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Method for Estimating the Isotopic Distributions of Metabolically Labeled Proteins by MALDI-TOFMS: Application to NMR Samples

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We have developed an efficient method of estimating metabolic incorporation of heavy isotopes into proteins, including those where a single amino acid carries the label. The protein is digested with trypsin, and the resulting peptide mixture is examined directly by MALDI-TOF mass spectrometry. Peptides are chosen for analysis if they contain one or more labeled atoms and also exhibit clearly separated mass spectra. The known atomic composition of the peptide is then used to simulate ion distributions for various proportions of heavy isotope incorporation, to obtain the best match to the observed ion distribution. We demonstrate the method by comparing simulated and observed mass spectra of tryptic peptides of *Escherichia coli* citrate synthase labeled with ^{15}N in several ways and show that the method is particularly applicable when only one amino acid is isotopically labeled.

Heavy isotope incorporation into proteins has major applications in NMR spectroscopy.[1−4] It is also an accepted way of differentiating between cells from variable growth conditions[5,6] and is useful in proteomics studies.[7−9] In all such experiments, the protein must be efficiently labeled, and the extent of labeling is an important parameter that must be known in order to interpret the experiment accurately.

The most obvious method of measuring labeling efficiency is to measure the average mass of the labeled protein by mass spectrometry and to compare it with the value for the unlabeled protein. Either electrospray ionization[10] or matrix-assisted laser desorption/ionization (MALDI)[1,3,11] may be used for this purpose. An average mass measurement of the intact protein is probably adequate if the protein is uniformly labeled, but the mass increase in the intact protein can be less than the error in its mass measurement if only one amino acid is labeled. If incorporation is less than ideal, even a slight heterogeneity broadens the mass peak so that the mass difference cannot be measured with sufficient precision. A better estimate of labeling efficiency, even for uniform labeling, can be found by analysis of the constituent peptides.[5,6,8,9] When only one amino acid is labeled, the mass shift of a peptide is ∼1 Da per incidence of that particular amino acid. At any incorporation rate less than 100%, there will be two populations of peptides (labeled and unlabeled) with overlapping ion distributions. Quantitation of the incorporation rate has been addressed,[12] but there is no general method available.

This report describes how to quantitate metabolic incorporation of labeled amino acids into any protein of known sequence. We demonstrate the method using the *Escherichia coli* enzyme citrate synthase (CS),[13,14] either labeled uniformly with ^{15}N or specifically labeled by an individual ^{15}N amino acid. For selected peptides, the observed ion distribution is compared with that predicted by an algorithm based on atomic mass and a range of ^{15}N incorporation. A least-squares method is used to determine which simulated ion distribution best matches the observed ion distribution for the peptide. This is a useful tool when preparing samples for NMR experiments, especially when only one amino acid is labeled. Although we show only ^{15}N incorporation, the analysis can also

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be applied to proteins prepared with ^{13}C, $^{13}C + {}^{15}N$, or perdeuterated amino acids.[15]

MATERIALS AND METHODS

For the labeling experiments described here, $^{15}NH_4Cl$, L-glycine-^{15}N, L-lysine-^{15}N, L-histidine-^{15}N, L-methionine-^{15}N, L-phenylalanine-^{15}N, and L-tryptophan-$^{15}N_2$ (each with an isotope purity of 98%) were purchased from Cambridge Isotopes Laboratories Inc. (Andover, MA). L-Histidine-$^{15}N_3$ was from Spectra Stable Isotopes (Maryland, CO). TPCK-trypsin and unlabeled L-amino acids were from Sigma (St. Louis, MO). Minimal M9 medium was used as the base.[16] M9 salts with or without NH_4Cl, 40% glucose solution, 5 M NaOH, 0.10 M $CaCl_2$, and 1 M $MgSO_4$ were autoclaved separately. Stock solutions of unlabeled amino acid mixtures, nucleotides, sodium acetate, thiamine, succinic acid, and other supplements[17] were filter sterilized. The final minimal medium was adjusted to pH 7.0 using 5 M NaOH. Just before inoculation, 1 mL each of kanamycin (25 mg/mL in H_2O) and chloramphenicol (34 mg/mL in ethanol) were added to each liter of media. For uniform labeling, $^{15}NH_4Cl$ (1 g/L) was added to the final minimal salts glucose mixture. For successful specific labeling, we followed the procedure outlined by LeMaster and Richards[17] with modifications as detailed below.

