Determination of protein synthesis in vivo using labeling from deuterated water and analysis of MALDI-TOF spectrum

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Xiao GG, Garg M, Lim S, Wong D, Go VL, Lee W-N. Determination of protein synthesis in vivo using labeling from deuterated water and analysis of MALDI-TOF spectrum. J Appl Physiol 104: 828–836, 2008. First published January 10, 2008; doi:10.1152/japplphysiol.00976.2007.—This paper describes a method of determining protein synthesis and turnover using in vivo labeling of protein with deuterated water and analysis of matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF) spectrum. Protein synthesis is calculated using mass isotopomer distribution analysis instead of precursor to product amino acid enrichment ratio. During protein synthesis, the incorporation of deuterium from water changes the mass isotopomer distribution (isotope envelop) according to the number of deuterium atoms (0, 1, 2, 3, etc.) incorporated, and the distribution of the protein with 0, 1, 2, 3, … atoms of deuterium follows a binomial distribution. A mathematical algorithm by which the distribution of deuterium isotopomers can be extracted from the observed MALDI-TOF spectrum is presented. Since deuterium isotopomers are unique to newly synthesized proteins, the quantitation of their distribution provides a method for the quantitation of newly synthesized proteins. The combined use of postsource decay sequence identification and mass isotopomer distribution analysis makes the use of in vivo labeling with deuterated water a precise method to determine specific protein synthesis.

mass isotopomer distribution analysis; isotopomer; spectral analysis; peptide sequence identification; matrix-assisted laser desorption time-of-flight mass spectrometer

The concentration of a protein in a cell depends on the rate of its synthesis and degradation. Thus the rate of protein turnover, the time required to synthesize a certain protein and maintain its concentration in a cell, is a sensitive indicator of cell physiology or its phenotype. Protein turnover is usually expressed as the rate of protein synthesis (in moles/unit time; or weight/unit time) or its half-life, which is the time required to achieve half of the maximum concentration of the protein. The determination of protein synthesis and turnover has been of great interest to biologists for more than two decades. Previously, radioactive tracers were used for the determination of protein synthesis and turnover. With availability of sensitive and precise mass spectrometers, the use of radioisotopes has been replaced by application of stable isotope tracers (18). The basic principle of protein turnover measurement using tracers relies on the measurement of the specific activity of the “precursor” (or the labeling agent, which can be deuterium in deuterated water, or specifically labeled amino acids such as [1-13C]leucine, [5,5,5-2H3]leucine) and the determination of the specific activity (SA) or enrichment (E) of the labeling agent (precursor) in the protein. The newly synthesized fraction (FNP) is provided by the formula:

\[ \text{FNP} = \frac{[\text{SA or E of the precursor in protein}]}{[\text{SA or E of the precursor}]} \]

Protein synthesis rate (PSR) is calculated by dividing the quantity of new protein by the time interval for the change using the equation:

\[ \text{PSR} = \frac{[\text{protein concentration}]}{(\text{FNP})/(\text{unit time})} \]

Examples of such methods for the determination of protein synthesis and turnover using tracers have been reviewed by Wolfe (21). Recently, Previs and colleagues (16) and Hellerstein and colleagues (3) separately introduced a novel method for the determination of protein synthesis using in vivo labeling with deuterated water (D2O) or heavy water. When cells or animals are given deuterium water, it is possible to maintain a constant high level of enrichment in water (0.5–2%). Plasma alanine rapidly incorporates the deuterium from water, changing its CH3 group to CD3, thus increasing its molecular weight depending on the deuterium enrichment in water (21). The enrichment in alanine can be determined by gas chromatography/mass spectrometry (GC/MS) after derivatization. To determine protein synthesis, the specific protein such as albumin is first isolated and hydrolyzed, and the enrichment in alanine isolated from the protein is similarly determined by GC/MS. FNP can be calculated using Eq. 1. The method of Hellerstein and colleagues (3) differs from the method of Previs and colleagues (16) in that a different experimental approach was used to determine amino acid enrichment using mass isotopomer distribution analysis (MIDA). It should be noted that these methods are variations of the basic precursor/product enrichment ratio method. They all require 1) determination of precursor SA or E, 2) isolation of specific protein of interest, 3) hydrolysis of the protein to separate the amino acids, 4) determining the enrichment in the specific amino acid of interest, and 5) application of Eqs. 1 and 2. A major disadvantage of this approach is that the accuracy of the calculated protein synthesis depends on the purity of the protein isolated.

