15N Metabolic Labeling of Mammalian Tissue with Slow Protein Turnover

Daniel B. McClatchy,† Meng-Qiu Dong,‡ Christine C. Wu,†‡ John D. Venable,§ and John R. Yates, III*†

Department of Cell Biology, 10550 North Torrey Pines Road, SR11, The Scripps Research Institute, LaJolla, California 92037

Received November 14, 2006

Abstract: We previously reported the metabolic 15N labeling of a rat where enrichment ranged from 94% to 74%. We report here an improved labeling strategy which generates 94% 15N enrichment throughout all tissues of the rat. A high 15N enrichment of the internal standard is necessary for accurate quantitation, and thus, this approach will allow quantitative mass spectrometry analysis of animal models of disease targeting any tissue.

Keywords: Brain • Proteomics • Quantification • Labeling • SILAM

Introduction

The measurement of ion signals by mass spectrometers is precise and quantitative. Sampling handling and ionization are subject to variations that need to be controlled and measured, and thus the use of internal standards to determine variations and errors is common. In addition, the measurement of relative changes in quantity requires an internal standard for every molecule to be compared. A general approach in quantitative mass spectrometry is to mix a protein sample containing only natural-abundance isotopes with an identical protein sample except the proteins are labeled with heavy stable isotopes (i.e., 2H, 13C, 15N, or 18O).1,2 The relative protein expression is calculated from the ion chromatograms of the labeled and unlabeled peptides.3-4

Several strategies have been used to introduce labels into proteins including covalent attachment of stable isotope labeled reporter groups,5-18 proteolysis in the presence of 18O water,11-13 and metabolic incorporation of stable isotope labeled amino acids.1,2,7-9,14-17 Compared to in vitro labeling methods, metabolic labeling ensures that every protein is enriched with a heavy stable isotope. Metabolic labeling is routinely performed with cultured cells ranging from bacteria, yeast to mammalian cells, and has been demonstrated in multicellular organisms such as Caenorhabditis elegans and Drosophila melanogaster.18 Ishihama et al. took a creative approach by using a metabolically labeled 15N Neuro2A mouse cell line as an internal standard for mouse brain.19 One drawback to this strategy is that the Neuro2A and brain proteome do not completely overlap. For example, neurons in vivo form synapses, while Neuro2A cultured cells do not. To pursue global quantification of complex mammalian tissues with mass spectrometry, we developed a technique to metabolically label a Sprague-Dawley rat by feeding it a 15N enriched protein diet for 44 days starting when the rat was 3 weeks old.20 We demonstrated that the labeled rat was healthy and phenotypically identical to an unlabeled rat. Using the proteins from the 15N labeled rat liver as internal standards, we quantified global changes in the liver induced by a sublethal dose of cyclohexamide.20

The introduction of stable isotope labeling of mammalians (SILAM) that serve as models for human physiology and disease creates an opportunity to study a wide range of diseases that affect tissues and organs. We observed, however, a striking difference in the levels of 15N enrichment in the various organs of the rat. While the average 15N enrichment of amino acids in liver proteins was 91%, the average 15N enrichment in brain was 74%.20 Although the nitrogen source is the same for all tissues, the amino acid precursor pools are not. Tissues with slower protein turnover rates will take longer for the 15N labeled amino acids to equilibrate with the normal amino acid precursor pool, which leads to a lower enrichment. Consistent with our measured levels of 15N enrichment, liver has a very high protein turnover rate, while brain has a very low turnover rate.21,22 The disadvantage of low 15N enrichment is that quantitation becomes less efficient and accurate.

In this report, we describe a method to more efficiently feed animals a 15N labeled protein diet and an improved metabolic labeling strategy to obtain high 15N protein enrichment of rat brain tissue. We designed and tested two different labeling protocols to determine the optimal procedure to ensure high 15N enrichment of brain. Furthermore, we show that 15N labeling of two generations of rats does not interfere with fetal development during gestation. In addition, we report that this protocol is also applicable to other slow protein turnover tissues besides the brain. This method allows quantitative mass spectrometry analysis of animal models of neurological disorders or other types of diseases.

