Measuring Proteome Dynamics *in Vivo*

AS EASY AS ADDING WATER?*

Nadia Rachdaoui‡, Leanne Austin§¶, Eric Kramer§, Michael J. Previs‖, Vernon E. Anderson, Takhar Kasumov‡‡, and Stephen F. Previs‡§ §§**

Proteomics investigations typically yield information regarding static gene expression profiles. The central issues that limit the study of proteome dynamics include how to (i) administer a labeled amino acid *in vivo*, (ii) measure the isotopic labeling of a protein(s) (which may be low), and (iii) reliably interpret the precursor/product labeling relationships. In this study, we demonstrate the potential of quantifying proteome dynamics by coupling the administration of stable isotopes with mass spectrometric assays. Although the direct administration of a labeled amino acid(s) is typically used to measure protein synthesis, we explain the application of labeled water, comparing ^2H_2O *versus* $H_2{}^{18}O$ for measuring albumin biosynthesis *in vivo*. This application emphasizes two distinct advantages of using labeled water over a labeled amino acid(s). First, in long term studies (*e.g.* days or weeks), it is not practical to continuously administer a labeled amino acid(s); however, in the presence of labeled water, organisms will generate labeled amino acids. Second, to calculate rates of protein synthesis in short term studies (*e.g.* hours), one must utilize a precursor/product labeling ratio; when using labeled water it is possible to reliably identify and easily measure the precursor labeling (*i.e.* water). We demonstrate that labeled water permits studies of protein synthesis (*e.g.* albumin synthesis in mice) during metabolic "steady-state" or "non-steady-state" conditions, *i.e.* integrating transitions between the fed and fasted state or during an acute perturbation (*e.g.* following a meal), respectively. We expect that the use of labeled water is applicable to wide scale investigations of proteome dynamics and can therein be used to obtain a functional image of gene expression *in vivo*. *Molecular & Cellular Proteomics 8:2653–2663, 2009.*

Proteomics investigations typically yield information regarding static gene expression profiles; *i.e.* current "state-of-the-art" research programs lack measurements of proteome dynamics (1–3). This deficiency is unfortunate because the ability to measure rates of protein synthesis and breakdown will likely facilitate the identification of biomarkers of disease and yield novel insight regarding underlying homeostatic abnormalities (3, 4). For example, by measuring the concentration of circulating aminotransferase and the synthesis/secretion of albumin, one might be able to determine the degree of liver damage and assess whether hepatic function is compromised, respectively (5). Also, it should be possible to determine the influence of specific factors on the regulation of protein synthesis; *e.g.* does a therapeutic agent stimulate insulin biosynthesis?

Classic studies of protein biosynthesis have measured the incorporation of a labeled amino acid(s) into a protein(s) of interest and estimated a synthesis rate by using a "precursor/product labeling ratio" (6). Because modern proteomics technologies can rapidly separate and quantify individual proteins from complex mixtures, investigators have started to exploit the use of stable isotope tracers in mass spectrometry-based studies of proteome kinetics. However, the ability to study protein dynamics *in vivo* presents unique challenges (3, 4, 7–13); *e.g.* how does one (i) administer an isotope (typically a labeled amino acid) over a prolonged period and (ii) determine the true precursor labeling (because the amino acid will be rapidly turned over and its labeling will be diluted)? We have demonstrated how to quantify protein synthesis using ^2H_2O *in vivo* (10, 11); the advantages are that the tracer can be given orally, body water is a homogeneous pool with a relatively slow turnover, and the organism will continuously generate ^2H-labeled amino acids (consequently one can study free living subjects, including humans (9, 11, 14)). The assumption of the method is that the equilibration between ^2H in body water and a free amino acid(s) is faster than the rate of incorporation of an amino acid(s) into a newly made protein(s); preferably, the labeling of a free amino acid(s) should remain constant regardless of the metabolic status. We have validated that assumption by measuring the time-dependent labeling of alanine *in vivo* during the administration of ^2H_2O and by measuring the incorporation of ^2H-labeled alanine into plasma albumin and total tissue proteins using gas chromatography-mass spectrometry methods (10, 11, 15). Subsequent reports support our observations (12, 13).