We describe here a novel approach for the determination of protein synthesis and turnover using 2H-labeling and protein...
mass spectrum. It is known that metabolic systems in cells of organisms are capable of synthesizing nonessential amino acids. In the process of amino acid synthesis, carbon, nitrogen, and hydrogen atoms are incorporated from their precursor substrates either directly or through exchanges (transamination). If these precursor substrates are enriched with one or more of these isotopes, the resultant amino acids are heavier than the “natural” amino acids. Protein molecules that are synthesized after the introduction of the heavy isotopes will contain the heavier amino acids, resulting in a mass shift in the corresponding spectrum (Fig. 1). The intensities of the mass peaks corresponding to the unlabeled (light) and the labeled (heavy) can be used for calculation of the newly synthesized fraction. The method described herein differs from previous methods in that it utilizes the intensities of all relevant mass peaks of the peptide spectrum instead of the intensities of unique mass peaks. The mass spectral peaks of the heavier protein are resolved from the lighter (natural) protein by a mathematical algorithm. The capability of resolving overlapping spectra makes it possible to label proteins with lower enrichment of stable isotopes. The need for highly enriched precursor substrates is obviated (22).

**MATHEMATICAL ALGORITHM**

**Binomial distribution model of mass isotopomers in peptide.** To be able to separate the “light” spectrum from that of the “heavy” or labeled spectrum, one has to fully understand the theory that governs the mass isotopomer distribution of a large molecule such as a peptide and how the incorporation of heavy isotope subsequently modifies such a distribution. Mass isotopomer distribution in a peptide spectrum is mostly generated from the presence of $^{13}$C at its natural abundance (see below for a more general discussion of isotopes in protein). The distribution of isotopomers in the “isotope envelop” can be modeled by a binomial distribution with parameters $N$ and $p$. In the case of $^{13}$C distribution in peptide $^1$, $N$ is the number of carbon atoms, and $p$ is the natural enrichment of $^{13}$C. Assum-

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$^1$ There are 2 parameters in a binomial distribution: the number of trials ($N$) and the probability of success ($p$). In the case of $^{13}$C distribution in peptide, $N$ is the number of carbon atoms, and $p$ is the natural enrichment. The natural abundance of $^{15}$N and $^{18}$O affects the accuracy of determining the number of carbon atoms in the peptide but not the overall isotopomer distribution in the isotope envelop.
Innovative Methodology

DETERMINATION OF PROTEIN SYNTHESIS USING MIDA

...molecules with 1 (m1) and 2 (m2), etc.,13C substitution. The monoisotopic 
.../-0.767/0.011 is shown by the open bars. 
...the molar fraction of the all the isotopomer species. The theoretical 
...distribution3 was calculated based on 
...fraction after tryptic digest (Fig. 2). The intensities of 
...incorporation from deuterated water into a peptide. The orig-
...carbon (C)-isotopomers and deuterium (d)-isotopomers as shown in Fig. 4. The 
...isotopomers of the whole molecules from the isotope 
...isotopomers of its component parts (Fig. 3) (11). For example, if 
...concatenation function (\(\oplus\)) of mass isotopomers. 
...composition is given by [\(a_0m_0, a_1m_1, a_2m_2, \ldots\)] and the isotopomers from deuterium is [\(b_0m_0, b_1m_1, b_2m_2, \ldots\)]. The isotopomers of the peptide that contains both components is given by [\(a_0b_0m_0, (a_0b_0 + a_1b_0)m_1, (a_1b_1 + a_2b_0 + b_2a_0)m_2, \ldots\)]. The sum of the coefficients for each component is equal to 1 (\(\sum a_i = 1\) and \(\sum b_i = 1\)). As illustrated by the example, the coefficient of the combined peptide is given by the sum of the products with subscript indexes equal to the mass isotopomer number. In this case, the coefficient of \(m_0\) is given by \(a_0b_0\) and \(m_1\) is given by \(a_0b_1 + b_2a_1\). In fact, the 
...concatenation function. 

Figure 4 shows a hypothetical example of deuterium incorporation from deuterated water into a peptide. The original peptide is represented by carbon isotopomers. When such a peptide is synthesized in the presence of deuterated water, each peptide molecule may incorporate 1, 2, or 3 deuterium atoms, depending on the enrichment in water. The final distribution of the heavy peptide is the result of concatenation of carbon-isotopomers and the deuterium isotopomers. In this example the distribution of carbon isotopomers is given by the binomial distribution with \(N(c) = 100\) and \(p(c) = 0.011\), and the deuterium isotopomer distribution is given by \(N(d) = 20\) and \(p(d) = 0.04\). These distributions are shown in Fig. 4, A and B. Using the concatenation operation on these two distributions, we arrived at the distribution of the combined distribution (see APPENDIX). Figure 4C shows the isotopomer distribution of the resultant peptide as observed in the mass 

\[\text{Concatenation Operation } \oplus\]

\[\text{C-isotopomers } \oplus \text{ d-isotopomers } \rightarrow \text{Isotopomers of peptide}\]

\[\text{Inverse Concatenation Operation } \ominus\]

\[\text{Isotopomers of peptide } \ominus \text{ C-isotopomers } \rightarrow \text{d-Isotopomers}\]

Fig. 3. Definition of concatenation operation and its inverse. Concatenation operation can be used to model the formation of heavy peptide from known carbon (C)-isotopomers and deuterium (d)-isotopomers as shown in Fig. 4. The inverse concatenation operation is used to generate the d-isotopomer distribution given the observed isotopomer distribution of a heavy peptide and its corresponding C-isotopomer distribution before labeling.
spectrum of the peptide. Figure 4D shows the changes in isotopomer distribution (mass spectrum) of the same peptide under 2% and 4% deuterium enrichment conditions.