Materials and Methods

Materials. 15N-Enriched (>99 atom % excess; ape) and unlabeled algal cells were purchased from Spectra Gases (Vista, CA). Digestion enzymes were purchased from Roche Applied
Animal Care.

by the American Association for Accreditation of Laboratory
Institutional Animal Research Committee and were accredited
purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise
purchased from Harlan (Indianapolis, IN). All chemicals were
purchased from Invitrosol (Invitrogen, Carlsbad, CA) contain-
ing 8 M urea and 1% sodium dodecyl sulfate (SDS). Tissue was
maintained in temperature-controlled (23 °C) facility with a
positive rate of 5% or less. The false
quadratic discriminant analysis to dynamically set XCorr and
DeltaCN thresholds for the entire data set to achieve a user-
specified false positive rate (5% in this analysis). The false
positive rates are estimated by the program from the number
and quality of spectral matches to the decoy database.

The percent atomic enrichment was estimated using a
previously published procedure. Briefly, the high-resolution
zoom scans were employed to determine the 15N peptide
isotopic distributions, which are characteristic of the 15N atomic
percent enrichment of the peptide. The 15N percent enrichment
for a tissue was calculated from one MudPIT analysis of 100
µg of tissue. The spectra were searched for 15N peptides, and an
algorithm was used to find the corresponding 15N peptide in the
MS1 zoom scan. The algorithm predicts isotope distributions
over a range of enrichments and compares the predicted
distributions to experimental peptide isotope distributions. In
addition, the algorithm generates a correlation factor between
the matching 15N and 13N peptide with zero being no correla-
tion and 1.0 being a perfect correlation. We used only 15N
peptides with a correlation factor greater than 0.7 to obtain
the average 15N enrichment for a given tissue. In addition, we
reduce the proteins. This mixture was then vortexed for 1 h at
room temperature. Next, iodoacetamide was added to a final
concentration of 10 mM and was incubated in the dark at room
temperature for 30 min to alkylate the sample. One hundred
millimolar Tris, pH 8.0, 2 mM CaCl2 was added to the mixture
to dilute the 5× Invitrosol to 1×. The mixture was divided into
two aliquots, and 4 µg of trypsin (1 µg/µL) was added to each
aliquot. The trypsin digestion was incubated at 37 °C for
24 h. The digestion was stored at −80 °C until mass spectrom-
ometry analysis.

Multidimensional Protein Identification Technology (Mud-
PIT). MudPIT was performed as previously described using an
LTQ linear ion trap mass spectrometer (ThermoFinnigan, San
Jose, CA) and a quaternary high-performance liquid chroma-
tography (HPLC) pump (Agilent, Foster City, CA) except for
the following modification in the acquisition of the mass spectra.24
A cycle of one full-scan mass spectrum (400–1400 m/z) followed
by six data-dependent spectra was repeated continu-
ously throughout each step of the multidimensional separation.
The first data-dependent event was a zoom scan from the most
intense peak to achieve high resolution of the isotope distribu-
tion. The second data-dependent event was an MS/MS scan of
the most intense peak at a 35% normalized collision energy.
The last four data-dependent events consisted of zoom scans
and MS/MS scans for the second and third most intense peaks.
All zoom scans were acquired using 25 microscans to improve
signal to noise. Application of mass spectrometer scan func-
tions and HPLC solvent gradients was controlled by the
Xcalibur data system (ThermoFinnigan, San Jose, CA).

Analysis of Tandem Mass Spectra. Tandem mass spectra
were analyzed using the following software analysis protocol.
Poor-quality spectra were removed from the data set using an
automated spectral quality assessment algorithm.25 Tandem
mass spectra remaining after filtering were searched with the
SEQUEST algorithm version 27.26 against the EBI-IPI_rat_
3.05_04-2005 database concatenated to a decoy database
in which the sequence for each entry in the original database
was reversed.27 All searches were parallelized and were per-
formed on a Beowulf computer cluster consisting of 100 1.2
GHz Athlon CPUs.28 No enzyme specificity was considered for
any search. SEQUEST results were assembled and filtered using
the DTASelect (version 2.0) program.29 DTASelect 2.0 uses a
quadratic discriminant analysis to dynamically set XCorr and
DeltaCN thresholds for the entire data set to achieve a user-
specified false positive rate (5% in this analysis). The false
positive rates are estimated by the program from the number
and quality of spectral matches to the decoy database.

Technical Notes

15N Metabolic Labeling of Mammalian Tissue

Science (Indianapolis, IN). All Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All methods involving animals were approved by the Institutional Animal Research Committee and were accredited by the American Association for Accreditation of Laboratory Animal Care.

