digestion in the presence of ^{18}O-labeled water, where the ^{18}O label is introduced at the C terminus of each peptide [26,27]. One of the first uses of isotopic labels in proteomics was for improved peptide sequence assignment by using endoproteinase trypsin- or Glu-C-catalyzed incorporation of ^{18}O during protein digestion [28]. The proteolytic ^{18}O labeling strategy has recently been adopted for quantitative proteomic studies as well [26,29]. Another established in vitro labeling approach for introducing stable isotope into a protein is a cell-free protein production system using primarily bacterial lysates [30]. The coupled transcription/translation activity enables the production of desired protein products from DNA templates, thereby allowing stable isotope-labeled amino acids to be

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[1] *Abbreviations used:* MS, mass spectrometry; PTM, posttranslational modification; SILAC, stable isotope labeling by amino acids in cell culture; NMR, nuclear magnetic resonance, SILIS, stable isotope-labeled internal standard; mAb, monoclonal antibody; TFA, trifluoroacetic acid; ACN, acetonitrile; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; IAA, iodoacetic acid sodium salt; NaCl, sodium chloride; Mes, 2-(*N*-morpholino)ethanesulfonic; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; SDS, sodium dodecyl sulfate; MW, molecular weight; rCE, reduced capillary electrophoresis; β-ME, β-mercaptoethanol; HPLC, high-performance liquid chromatography; SE, size exclusion; CEX, cation exchange; UV, ultraviolet; TOF, time-of-flight; ESI, electrospray ionization; LC, liquid chromatography; MS/MS, tandem MS.

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incorporated into the product protein when the cell-free reactions are carried out in lysate containing labeled amino acids. This approach has the advantage that only the protein of interest is synthesized and labeled [31,32].

The in vivo labeling is accomplished metabolically by growing cells in the presence of stable isotope-labeled nutrient sources. This approach has gained wide popularity in the form of the stable isotope labeling by amino acids in cell culture (SILAC) approach introduced by Mann and coworkers [20,33]. Since then, SILAC has been widely adopted in the quantitative proteomic field for quantitation of protein and protein PTMs. In contrast to most of the in vitro labeling approaches, the in vivo metabolic labeling usually introduces the label early in the process (during cell culture), thereby minimizing the experimental variation, which is particularly important for the quantification of differences between two or more physiological states of a biological system. In general, the introduction of stable isotopes does not affect the physicochemical properties of a protein [33]. Therefore, no distinction is made between "light" and "heavy" versions of the same protein during the protein synthesis, protein extraction, separation, proteolytic digestion, and peptide fragmentation. It is only when performing detailed data analysis using MS that a distinction can be made between the heavy and light versions of the same peptide.

For MS-based quantitative analysis, the metabolic labeling commonly relies on incorporation of a given light or heavy form of particular amino acids into the proteins [33–37]. Incorporation of stable isotopes at every amino acid has been widely used in nuclear magnetic resonance (NMR) studies, in which all ^{14}N atoms are replaced by ^{15}N, to determine phase shifts [32,38]. Oda and coworkers [39] used the whole-cell ^{15}N labeling (via ^{15}N-labeled cell growth medium) for the purpose of quantifying differences in protein expression and modification using yeast as the host cell. The level of incorporated ^{15}N label was estimated to be 93% for one peptide of interest when comparing the experimental isotopic distribution with the theoretical distribution of the ^{15}N-labeled peptide [39]. Enhanced and uniform stable isotope labeling in mammalian cells was also recently reported, where cell culture media based on stable isotope-labeled autolysates and lipid extracts of various microbiological origin were used [40]. The stable isotope enrichment was, in that case, estimated to be at levels greater than 90%. However, detailed biochemical characterization of labeled material has not been reported so far. There remains a pressing need to develop analytical tools for comprehensive assessment of stable isotope-labeled proteins. In recent articles [17,18], we have described a strategy for accurate quantification of PTMs and structural variants in recombinant proteins by introducing a stable isotope-labeled internal standard (SILIS). The SILIS for any given protein requires uniform incorporation of the stable isotopes into each amino acid along the protein sequence [17,18].