In this study, we demonstrate (as a model example) the application of our ^2H_2O-based approach for measuring albumin biosynthesis *in vivo* in mice during long term and short term investigations. Namely, we recently demonstrated how to obtain relatively precise measurements of mass isoto-

From the Departments of ‡Medicine, §Nutrition, and **Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, ‖Cell and Molecular Biology Program, University of Vermont, Burlington, Vermont 05405, and ‡‡Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44106

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pomer profiles of peptides and other relatively large molecules by developing a novel approach for integrating the data (16, 17). Our method allowed us to detect shifts in the isotope distribution profile of albumin-derived peptides from mice given ^2H_2O (17). In the current report, parallel studies examined the use of $H_2{}^{18}O$ because it offers potential advantages over ^2H_2O, especially during acute studies that involve perturbations such as consumption of a meal. For example, the cleavage of a protein will immediately add a labeled oxygen atom into the carboxyl group of a free amino acid; resonance effects will distribute the label over both carboxyl oxygens. Although repeated cleavage is required to achieve maximal labeling of both oxygens, cleavage of tRNA-bound amino acids will also contribute to the labeling of the carboxyl oxygen (18–21). The synthesis of a new protein(s) then results in the stable incorporation of ^{18}O into the peptide bond; indeed, the oxygen in peptide bonds accounts for a majority of the total oxygen in a protein (18, 19), making it potentially easier to describe precursor/product labeling relationships (6). Finally, during the development of this work pitfalls were identified; thus we discuss strategies to circumvent potential problems.

MATERIALS AND METHODS

Supplies

Unless noted, chemicals and reagents were purchased from Sigma-Aldrich. ^2H_2O (99%) was purchased from Cambridge Isotopes (Andover, MA), and $H_2{}^{18}O$ (95%) was purchased from Isotec (Miamisburg, OH). Gas chromatography and mass spectrometry supplies were purchased from Agilent Technologies (Wilmington, DE). Sequencing grade trypsin (catalog number V5111) was purchased from Promega (Madison, WI).

Biological Methods

Male C57BL/6J mice (~25 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and fed standard rodent chow for 5 days before initiating an experiment; mice were housed four or five per cage. The long term and short term experimental protocols were approved by, and conducted in compliance with the policies of, the Case Western Reserve University Institutional Animal Care and Use Committee.

In the long term studies, mice were randomized to receive an intraperitoneal bolus of ^2H_2O or $H_2{}^{18}O$ (14 μl of ^2H_2O or 12 μl of $H_2{}^{18}O$/g of body weight) saline (9 g of solid NaCl dissolved/1000 ml of labeled water). Following the injection, mice were returned to their cages and allowed to eat and drink *ad libitum*. In the mice given ^2H_2O, the drinking water was enriched to twice that of body water (*i.e.* we expected that the priming bolus would achieve ~2% ^2H labeling; therefore the drinking water was labeled at 4% ^2H_2O), whereas in mice given $H_2{}^{18}O$, the drinking water was maintained at 3.5% $H_2{}^{18}O$. The rationale for labeling the drinking water more than the expected labeling of body water is based on previous studies (22, 23). For example, the labeling of ^2H in body water can be diluted by several sources, including water intake/exchange via respiration and water generated during metabolism (*i.e.* the reduction of molecular oxygen). We expected that the dilution would be slightly greater for ^{18}O *versus* ^2H because oxygen labeling is diluted via the equilibration/exchange with CO_2 in addition to the dilution factors noted above for ^2H (14). Food intake, water consumption, and body weights were measured throughout the studies. Mice were killed at various points to obtain blood samples, which were collected in heparinized capillary tubes. Samples were centrifuged, and the plasma was frozen at −20 °C.

For the short term studies, mice were trained for 7 days to eat during a 2-h period of time (9 and 11 a.m.). On the day of the experiment, mice were given an intraperitoneal bolus of labeled water (21 or 25 μl of ^2H_2O or $H_2{}^{18}O$/g of body weight to achieve ~3% ^2H or ~3.5% ^{18}O labeling in body water, respectively) immediately before being randomized to either a fasted or a fed group (9 and 11 a.m.). Mice were killed at various intervals after the feeding session (*i.e.* 45, 90, 180, and 360 min), and blood and liver samples were collected as described above. Note that $n = 1$ fed and $n = 1$ fasted mouse were killed at 45, 90, 180, and 360 min to determine the ^2H labeling profiles of free amino acids, and $n = 5$ fed mice were killed at 360 min to quantify the ^2H or the ^{18}O labeling of plasma albumin. Finally, a group of control mice ($n = 5$) was killed to determine the natural isotopic background labeling.

Analytical Methods

^2H Labeling of Body Water—The ^2H labeling of body water was determined by exchange with acetone (24, 25). Briefly, 10 μl of sample or standard was incubated with 2 μl of 10 N NaOH and 4 μl of a 5% (v/v) solution of acetone in acetonitrile for 24 h. Acetone was extracted by addition of 600 μl of chloroform followed by addition of ~0.5 g of Na_2SO_4. Samples were vigorously mixed, and a small aliquot of the chloroform was transferred to a GC[1]-MS vial.