Preparation of 15N Enriched Diet. Ten grams of spirulina
biomass (algae), unlabeled or 15N labeled, was mixed well with
30 g protein-free diet powder (Harlan TD 93328). Initially, rats
were fed the powder mixture directly; however, we found this
method to be cumbersome and inefficient protocol to feed
the rats. To improve our feeding protocol, we created 15N
enriched food pellets. To make the pellets, we sprinkled 6 mL
(for unlabeled) or 6.5 mL (for 15N labeled) of H2O in the
powdered food, stirred, and worked the mixture into a dough.
The dough was shaped into a long cylinder about 2-cm thick.
The cylinder was cut into 2-cm pieces lengthwise. The pellets
were dried at 60 °C for 2–4 h and then at 35 °C overnight using
an Excalibur food dehydrator.

Metabolic Labeling with 15N-Labeled Algal Cells. Protocol
1. Two female Sprague–Dawley rats were delivered to the
animal facility on pregnancy day 2. One rat was immediately
fed a specialized diet consisting of a protein-free rodent diet
(Harlan Teklad, TD93328) supplemented with algal cells con-
taining only natural abundance isotopes (control diet), and the
other rat was immediately placed on the 15N diet.

Protocol 2. Four female rats (littermates) arrived at the
animal facility immediately after weaning. Two rats were
immediately placed on the 15N diet and were housed in the
same cage. The other two were placed on the control diet and
were housed in the same cage. Once the female rats reached
sexual maturity, one male rat was placed in the female cage
from 6 p.m. to 6 a.m. until pregnancy, which was determined
by the observation of a vaginal plug. Before pregnancy, the
female rats were offered the 15N diet or the control diet in
separate cages. The other two were placed on the control diet and
were housed in the same cage during the pregnancy period.

For both protocols, pregnant female rats were received
pregnancy day 2. One rat was immediately
fed a specialized diet consisting of a protein-free rodent diet
(Harlan Teklad, TD93328) supplemented with algal cells con-
taining only natural abundance isotopes (control diet), and the
other rat was immediately placed on the 15N diet.

After pregnancy, the female rats were offered the 15N diet or the control diet in
powder form for 45 min every 6 h (6 a.m., 12 p.m., 6 p.m., and
12 a.m.). The male rats were removed from the cage when the
female rats were given the powdered food. The male rats were
given standard Harlan rodent diet ad libitum when caged
separately from the female rats.

For both protocols, pregnant female rats were caged sepa-
rately and the food (in the form of dry pellets) was given to
the rats ad libitum. After weaning, the pups were given food
ad libitum. All rats had ad libitum access to water. All rats were
maintained in temperature-controlled (23 °C) facility with a
12-h light/dark cycle. Taking the cost of the labeled algae into
consideration, the resultant 15N labeled tissue usable as a
quantitative internal standard is ~$1/mg of protein.

Sample Preparation. Tissues were removed and stored as
previously described.21 Frozen tissues were homogenized in ice-
cold homogenization buffer (4 mM HEPES, pH 7.4, 0.32 M
succrose) using 30 strokes in a tight-fitting Dounce homogenizer.
Protein concentration was determined using the BCA protein
assay (Pierce, Rockford, IL). One hundred micrograms of the
15N tissue and the corresponding 14N tissue were mixed. For
example, 15N labeled brain homogenate from a p1 pup was
mixed with 14N labeled brain homogenate from a p1 pup. The
sample was then centrifuged at 14 000 g for 15 min, and the
supernatant was discarded. One hundred twenty microliters of
5× Invitrosol (Invitrogen, Carlsbad, CA) containing 8 M urea
was added to the pellet, and tris(2-carboxyethyl)-phosphine
hydrochloride was added at a final concentration of 5 mM to
reduce the proteins. This mixture was then vortexed for 1 h at
room temperature. Next, iodoacetamide was added to a final
concentration of 10 mM and was incubated in the dark at room
temperature for 30 min to alkylate the sample. One hundred
millimolar Tris, pH 8.0, 2 mM CaCl2 was added to the mixture
to dilute the 5× Invitrosol to 1×. The mixture was divided into
two aliquots, and 4 µg of trypsin (1 µg/µL) was added to each
aliquot. The trypsin digestion was incubated at 37 °C for
24 h. The digestion was stored at −80 °C until mass spectrom-
etry analysis.
determined that at least 100 $^{15}$N peptides are required to determine an accurate $^{15}$N average percent enrichment of a tissue sample.