The mathematical algorithm of the inverse operation \( \otimes \) takes the observed spectrum and the unlabeled spectrum and converts it to a deuterium isotopomer spectrum using regression analysis. It can be shown that the \( \otimes \) operation on a peptide without deuterium label (pre-existing protein) returns the result \( m_0 = 1 \) or unlabeled\(^4\). Since \( m_0 \) of the deuterium spectrum can come from the newly synthesized peptide or the preexisting peptide, the deuterium isotopomers of the deuterated protein is diluted by \( m_0 \) contributed by the unlabeled protein. The ratio between the observed and the theoretical molar fractions of \( m_1, m_2 \), etc., from the deuterium isotopomers of the newly synthesized peptide give the fraction of newly synthesized peptide (11). (Fig. 5).

**MATERIALS AND METHODS**

**Animal protocol.** The animal study was carried out according to a protocol approved by Animal Research Committee at UCLA. Sprague-Dawley rats (250 g) were treated with a priming dose of \( D_2O \) equal to 6% body water and maintained on drinking water enriched with 4% deuterium as previously described (10). Serum was obtained before and on the 8th day of deuterated water treatment. Serum albumin was purified from these samples using AffiGel-Blue affinity column. The albumin fraction was eluted with 1.4 M NaCl in 20 mM Tris buffer (pH 7.1). Protein concentration in the albumin fraction was determined by its optical density (OD\(_{280}\)). Pretreatment sample and posttreatment samples were mixed in the proper proportions of protein concentrations to simulate 10, 25, 50, and 75% synthesis. The proteins in these samples were precipitated using 10% trichloroacetic acid. The desalted sample was concentrated before trypsin digestion and analysis by mass spectrometer.

\(^4\) [carbon isotopomers] \( \otimes \) [carbon isotopomers] returns a coefficient for \( m_0 = 1 \), and for other isotopomers, coefficients = 0.
Acquisition of mass spectra by MALDI-TOF. Trypsin digestion was performed by following the standard protocol (17, 23) with minor modifications. The purified albumin fractions were prepared in 50 mM NH₄HCO₃. A 50-ml aliquot was removed and mixed with 50 ml of ACN to improve proteolysis (17). Trypsin (Trypsin Gold, Promega) was added to a final protease-to-protein ratio of 1:80, and the digestion mixture was incubated at 37°C for 24 h. The digested peptides were lyophilized and resuspended in 50% acetonitrile/0.3% trifluoroacetic acid (TFA), and purified by using C₁₈ zip tips (Millipore) before MALDI-TOF profiling and postsource decay sequence identification using a Voyager DE (Voyager-DE workstation, Applied Biosystems, Forster, CA) (23).

The purified peptides using C₁₈ zip tip was directly eluted to MALDI plate with 2 μl of 10 mg/ml a-cyano-4-hydroxyquinamic acid (CHCA), and allowed to dry. The molecular weight of each sample was measured on a MALDI mass spectrometer with positive mode using Voyager DE reflector mode MALDI-time-of-flight (TOF). The instrument was calibrated with insulin standard (ProteoMass Insulin MALDI-MS standard [5,729,6089] and ACTH standard (ProteoMass ACTH fragment 18–39 MALDI-MS standard [2,464,1989]) (Sigma, St. Louis, MO). The mean molecular mass was calculated from multiple independent measurements. For the extraction of the protein from the MALDI plate, high laser power was applied (2,175 vs. 1,970 U for the standards). The instrument operated in positive ion delayed extraction mode with a low-mass gate of 500 Da. The accelerating voltage was 25,000 V (nondigested insulin and glucagons) or 20,000 V (trypsin-digested insulin and glucagons), the grid voltage was set to 65%, and the delayed time is 150 ns (trypsin-digested insulin and glucagons) or 285 ns (nondigested insulin and glucagons). Each spectrum was the average of ~200 scans, calibrated using external ACTH and insulin calibration files generated during the experiment.

Identification of tryptic digest by postsource decay. Tryptic digest of rat albumin was identified using postsource decay (PSD) matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (6, 7). m/z, mass to charge ratio.