Protein Preparation. In our usual expression system, the *E. coli* CS gene, *gltA*, is regulated by its native promoter,[18] but it produces very little CS protein when the host cells are grown in minimal salts glucose medium (M. Bidinosti, unpublished observations). Consequently, we used polymerase chain reaction to introduce new restriction sites into the original plasmid, pES*glt*A,[19] and subcloned the gene into vector pET 24b+ (Novagen), which places the *gltA* gene under control of the T7 expression system. For protein production, the plasmid was transformed into strain BL21(DE3)/pLysS[20] and grown at 37 °C with vigorous shaking. An auxotrophic strain was used only for the ^{15}N-lysine experiments.

For uniform ^{15}N labeling, a single colony was transferred into 2 mL of Luria−Bertani broth (LB)[16] and grown overnight. This culture was directly inoculated into 100 mL of complete minimal glucose medium with unlabeled NH_4Cl. The cells were collected by centrifugation and transferred into 3 L of minimal glucose with unlabeled NH_4Cl. When the culture reached an A_{600} value of ~0.5, the cells were collected by centrifugation and resuspended into 2 L of minimal glucose medium with $^{15}NH_4Cl$. After 1 h, expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to give 1 mM final concentration. Cells were harvested after 8−10 h further growth.

For single amino acid labeling, a single colony was transferred into 2 mL of LB as above. The next day, this culture was inoculated into 100 mL of minimal glucose medium containing the desired ^{15}N-labeled amino acid amid a pool of 19 unlabeled amino acids and other components,[17] but not containing NH_4Cl.[21] The cells were collected by centrifugation and resuspended in 2 L of minimal glucose medium lacking NH_4Cl and the labeled amino acid, but containing the 19 unlabeled amino acids and other components as required. When $A_{600} \sim 0.3$, the ^{15}N-labeled amino acid was then added at 50% of the normal requirement, a rate-limiting concentration,[22] and then expression was induced at $A_{600} \sim 0.5$ by addition of IPTG to 1 mM as above. The culture was harvested after 8−10 h further growth at 37 °C. The pool of 19 unlabeled amino acids ensures growth and should repress transaminase activities in the cell.[22] Protein was purified as described previously.[23] NMR samples were prepared in a buffer of 20 mM potassium phosphate at pH 7.0, 1 mM EDTA, and 0.04% NaN_3.

Mass Spectrometry. Initially, pure protein was dialyzed into 50 mM ammonium bicarbonate buffer,[24] diluted to 1 mg/mL, and incubated overnight with 1:100 w/w TPCK-trypsin. However, we found later that protein could be diluted directly from the NMR buffer into digest conditions to give a reasonable MALDI spectrum, so this procedure was used routinely. Aliquots of 0.5 μL each of digest and 2,5-dihydroxybenzoic acid matrix solution were applied to a metal target and analyzed on a MALDI QqToF instrument.[25,26] Peptides that covered a wide range in mass were chosen for analysis as long as they were clearly defined and contained the amino acid of interest.

Simulations. In each numerical simulation, the isotope mass distribution of a given peptide is calculated through a number of trials. The peptide mass for each trial is determined by constructing the peptide atom by atom, using a program that produces random numbers between 0 and 100 to select each atom's mass in accordance with its assumed abundance in the sample. For example, for each nitrogen in an unlabeled sample, the mass for ^{14}N is chosen if the random number is less than 99.634; otherwise, the mass for ^{15}N is added. For a sample enriched to 80% ^{15}N, the mass of ^{15}N is added if the random number is less than 80 and the mass of ^{14}N otherwise. The resulting peptide masses from 30 000 trials (1000 for Figure 1) are binned at 1-Da resolution to obtain the histograms reported here. A worked example and the algorithm are in files S 1−3 (Supporting Information). The code was written in perl 5.8.6 for Mac OS-X 10.4 and is sufficiently portable that it should work on a wide range of platforms (Windows, Unix, etc.) with few or no modifications.

The peptides used for analysis are listed in Table 1. Figure 1 shows simulated ion distributions for a typical peptide, LMGFGHR ($C_{36}H_{56}N_{12}O_8S$, peptide 1), first for natural isotopic abundance and then for cases where a single nitrogen atom has been labeled with ^{15}N to various degrees. This simulation applies directly to labeling of peptide 1 with leucine (L), methionine (M), or phenylalanine (F), since the peptide contains one of each residue, and each residue contains one nitrogen atom. The input will be modified if glycine (G) is labeled, since the peptide contains two G residues,