**Statistical Analysis.** We performed unpaired t-tests (two-tailed) to determine significant differences between enrichment protocols. The sample size equaled the number of peptides for which we were able to determine a confident $^{15}$N enrichment percentage (correlation value > 0.7). All statistical analyses were performed using Prism 4.0 software (GraphPad Software, San Diego, CA).

**Results and Discussion**

**Enrichment Protocols.** We designed two protocols for which rats were fed a $^{15}$N enriched diet for two generations (Figure 1). This diet was formulated by supplementing a protein-free Harlan rodent diet with $^{15}$N enriched algal cells included as the sole protein source. Control rats were fed the same diet formulated with algae containing only natural abundance isotopes. In protocol 1, a pregnant rat was placed on the $^{15}$N enriched diet on its second day of pregnancy (E2). This rat remained on the $^{15}$N enriched diet after parturition and until it finished weaning its pups after 20 days. In protocol 2, a female rat was placed on the $^{15}$N diet immediately after it was weaned. The rat remained on the diet during mating and pregnancy. For both protocols, the mother was sacrificed after it weaned its pups and the pups remained on the $^{15}$N diet for an additional 25 days (Figure 1).

We previously demonstrated that a rat on a $^{15}$N diet lasting 44 days had no adverse physiological or histological consequences on a 3-week old rat. One potential concern, however, is the effect of $^{15}$N labeling on fertilization, fetal development, gestation, or parturition. All the litters of rats on the $^{15}$N diet were within the range of the average litter size for a rat, which is 7–11 pups (data obtained from Harlan, our rodent supplier) (Figure 2A). In protocol 1, the mother was on the $^{15}$N diet for 50 days while the mother from protocol 2 was on the $^{15}$N diet for 107 days. Accordingly, we observed a lower enrichment of $^{15}$N in tissues from mother1 (liver 86.0% ± 4.69; brain 71.9% ± 8.13) compared to mother2 (liver 94.4% ± 4.24; brain 83.3% ± 6.09) (Figure 2B). As might be expected, $^{15}$N enrichment in the liver and brain increased with the longer labeling times and, consistent with our previous report, the $^{15}$N enrichment in the brain was lower than the enrichment in the liver in both protocols.

We analyzed the $^{15}$N enrichment of tissues from the pups. We determined the enrichment levels of $^{15}$N for the pups at p1 (postnatal day 1), p20, and p45. In liver, protocol 2 had an enrichment of 70.8% ± 6.48 and 88.8% ± 1.37 at p1 and p20, respectively. This is significantly higher $^{15}$N enrichment compared to the liver in the p1 (66.3% ± 2.77) and p20 (76.6% ± 2.68) pups of protocol 1 (Figure 3A). This is likely due to the differences in the amount of $^{15}$N labeled protein obtained while breast feeding. At p1 and p20, the sole source of protein for these pups had been breast milk from the mother, and the enrichment of $^{15}$N in proteins was lower in the mother from protocol 1 (liver 86.0% ± 4.69; brain 71.9% ± 8.13) compared to the mother from protocol 2 (liver 94.4% ± 4.24; brain 83.3% ± 6.09) (Figure 2B). Since the enrichment in the pups cannot exceed their $^{15}$N source, the pups from protocol 1 would be expected to have less $^{15}$N enrichment than the pups from protocol 2. After weaning, however, the pups from both protocols were placed on the same diet enriched with 99% $^{15}$N. After 25 days, the $^{15}$N enrichment of pups from protocol 1 (96.2% ± 2.34) reached the level of the pups from protocol 2 (96.5% ± 5.07). These data suggest that 96% is the maximum...
tissue enrichment for two generations of rats on a diet consisting of 99% $^{15}$N.