Table 1. Peptides identified with MALDI-TOF PSD

<table>
<thead>
<tr>
<th>m/z</th>
<th>Sequences</th>
<th>Protein Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,299.4</td>
<td>HPDYSVSLLLRR</td>
<td>Rat serum albumin</td>
</tr>
<tr>
<td>1,393.4</td>
<td>MPLEKLTBDGDM</td>
<td>Rat AMP deaminase</td>
</tr>
<tr>
<td>1,479.5</td>
<td>LGYESGPNAILUR</td>
<td>Rat serum albumin</td>
</tr>
<tr>
<td>1,609.4</td>
<td>DVFGLTFVLEYSK</td>
<td>Rat serum albumin</td>
</tr>
<tr>
<td>1,681.7</td>
<td>IRENGSMT</td>
<td>Rat alpha2u-globulin</td>
</tr>
</tbody>
</table>

Table 2 shows the normalized spectra of peptides isolated from serum after 8 days of deuterated water treatment. The incorporation of deuterium in these peptides results in small shifts in the average mass. The magnitude of the shift due to deuterium incorporation depends on the enrichment in water, the number of nonessential amino acids, and the fraction of new protein. The fraction of new protein was calculated using the inverse concatenation operation. Examples of such a calculation are shown in the Appendix. Since the operation utilizes information from all spectral peaks, an estimate of the accuracy (SD) can be obtained from a single spectrum in m/z 1,681. From the magnitude of mass shift and the calculated new fraction, the number of possible deuterium substitution [N(d)] can be estimated. It is to be expected that peptides of the same protein have the same synthesis rate and new fraction, which is shown in the albumin peptides in Table 2. Peptides from other proteins may have different synthesis rates and have different new fractions.

The fraction of new protein can also be determined from the peptide spectrum when the spectra of the labeled and unlabeled peptides are known. In the example of AMP deaminase, we found that the protein was almost completely labeled (100% new synthesis) by day 8 of deuterated water treatment. Protein mass spectrometry containing albumin and AMP deaminase from sera obtained before deuterium labeling and on day 8 after deuterium labeling were mixed in to simulate 10, 25, 50, and 75% synthesis. The different mixtures were subject to trypsin digest and MALDI-TOF analysis. The observed spectra are shown in Table 4.

RESULTS

Serum was collected from a Sprague-Dawley rat after 8 days of deuterated water treatment. Albumin fraction was purified by AffiGelBlue affinity column. After tryptic digest, the sample was analyzed by MALDI-TOF for peptides in the range of 1,000–2,000 Da. Five peptides were identified using MALDI-TOF and postsource decay analysis. Their sequences were matched to rat serum albumin, AMP deaminase, and alpha 2u-globulin (Table 1). Despite our attempt to purify albumin, the MALDI-TOF spectrum indicated contamination of albumin by other serum proteins. The corresponding normalized spectra of these peaks from the sample before deuterated water treatment (unlabeled spectra) are shown in Table 2. Average mass was calculated using method of Blom (2).

Table 3 shows the normalized spectra of peptides isolated from serum after 8 days of deuterated water treatment. The incorporation of deuterium in these peptides results in small shifts in the average mass. The magnitude of the shift due to deuterium incorporation depends on the enrichment in water, the number of nonessential amino acids, and the fraction of new protein. The fraction of new protein was calculated using the inverse concatenation operation. Examples of such a calculation are shown in the Appendix. Since the operation utilizes information from all spectral peaks, an estimate of the accuracy (SD) can be obtained from a single spectrum in m/z 1,681. From the magnitude of mass shift and the calculated new fraction, the number of possible deuterium substitution [N(d)] can be estimated. It is to be expected that peptides of the same protein have the same synthesis rate and new fraction, which is shown in the albumin peptides in Table 2. Peptides from other proteins may have different synthesis rates and have different new fractions.

The fraction of new protein can also be determined from the peptide spectrum when the spectra of the labeled and unlabeled peptides are known. In the example of AMP deaminase, we found that the protein was almost completely labeled (100% new synthesis) by day 8 of deuterated water treatment. Protein fractions containing albumin and AMP deaminase from sera obtained before deuterium labeling and on day 8 after deuterium labeling were mixed in to simulate 10, 25, 50, and 75% synthesis. The different mixtures were subject to trypsin digest and MALDI-TOF analysis. The observed spectra are shown in Table 4. Using multiple linear regression analysis, we determined the coefficients for labeled and unlabeled peptides that would give the observed distributions. The coefficient representing percent synthesis and its SD were provided by the regression analysis (Table 4). Linear response of isotopomer distribution due to mixing of labeled and unlabeled peptides is shown in Fig. 6. As expected, the SD for each measurement increases as percent synthesis decreases. Therefore, protein synthesis and protein concentration can be determined from proteins that are maximally enriched (i.e., 100% newly synthesized).