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Table 1. Tryptic Peptides from *E. coli* CS Used in the Simulations Shown in Figures 1−3

peptide	sequence	site in protein	monoisotopic mass	formula
1	LMGFGHR	300−306	816.406	$C_{36}H_{56}N_{12}O_8S$
2	HIPEFVRR	283−290	1052.588	$C_{48}H_{76}N_{16}O_{11}$
3	QLYTGYEKR	410−418	1156.588	$C_{52}H_{80}N_{14}O_{16}$
4	HTMIHEQITR	110−119	1264.635	$C_{53}H_{88}N_{18}O_{16}S$
5	YSIGQPFVYPR	178−188	1325.677	$C_{64}H_{91}N_{15}O_{16}$
6	ITFIDGDEGILLHR	56−69	1597.846	$C_{72}H_{115}N_{19}O_{22}$
7	TVGWIAHWSEMHSDGMK	388−404	1970.877	$C_{87}H_{126}N_{24}O_{25}S_2$

or if histidine (H, 3 nitrogens) or arginine (R, 4 nitrogens) is labeled.

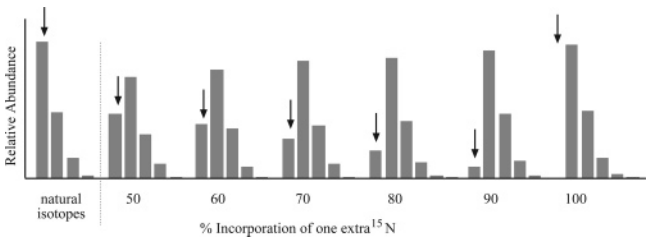

Figure 1. Simulated ion distributions (1000 trials) for peptide 1 (LMGFGHR, $C_{36}H_{56}N_{12}O_8S$), at natural abundance, and for 50−100% incorporation of ^{15}N in a single nitrogen atom. The arrow indicates the position of the monoisotopic ion at *m*/*z* 817.

As expected, Figure 1 shows that the ion at m/z 817 is dominant for natural isotopic abundance, but it progressively loses intensity compared to m/z 818 as the ^{15}N content increases. In this case, we have shown 1000 trials at 10% increments without normalization but note that succeeding histograms represent 30 000 trials. The algorithm allows for any increment value and even more trials, which may be necessary in order to acquire adequate statistics for larger peptides. However, an improved simulation simply requires more computer time, so the error can easily be made smaller than the statistical error in the measured mass spectrum, which usually dominates.

Comparison with Experiment. Figure 2A, B shows a comparison of real MALDI m/z spectra with the corresponding simulations for natural isotopic abundance. For the three peptides shown, there is an excellent match between the shapes of the ion distributions for the simulations and the real peptides. When we look at the same three peptides from protein prepared with $^{15}NH_4Cl$ (Figure 2C), the mass shift of the most abundant ion is equal to the number of nitrogens in the peptide, as has been quantitated by others using different methodology.[8,9] Calculation of the labeling efficiency (shown for peptide 1 in Figure 4A) has the best fit at 96% incorporation of ^{15}N. Simulations of ion distributions of these same three peptides with 96% incorporation of ^{15}N (Figure 2B) have the same shape as those in the real spectrum.

Figure 3 shows a comparison of real MALDI m/z spectra with the corresponding simulations when only one amino acid is labeled: phenylalanine (F) in (A) and (B), glycine (G) in (C) and (D), and histidine (H) in (E) and (F). The calculated labeling efficiency for peptide 1 is graphed in Figure 4B (phenylalanine), C (glycine), and D (histidine). The goodness of fit was measured as the least difference between observed and expected values.