Determination of $^{15}$N enrichment in the brain revealed that p1 (69.6% ± 4.08) and p20 (87.3% ± 2.40) pups of protocol 2 had significantly higher $^{15}$N enrichment in proteins compared to the p1 (66.4% ± 2.27) and p20 (74.9% ± 2.34) pups of protocol 1 (Figure 3B). This is consistent with the observation from the liver. Furthermore, the $^{15}$N enrichment of liver and brain is comparable in both protocols in the p1 and p20 pups. After approximately 40 days of metabolic labeling starting at fertilization, p20 pups in protocol 2 demonstrated a greater $^{15}$N enrichment in brain tissue (>85%) compared to our previous attempt (74%) of labeling a 3-week old rat for 44 days.20 Thus, beginning the labeling process earlier increases $^{15}$N enrichment in the brain. This is consistent with reports demonstrating protein turnover in muscle tissue decreases with age.31,32 We did observe, however, a difference in $^{15}$N enrichment between liver and brain in the p45 pups. In protocol 1, the $^{15}$N brain enrichment was 87.0% ± 8.95 at p45, while in protocol 2, the $^{15}$N brain enrichment was 95% ± 2.25 at p45, but in liver there was no significant difference between the $^{15}$N enrichment of the two protocols at p45. Consistent with reported changes in mass spectra as the enrichment decreases,30 the mass spectra of the same peptide from the p45 and p1 brain samples are quite different (Figure 4). As a result, the signal-to-noise ratio will decrease, which causes two problems. First, identification of $^{15}$N labeled peptides becomes difficult. Since the labeled and unlabeled protein samples are mixed 1:1, low enrichment will result in fewer proteins identified and then fewer proteins quantified. Second, low-signal intensity hinders the precision of extracting the ion chromatogram of the $^{15}$N peptide, which leads to inaccurate quantification. Therefore, labeling for two generations is preferred to adequately enrich brain tissue.

Figure 3. The percent $^{15}$N enrichment of liver and brain tissues from rat pups during development. (A) The percent $^{15}$N enrichment was determined for liver tissue at three developmental ages, p1, p20, and p45. There were significant differences (**p < 0.001) detected between the two enrichment protocols at p1 and p20, but no significant differences were detected at p45. (B) The percent $^{15}$N enrichment was determined for brain tissue at three developmental ages, p1, p20, and p45. There were significant differences (**p < 0.001) detected between the two enrichment protocol at p1 and p20. In contrast to the liver tissue, there was a significant difference detected at p45 between the two enrichment protocols. Values represent averages ± SD.

Figure 4. The $^{15}$N enrichment affects the isotopic distribution. The peptide, K.EITALAPSTMK.I, was identified in both p45 (A) and p1 (B) brain samples from protocol 2. The peptide was measured to be 95% enriched in the p45 sample and 68% enriched in the p1 sample. The isotopic distribution of the $^{14}$N peptide is identical in both mass spectra with the major peak at 1161.5 m/z, but the isotopic distribution for the $^{15}$N peptide is different between the two samples. The major isotopic peak of the $^{15}$N peptide in p45 is at 1173.5 m/z, while it is at 1170.5 m/z in p1. In addition, the $^{15}$N isotopic distribution is broader and less intense in p1 compared to p45. The y-axis is relative abundance and the x-axis is mass/charge.
addition, the mouse is another important disease animal model and initial experiments have demonstrated that this protocol is also applicable to this species.33 Quantitative mass spectrometry has the potential to provide novel insight into these complex neurological disease models, and the ability to metabolically label rats with 13N to a high degree of enrichment will allow more accurate and precise measurement of protein expression to better understand these diseases. We conclude here that labeling rats for two generations generates high 15N enrichment of all tissues regardless of their intrinsic protein turnover rate. This SILAM strategy will be important in applying quantitative mass spectrometry to more complex systems such as tissues and organs with low protein turnover rates such as the brain.

Acknowledgment. The authors would like to acknowledge financial support from National Institutes of Health grants 5R01 MH067880-02 and P41 RR11823-10.

Supporting Information Available: (1) A list of all the peptides and their calculated percentage 15N enrichment for liver, brain, and muscle tissues of the rat pups from both protocols. In addition, the table for brain tissue from protocol 1 includes the protein from which the peptides where derived from and the spectrum count of that protein. Since spectrum count has previously been demonstrated to correspond to protein abundance, we demonstrate high enrichment of both high and low abundant proteins. (2) The distribution of the percentage 15N enrichment measurements for liver, brain, and muscle tissues of the rat pups from both protocols. (3) Three additional spectra of 15N enriched peptides. The y-axis is relative abundance and the x-axis is mass/charge. This material is available free of charge via the Internet at http://pubs.acs.org.

References


Figure 5. The 15N enrichment of skeletal muscle. (A) The percent 15N enrichment was determined for skeletal muscle from the maternal rats. Consistent with other maternal tissues, the percent enrichment of skeletal muscle was greater from the maternal rat from protocol 2 compared to protocol 1. (B) The percent 15N enrichment was determined for skeletal muscle at p20 and p45. There were significant differences (** p < 0.001) detected between the two enrichment protocols both at p20 and p45. Values represent averages ± SD.