DISCUSSION

Mass isotopomer distribution analysis has been used to determine protein synthesis and turnover. Specifically labeled

5 N(d) is the theoretical number of deuterium atoms incorporated when deuterated water enrichment is 100%.
Determining protein synthesis using MIDA

Table 2. Mass isotopomer distributions of the identified peptides before deuterium labeling

<table>
<thead>
<tr>
<th>m/z 1,299</th>
<th>m/z 1,393</th>
<th>m/z 1,479</th>
<th>m/z 1,609</th>
<th>m/z 1,681</th>
</tr>
</thead>
<tbody>
<tr>
<td>m0</td>
<td>0.4892 ± 0.0038</td>
<td>0.4466 ± 0.0101</td>
<td>0.4239 ± 0.0029</td>
<td>0.3802 ± 0.0016</td>
</tr>
<tr>
<td>m1</td>
<td>0.3418 ± 0.0074</td>
<td>0.3393 ± 0.0047</td>
<td>0.3349 ± 0.0056</td>
<td>0.3343 ± 0.0022</td>
</tr>
<tr>
<td>m2</td>
<td>0.1250 ± 0.0089</td>
<td>0.1448 ± 0.0024</td>
<td>0.1480 ± 0.0030</td>
<td>0.1807 ± 0.0034</td>
</tr>
<tr>
<td>m3</td>
<td>0.0368 ± 0.0010</td>
<td>0.0474 ± 0.0110</td>
<td>0.0581 ± 0.0056</td>
<td>0.0724 ± 0.0010</td>
</tr>
<tr>
<td>m4</td>
<td>0.0060 ± 0.0039</td>
<td>0.0127 ± 0.0044</td>
<td>0.0247 ± 0.0025</td>
<td>0.0217 ± 0.0017</td>
</tr>
<tr>
<td>m5</td>
<td>0.0007 ± 0.0004</td>
<td>0.0051 ± 0.0025</td>
<td>0.0105 ± 0.0038</td>
<td>0.0106 ± 0.0023</td>
</tr>
<tr>
<td>Average mass</td>
<td>1,300.1348</td>
<td>1,394.2474</td>
<td>1,479.4711</td>
<td>1,610.4081</td>
</tr>
</tbody>
</table>

The averages and SD were calculated from triplicate MALDI-TOF analyses. We had only 1 analysis for the peak at m/z 1.681. Average mass was calculated according to Blom (2). m0, molar fraction of monoisotopic species; m1, m2, m3, m4, and m5 are molar fraction of isotopomer molecules with 1, 2, 3, 4, and 5 isotopic substitutions, respectively.

Amino acids of high enrichment are required. Papageorgopoulos et al. (15) attempted to determine protein synthesis in vivo using mass isotopomer distribution analysis (MIDA) based on their method for the determination of synthesis of biopolymers (9, 14). As a demonstration, rats were infused with [5,5,5-2H3]leucine (99% enriched) via the jugular catheter for 24 h at a rate of ~ 50 mg·kg⁻¹·h⁻¹ using a minipump. Muscle was harvested and creatine kinase (CK) was isolated. Trypsin digest of the protein was analyzed using an electrospray ionization/magnetic sector mass spectrometer. Mass isotopomers containing leucine isotope in peptides rich in leucine were determined. Incorporation of [5,5,5-2H3]leucine would result in mass shift of +3, +6, etc., depending on the number of leucine in the peptide and the enrichment of intramyocyte leucine. However, because of the low protein turnover rate, insufficient amount of the +3 or +6 isotopomers was detected. Even though the MIDA method is theoretically possible in such an application, it is not feasible to maintain a high infusion of the labeled precursor to determine synthesis of proteins that turn over slowly. Because of the high enrichment of specifically labeled leucine required, the MIDA method as described by Papageorgopoulos et al. (15) for in vivo study is not a practical one. Another method using mass spectrum for the determination of protein synthesis is that of Cargile et al. (4). The method introduces 13C carbon into amino acids from [U-13C6]glucose (final enrichment ~50%) via amino acid synthesis in bacteria. In organisms that can synthesize essential and nonessential amino acids from glucose and nitrogen, [U-13C6]glucose effectively replaces 12C with 13C in protein, creating a heavy protein that can be separated by mass spectrometry. By quantitating the intensity of the labeled and the unlabeled peaks, a synthesis/degradation ratio was calculated to represent relative protein turnover dynamics. Such a method is of limited use in higher organisms, which can only synthesize their nonessential amino acids from glucose. Furthermore, such an approach is expensive because of the large amount of highly enriched glucose required.

There are major differences distinguishing the described mass isotopomer analysis from previous in vivo methods. The present method differs from those of Previs et al. (16) and Busch et al. (3) in that 1) determination of isotope enrichment in specific amino acid in protein is not required, and 2) the fraction of new protein is determined from the mass spectrum of the protein or its fragments after enzyme digestion. A major weakness of previously published deuterated water methods is their dependence on the use of Eq. 1. It is well known that the isotope enrichment of plasma amino acids usually does not represent the enrichment in the intracellular amino acids, which are the immediate precursors for protein synthesis. As shown in Table 1, it is difficult to be certain of the purity of the protein isolated before hydrolysis. Any contamination by other proteins in the purification potentially introduces error in the determination of amino acid enrichment in the isolated protein. The described method permits the separate identification of peptide by postsource decay sequence identification. Thus protein synthesis determined from a specific peptide is truly the synthesis of the protein without ambiguity.