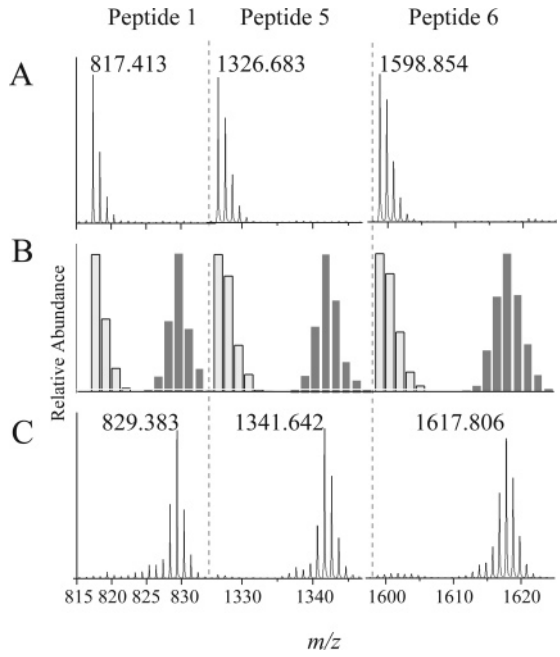

Figure 2. Comparison of simulated ion distributions with the *m*/*z* spectra from real protein digests for three of the peptides listed in Table 1. Peptide 1 (LMGFGHR, $C_{36}H_{56}N_{12}O_8S$), peptide 5 (YSIGQPFVYPR, $C_{64}H_{91}N_{15}O_{16}$), and peptide 6 (ITFIDGDEGILLHR, $C_{72}H_{115}N_{19}O_{22}$). (A) Normalized MALDI spectra from a digest of unlabeled CS. (B) Simulations of the ion distributions for natural isotopic abundance □, and for 96% incorporation of ^{15}N into all nitrogens in each peptide ■. (C) Normalized MALDI spectra from a digest of CS protein prepared from $^{15}NH_4Cl$. The variation of fit with simulated ^{15}N content for peptide 1 is shown in Figure 4A.

Phenylalanine (F) was the only amino acid where four readily identified peptides each had only one copy of the amino acid. However, as the simulations show, ^{15}N-phenylalanine incorporation was poor, at ∼50% (the fit for peptide 1 is shown in Figure 4B). Peptides without phenylalanine (peptides 3, 4, and 7) had natural abundance isotopic distributions, showing that the ^{15}N label was probably not lost by metabolic transfer to another amino acid during cell growth. Instead, it appears that the protein acquired about half of its phenylalanine from residual unlabeled material carried over from the early stages of cell culture. In this case, a weak NMR signal could be directly attributed to the high proportion of unlabeled protein. Changes in cell growth conditions in a subsequent experiment improved the labeling efficiency, but only to ∼ 60%.

Only two peptides have single glycine (G) residues, and two others each had two. The algorithm was adjusted for the

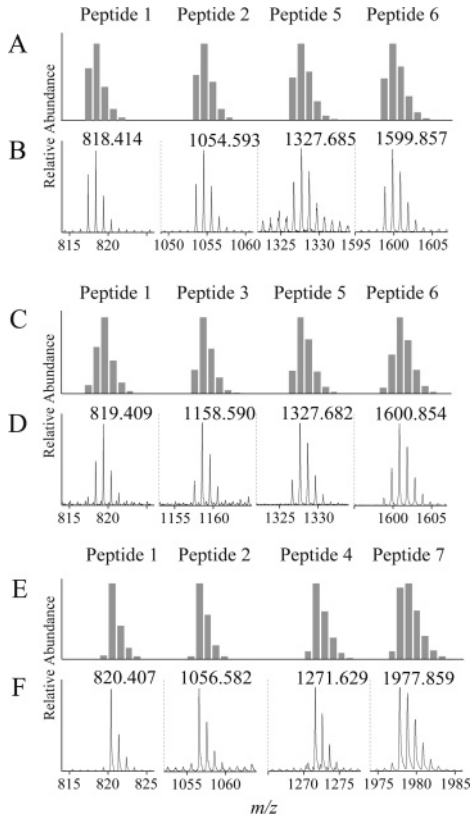

Figure 3. Comparison of the ion distributions from simulations (A, C, E) and real MALDI *m*/*z* spectra (B, D, F) for peptides from three different experiments where only one species of amino acid was labeled with ^{15}N. (A, B) For protein prepared with ^{15}N-phenylalanine. The simulations were calculated for an incorporation rate of 50%; see the fit for peptide 1 shown in Figure 4 B. (C, D) For protein prepared with ^{15}N-glycine. The simulations were calculated for an incorporation rate of 72%; see the fit for peptide 1 shown in Figure 4C. (E, F) For protein prepared with $^{15}N_3$-histidine. The simulations were calculated for an incorporation rate of 98%; see the fit for peptide 1 shown in Figure 4D.