Here we describe a set of analytical tools developed and applied for accurately evaluating the levels and distribution of stable isotopes incorporated into recombinant proteins. Several labeling approaches were studied, including the use of ^{13}C and ^{15}N. The ^{13}C labeling was applied to a monoclonal antibody (mAb) expressed in mammalian cells via feeding ^{13}C-labeled glucose. The mammalian expressed mAb was also labeled with ^{15}N evaluating two different approaches: (i) using ^{15}N-labeled Gln at both amide and amine nitrogen positions and (ii) using solely ^{15}N-labeled amino acids with ^{15}N at all nitrogen positions. In addition to the labeled mAb, an Fc fusion protein expressed in microbial cells was labeled using ^{15}N-labeled Celtone (^{15}N–Celtone) medium. Different levels of stable isotope incorporation have been observed with different labeling approaches. We have demonstrated that the developed tools may provide valuable insights into examining stable isotope incorporation, studying amino acid metabolism, and performing energy flux analysis during cell culture, especially in cases where limited incorporation of stable isotopes was obtained.

Materials and methods

Materials

Both the mAb (IgG_2 subtype) and the Fc fusion protein were produced and purified at Amgen (Thousand Oaks, CA, USA).

All labeled raw materials, including ^{13}C-labeled glucose ($^{13}C_6$–glucose, 98% $^{13}C_6$), ^{15}N-labeled amino acids (at all N positions), and ^{15}N-labeled Celtone medium, were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Endoproteinase trypsin and Glu-C were obtained from Roche Diagnostics (Penzberg, Germany). Trifluoroacetic acid (TFA) and acetonitrile (ACN) were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Guanidine hydrochloride (GuHCl) was obtained from ICN Biomedicals (Aurora, OH, USA). Dithiothreitol (DTT), iodoacetic acid sodium salt (IAA), sodium phosphate monobasic and dibasic solutions, and sodium chloride (NaCl) solutions were obtained from Sigma–Aldrich (St. Louis, MO, USA). 2-(*N*-Morpholino)ethanesulfonic (Mes) acid and base were purchased from Calbiochem (Darmstadt, Germany). NAP-5 columns were obtained from GE Healthcare (Pittsburgh, PA, USA). The AccQ Tag Ultra Derivatization Kit used for amino acid analysis was purchased from Waters (Milford, MA, USA). This methodology allows derivatization of the amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). Hydrochloric acid (HCl, 6 N) solution used in amino acid analysis was obtained from Thermo Scientific (San Jose, CA, USA). The sodium dodecyl sulfate (SDS)–molecular weight (MW) analysis kit used for reduced capillary electrophoresis (rCE)–SDS analysis, containing bare fused silica capillary (57 cm length, 50 μm i.d.), SDS–MW gel buffer, SDS sample buffer, β-mercaptoethanol (β-ME), 0.1 N HCl, and 0.1 N sodium hydroxide (NaOH) were purchased from Beckman Coulter (Brea, CA, USA).

Stable isotope labeling and purification of mAb

Chinese hamster ovary (CHO) cell lines were the host used to produce the mAb. Batch production cultures were performed. Cultures were inoculated in a chemically defined batch medium. The cells were fed with chemically defined growth medium and glucose and were harvested on either day 7 or day 11. Harvested conditioned medium suspensions were centrifuged, and the supernatants were filtered through a 0.2-μm Pall Supor membrane before further purification.

For those experiments using stable isotope labeling, the same protocol as for nonlabeled control cell growth was followed. Cells were labeled by three different approaches: one for ^{13}C-labeled protein and two for ^{15}N-labeled protein. To produce ^{13}C-labeled mAb, the cell culture contained labeled ^{13}C–glucose (at all C positions). The batch medium consisted of 5.5 g/L ^{13}C–glucose and 0.5 g/L ^{12}C–glucose. Because the daily feeds contained exclusively ^{13}C–glucose, the ^{12}C–glucose was depleted during the course of the cell culture process. To produce the ^{15}N-labeled mAb, one approach was to replace Gln in the growth and feed media with ^{15}N-labeled Gln (^{15}N on both amine and amide groups of Gln), whereas the other approach was to use both growth and feed media containing exclusively ^{15}N-labeled amino acids, all bearing ^{15}N at every nitrogen position.

The filtered conditioned medium was purified by a protein A column (HiTrap rProtein A Fast Flow 5-ml column, 1.6 × 2.5 cm, GE Healthcare) using an Akta high-performance liquid chromatography (HPLC) system. The equilibration buffer was 25 mM Tris and 100 mM NaCl (pH 7.4), and the elution buffer was 100 mM acetic