With the advent of high-resolution mass spectrometer capable of resolving molecules with molecular weights (m/z) > 2000 Da, many isotope labeling approaches have been devised to quantify proteins, determine relative protein expression, and

Table 3. Mass isotopomer distributions of the identified peptides after deuterium labeling

<table>
<thead>
<tr>
<th>m/z 1,299</th>
<th>m/z 1,393</th>
<th>m/z 1,479</th>
<th>m/z 1,609</th>
<th>m/z 1,681</th>
</tr>
</thead>
<tbody>
<tr>
<td>m0</td>
<td>0.3409 ± 0.0089</td>
<td>0.2748 ± 0.0044</td>
<td>0.2672 ± 0.0073</td>
<td>0.2757 ± 0.0026</td>
</tr>
<tr>
<td>m1</td>
<td>0.3418 ± 0.0061</td>
<td>0.3392 ± 0.0090</td>
<td>0.3159 ± 0.0127</td>
<td>0.3266 ± 0.0120</td>
</tr>
<tr>
<td>m2</td>
<td>0.1931 ± 0.0084</td>
<td>0.2205 ± 0.0063</td>
<td>0.2089 ± 0.0023</td>
<td>0.2266 ± 0.0028</td>
</tr>
<tr>
<td>m3</td>
<td>0.0726 ± 0.0110</td>
<td>0.1025 ± 0.0028</td>
<td>0.1201 ± 0.0073</td>
<td>0.1082 ± 0.0057</td>
</tr>
<tr>
<td>m4</td>
<td>0.0192 ± 0.0023</td>
<td>0.0395 ± 0.0014</td>
<td>0.0550 ± 0.0030</td>
<td>0.0451 ± 0.0044</td>
</tr>
<tr>
<td>m5</td>
<td>0.0013 ± 0.0056</td>
<td>0.0160 ± 0.0022</td>
<td>0.0329 ± 0.0072</td>
<td>0.0178 ± 0.0042</td>
</tr>
<tr>
<td>Average mass</td>
<td>1,300.4942</td>
<td>1,394.7257</td>
<td>1,479.9301</td>
<td>1,610.7297</td>
</tr>
<tr>
<td>ΔMass</td>
<td>0.3594</td>
<td>0.4783</td>
<td>0.522</td>
<td>0.3211</td>
</tr>
<tr>
<td>N(d)</td>
<td>21</td>
<td>20</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>New fraction</td>
<td>0.6960 ± 0.0827</td>
<td>0.6918 ± 0.0158</td>
<td>0.6994 ± 0.0442</td>
<td>0.7380 ± 0.0131</td>
</tr>
</tbody>
</table>

The averages and SD were calculated from triplicate MALDI-MOF analyses. We had only 1 analysis for the peak at m/z 1.681. N(d), p, and q are defined in APPENDIX. The observed mass isotopomer distributions were first converted to their corresponding deuterium isotopomer distribution (d-isotopomers) using the inverse concatenation function. The new fractions were then calculated as in the Appendix. Average mass was calculated according to Blom (2). The change in average mass is given by N(d) × p × (new fraction).
Isotopomer analysis after partial purification is certainly a useful approach in the studies of protein turnover and protein expression in proteomics.

### APPENDIX

The concatenation operation $\odot$ of mass isotopomers. In a molecule that contains more than one element containing isotope species, the molecular spectrum can be considered as a complex mixture of its components. For example, the distribution of molecules affected by $^{13}$C natural abundance is approximated by a binomial distribution with $N(c)$ being the number of carbon and $p(c)$ being the natural enrichment (1.1%). This distribution is reflected in the isotopomer distribution reflected in the isotope envelop. In a protein that is synthesized in the presence of deuterium (e.g., 4.0% deuterated water), when the contribution of other isotopes is ignored, the distribution of molecules having 0, 1, 2, 3... deuterium substitutions is given by the binomial distribution with $N(d)$ being the number of possible deuterium substitution and $p(d)$ the deuterium enrichment in water. The isotopomer distribution in these components is denoted as (C-isotopomers) and (d-isotopomers). The protein spectrum can be considered as the weighted sum of a series of spectra having 0, 1, 2, 3... deuterium substitutions, which can be derived from (C-isotopomers) and (d-isotopomers) using the concatenation operation $\odot$.

(C-isotopomers) $\odot$ (d-isotopomers) = observed mass isotopomers in protein.