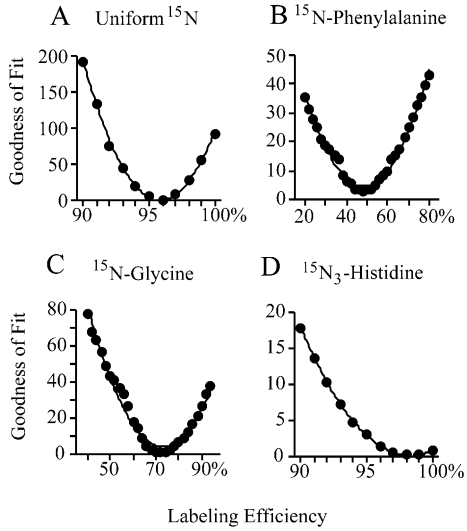

Figure 4. Demonstration of the calculated labeling efficiency for peptide 1 (LMGFGHR). A least-squares method is used to compare the relative abundance of ions from the real spectrum to those from a set of theoretical spectra. The goodness of fit, *y*, is calculated from the equation $y = [\sum_{i=1}^{n}[\text{mass}_i(\text{theoretical}_i - \text{measured}_i)^2]]/[N\sum_{i=1}^{n}(\text{mass}_i)]$, where *i* is any ion and *N* is the number of compared points (cf. ref 8) and is plotted as a function of the labeling efficiency (on the *x* coordinate). (A) Uniform ^{15}N label (Figure 2B, C). Minimum is at 96%. (B) Incorporation of one ^{15}N in phenylalanine (Figure 3A, B). Minimum is at 50%, (C) Incorporation of ^{15}N in two glycines, (Figure 3C, D). Minimum is at 72%. (D) Incorporation of ^{15}N in the three nitrogens of histidine (Figure 3E, F). Minimum is at 98%.

appropriate number of substitutions, and the simulations at 72% ^{15}N (the fit for peptide 1 is in Figure 4C) agree well with the real peptide ion distributions. This shows that we had decreased the amount of carryover of unlabeled protein, and in this case, the NMR signal was better than in the experiment with ^{15}N-phenylalanine. If there were significant metabolic transfer of the ^{15}N from glycine to serine during cell growth,[22,27] the ion distribution for peptide 6 would be expected to show the influence of a third ^{15}N; this was not observed. Three other peptides that have serine residues and no glycines had ion distributions that matched the unlabeled control (data not shown).

A further demonstration of our algorithm was in the analysis of a spectrum where the protein was prepared from $^{15}N_3$-histidine (the fit for peptide 1 is shown in Figure 4D). This shows very efficient labeling, consistent with 98% incorporation of ^{15}N. Note that peptides 1 and 2 each have three, and peptides 4 and 7 each have six, ^{15}N. Other experiments with ^{15}N-lysine, ^{15}N-histidine, and ^{15}N-methionine were equally good, all with incorporation efficiencies greater than 95% (data not shown). The exceptions were ^{15}N-leucine (75%) and $^{15}N_2$-tryptophan (85%). Very little tryptophan is required in the minimal growth medium,[17] and it is possible that this amino acid was not depleted sufficiently before induction. In all the single amino acid labeling experiments, the growth medium was supplemented with unlabeled amino acids,[22] and no NH_4Cl was included.[21] This method is expected to limit dilution of the labeled amino acid by endogenous synthesis or transamination and effectively dilute out any ^{15}N transferred to a different amino acid.[3,22] We did not find any evidence for transfer of ^{15}N to amino acids other than the intended one. Direct metabolic transfer may be more of a problem with nonbacterial cell lines,[7,12,27] and we avoided the amino acids where exchange would be substantial, such as glutamate−glutamine and aspartate−asparagine.[3,22]

In all cases, the amount of labeling was observed to be uniform and specific to the chosen amino acid for any protein preparation. The relative amount of ^{15}N was the same for all peptides having the residue of interest and was the same as natural abundance for those peptides without that particular amino acid. Our peptides span the length of the protein, so this clearly demonstrates that there is no preferential or nonuniform labeling.

Although we have shown only ^{15}N labeling, the simulations work equally well with ^{13}C, or both, and can also be applied to analysis of exotic amino acids.[15,28,29] Peptides from proteins prepared in D_2O are more difficult to analyze because the exchange between H and D varies among amino acids, and therefore, each peptide must be evaluated individually. We prefer

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to analyze the peptides using MALDI because there is a clear definition of the singly charged ions. However, the simulation works equally well for the multiply charged ions of electrospray ionization provided the charge state is known. The program can be adjusted to deal with any kind of modification. For our purposes, it has given us a reliable method of determining the ^{15}N incorporation into proteins prepared specifically for NMR experiments. This measurement is independent of both the total protein concentration and the NMR signal.

A potential limitation of the method is that imposed by the protein sequence on what peptides may be available for analysis and whether they are well defined. This difficulty might be circumvented somewhat by using different proteases. As for any statistical analysis, more data are required for the larger peptides. The simulations can easily be run for more cycles, and that improves the curves shown in Figure 4, but the fundamental limitation is normally imposed by the number of counts in the experimental spectrum.

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SUPPORTING INFORMATION AVAILABLE

Three text files: the algorithm, the input data used for Figure 2B, and the output data, part of which is shown in Figure 4A. This material is available free of charge via the Internet at http://pubs.acs.org.

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