If distribution of isotopomer in (C-isotopomers) is represented by $(a_0m0, a_1m1, a_2m2,...)$ and distribution of isotopomer in (d-isotopomers) by $(b_0m0, b_1m1, b_2m2,...)$, the distribution in the protein or peptide is given by $(a_0b_0m0, (a_0b_1 + a_1b_0)m1, (a_0b_2 + a_1b_1 + a_2b_0)m2,...$), where $m0$ represents the monoisotopic species and $m1$, $m2$, $m3$, etc., are the isotopomers containing 1, 2, 3... $^{13}$C or $^2$H.

### Table 4. Determination of percent synthesis using “labeled” and “unlabeled” spectra and multiple linear regression analysis

<table>
<thead>
<tr>
<th>%Synthesis</th>
<th>Unlabeled</th>
<th>10% Labeled</th>
<th>25% Labeled</th>
<th>50% Labeled</th>
<th>75% Labeled</th>
<th>100% Labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5%</td>
<td>0.4466±0.0101</td>
<td>0.4333±0.0072</td>
<td>0.4061±0.0106</td>
<td>0.3550±0.0106</td>
<td>0.3054±0.0106</td>
<td>0.2748±0.0044</td>
</tr>
<tr>
<td>3.3%</td>
<td>0.3393±0.0047</td>
<td>0.3402±0.0036</td>
<td>0.3360±0.0222</td>
<td>0.3280±0.0106</td>
<td>0.3247±0.0106</td>
<td>0.3392±0.0090</td>
</tr>
<tr>
<td>6.6%</td>
<td>0.1448±0.0024</td>
<td>0.1574±0.0014</td>
<td>0.1667±0.0082</td>
<td>0.1813±0.0106</td>
<td>0.1963±0.0106</td>
<td>0.2205±0.0063</td>
</tr>
<tr>
<td>12.5%</td>
<td>0.0474±0.0110</td>
<td>0.0549±0.0021</td>
<td>0.0576±0.0037</td>
<td>0.0774±0.0106</td>
<td>0.0858±0.0106</td>
<td>0.1025±0.0028</td>
</tr>
<tr>
<td>25%</td>
<td>0.0127±0.0044</td>
<td>0.0095±0.0038</td>
<td>0.0179±0.0026</td>
<td>0.0309±0.0106</td>
<td>0.0459±0.0106</td>
<td>0.0395±0.0014</td>
</tr>
<tr>
<td>50%</td>
<td>0.0051±0.0025</td>
<td>0.0047±0.0029</td>
<td>0.0157±0.0094</td>
<td>0.0208±0.0106</td>
<td>0.0249±0.0106</td>
<td>0.0160±0.0022</td>
</tr>
</tbody>
</table>

Protein fractions containing albumin and AMP deaminase from sera obtained before deuterium labeling and on day 8 after deuterium labeling were mixed in to simulate 10, 25, 50, and 75% synthesis. The averages and SD of peptide $m_0$, 1,393 were calculated from triplicate MALDI-TOF analyses for the different mixtures.

### Table 5. Results from multiple linear regression analysis using an Excel spreadsheet program

<table>
<thead>
<tr>
<th>Intercept</th>
<th>Coefficients</th>
<th>SE</th>
<th>t Statistic</th>
<th>P Value</th>
<th>Predicted Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>X Variable 1</td>
<td>0.6154</td>
<td>0.0043</td>
<td>120.5862</td>
<td>0.0031</td>
<td>0.6027</td>
</tr>
<tr>
<td>X Variable 2</td>
<td>0.2919</td>
<td>0.0038</td>
<td>77.6582</td>
<td>0.0082</td>
<td>0.3091</td>
</tr>
<tr>
<td>X Variable 3</td>
<td>0.0727</td>
<td>0.0038</td>
<td>18.9608</td>
<td>0.0335</td>
<td>0.0753</td>
</tr>
<tr>
<td>X Variable 4</td>
<td>0.0138</td>
<td>0.0038</td>
<td>3.6419</td>
<td>0.1706</td>
<td>0.0116</td>
</tr>
<tr>
<td>X Variable 5</td>
<td>0.0078</td>
<td>0.0038</td>
<td>2.5213</td>
<td>0.2404</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

Using the ratio of consecutive isotopomers relationship that $m_2/m_1 = ([N(d)] - 1)/2 \times p(1 - p)$, we determined the theoretical $N(d)$ to be 20. The theoretical deuterium isotopomers of (20, 0.025) are shown in the last column corresponding to fractions of molecules with 0, 1, 2, 3, and 4 deuterium substitution. The coefficients for $m_0$, $m_1$, $m_2$, ... are represented by $X variable 1$, $X variable 2$, etc.
isotopes. The use of $m_i$ notation allows the comparison of peptide peaks of different masses.

(C-isotopomers) distribution is given by $(a_0, a_1, a_2, a_3, \ldots)$

(d-isotopomer) distribution is given by $(b_0, b_1, b_2, b_3, \ldots)$

isotopomers of the protein is given by $(c_0, c_1, c_2, c_3, \ldots)$

$(a_0, a_1, a_2, a_3, \ldots) \oplus (b_0, b_1, b_2, b_3, \ldots) = (c_0, c_1, c_2, c_3, \ldots)$

The concatenation operation is defined by the matrix multiplication below. The C-isotopomers are arranged in a $(n+1)$ by $(m+1)$ matrix, and the d-isotopomers by a $(m+1)$ by 1 column vector. The product is the C-isotopomers in the form of a $(n+1)$ by 1 column vector:

$$
\begin{bmatrix}
a_0 & 0 & 0 & 0 & \cdots & 0 & b_0 & c_0 \\
a_1 & a_0 & 0 & 0 & \cdots & 0 & b_1 & c_1 \\
a_2 & a_1 & a_0 & 0 & \cdots & 0 & b_2 & c_2 \\
a_3 & a_2 & a_1 & a_0 & \cdots & 0 & b_3 & c_3 \\
a_4 & a_3 & a_2 & a_1 & a_0 & \cdots & 0 & b_4 & c_4 \\
\vdots & \vdots & \vdots & \vdots & \ddots & \vdots & \vdots & \vdots & \vdots \\

\end{bmatrix}
$$

The inverse operation $\ominus$ of the concatenation operation is the inverse of the above algorithm, which is to determine the d-isotopomer distribution given column matrix of observed-isotopomers and square matrix of the C-isotopomers.

$$(c_0, c_1, c_2, c_3, \ldots) \ominus (a_0, a_1, a_2, a_3, \ldots) = (b_0, b_1, b_2, b_3, \ldots)$$

It is clear by inspection that d-isotopomer distribution can be determined using multiple linear regression analysis. The coefficients obtained from linear multiple regression analysis is the deuterium isotopomer distribution $(b_0, b_1, b_2, b_3, \ldots)$. Because of the low enrichment of deuterium used in animal studies, there is a good likelihood that some of the newly synthesized peptide does not have any deuterium incorporation. In such experiments, $m_0$ represents the sum of preexisting and newly synthesized peptide. However, the fractions represented by $m_1$ and $m_2$ are the results of deuterium incorporation. Thus, using the consecutive mass isotopomer equation, we can determine the $N(d)$ and $p(d)$, using the equation $m_2/m_1 = (N - 1)/2 \times p/q$, where $q = 1 - p$. After determining deuterium enrichment, we can estimate $N(d)$ and fraction of newly synthesized protein by dividing the observed $m_1$ by the theoretical $m_1$. This is illustrated in Fig. 5.

Steps in the calculation of new fraction. We first estimated the deuteration enrichment in water to be 2.5% using deuterium incorporation into palmulite (data not shown) (Ref. 11). Using results of $m/z \ 1,393$ in Tables 1 and 2, we set up the algorithm for the determination of deuterium isotopomers of the protein using the inverse concatenation operation. The regression matrices of the operation are shown below.

$$
\begin{bmatrix}
0.4466 & 0.0000 & 0.0000 & 0.0000 & 0.0000 \\
0.3393 & 0.4466 & 0.0000 & 0.0000 & 0.0000 \\
0.1448 & 0.3393 & 0.4466 & 0.0000 & 0.0000 \\
0.0474 & 0.1448 & 0.3393 & 0.4466 & 0.0000 \\
0.0127 & 0.0107 & 0.0247 & 0.0748 & 0.0395 \\
0.0501 & 0.0017 & 0.0274 & 0.0748 & 0.0160 \\

\end{bmatrix}
$$

Using a spreadsheet program (Excel), the output of such a multiple linear regression is shown below in Table 5, and the coefficients represent the distribution of mass isotopomers due to deuteration incorporation.

Thus 61.5% of the molecules did not have any deuterium, 29.2% were labeled with one deuterium, and 7.3% were labeled with 2 deuterium atoms. Using the ratio of consecutive isotopomers relationship that $m_2/m_1 = [(N - 1)/2] \times p/q$, we determined the theoretical $N(d)$ to be 20. The theoretical deuterium isotopomers of $m/z \ 20, 0.025$ are $(0.6027, 0.3091, 0.0753, 0.0716, 0.0013)$ corresponding to fractions of molecules with 0, 1, 2, 3, and 4 deuterium substitutions. This is the theoretical distribution of deuterium isotopomers in the newly synthesized protein. It can be shown by multiple linear regression that observed distribution $(X_1, X_2, X_3, \ldots)$ accounts for 96.2% of the deuterium distribution in $m/z \ 1,393$ or the new fraction is 0.9619.

The $m_2/m_1$ ratios were determined similarly for the other peptides of Table 3. The corresponding theoretical distributions were calculated. The new fraction of the peptide is the contribution of the theoretical distribution as a fraction of the observed d-isotopomer distribution.

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