Acetone was analyzed using an Agilent 5973N-MSD instrument equipped with an Agilent 6890 GC system. A DB-17MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The temperature program was as follows: 60 °C initial, increase by 20 °C/min to 100 °C, increase by 50 °C/min to 220 °C, and hold for 1 min. The sample was injected at a split ratio of 40:1 with a helium flow of 1 ml/min. Acetone eluted at ~1.5 min. The mass spectrometer was operated in the electron impact mode (70 eV). Selected ion monitoring of *m/z* 58 and 59 was performed using a dwell time of 10 ms per ion.

^{18}O Labeling of Body Water—The ^{18}O labeling of body water was determined following conversion to trimethylphosphate (TMP) as follows (26). Plasma or standards (5 μl) were added to 12 × 75-mm glass tubes and incubated with ~3 mg of PCl_5 to generate phosphoric acid, and samples were allowed to stand for 20 min. Next, 100 μl of trimethylsilyldiazomethane (Sigma-Aldrich) was added to the sample, which was allowed to stand for 30 min. TMP was extracted by addition of 100 μl of water and 300 μl of chloroform, samples were vigorously mixed, and a small aliquot of the chloroform was transferred to a GC-MS vial.

GC-MS analyses of the TMP derivative were performed using an Agilent 5973N-MSD instrument equipped with an Agilent 6890 GC system. A DB-17MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The temperature program was as follows: 90 °C initial, increase by 30 °C/min to 240 °C, and hold for 1 min. The split ratio was 20:1 with helium flow at 1 ml/min. TMP eluted at ~2.4 min. The ^{18}O enrichment was determined using electron impact ionization (70 eV) and selected ion monitoring (10-ms dwell time) of *m/z* 140 and 142.

Gas Chromatography-Mass Spectrometry of Amino Acids—Liver samples (~0.5 g) were homogenized by addition of 6% perchloroacetic acid (~2.5 ml). The supernatant was run over an ion exchange column (AG 50W-X8 resin, hydrogen form). The column was first

[1] The abbreviations used are: GC, gas chromatography; TMP, trimethylphosphate; FSR, fractional synthesis rate; SILAC, stable isotope labeling by amino acids in cell culture.

washed with water (~10 ml), and amino acids were then eluted by washing with 3 M ammonium hydroxide (~20 ml). To generate the *tert*-butyldimethylsilyl derivatives the effluent was evaporated to dryness and incubated with 200 μl of *tert*-butyldimethylsilyl trifluoroacetamide + 10% trimethylchlorosilane (Pierce) + 50 μl of acetonitrile at 100 °C for 90 min (27–29).

GC-MS analyses of the *tert*-butyldimethylsilyl derivatives were performed using an Agilent 5973N-MSD instrument equipped with an Agilent 6890 GC system (27). A DB-17MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The temperature program was as follows: 70 °C initial, hold for 3 min, increase by 10 °C/min to 290 °C, and hold for 3 min. The split ratio was 10:1 with helium flow at 1 ml/min. The ^{2}H enrichment of individual amino acids was determined using electron impact ionization (70 eV) and selected ion monitoring (10-ms dwell time). To ensure that a sufficient number of data points were collected across a given signal, a selected ion monitoring routine was used; the instrument was programmed to acquire a maximum of four ions per time window (10-ms dwell per ion).

Proteomics-based Assay of Albumin Biosynthesis—Albumin was isolated from plasma as described previously (10, 11). Briefly, 50 μl of plasma was treated with 500 μl of 10% trichloroacetic acid, samples were mixed well and centrifuged, and the supernatant was removed and discarded. Albumin was extracted following addition of 100% ethanol (200 μl). Following evaporation of the ethanol, the residue was dissolved in 25 μl of trypsin solution (*i.e.* 20 μg of trypsin in 0.5 ml of 25 mM ammonium bicarbonate buffer). Samples were incubated for 18 h at 40 °C, then evaporated to dryness, and redissolved in 0.1% trifluoroacetic acid. Peptide extracts were desalted using a ZipTip$_{C18}$ (Millipore) and eluted with 2 μl of matrix solution (4 mg of α-cyano-hydroxycinnamic acid dissolved in 1 ml of acetonitrile containing 20 μl of trifluoroacetic acid).

MALDI-TOF analyses were done using a prOTOF 2000 instrument (PerkinElmer Life Sciences). The data were acquired by taking 100 laser shots over 42 s; the instrument was programmed to take a six-point circle pattern and record two spectra per location. The acquisition parameters were as follows: laser energy, 75%; laser rate, 100 Hz; declustering voltage, 30 V; cooling flow, 190.0 ml/min; and record a range of 700–5000 Da in the reflectron mode.

We previously reported a novel data processing method that allows us to reliably quantify the isotopic labeling of peptides (16, 17); this method was used to process the spectra. Briefly, the isotope profiles of a given peptide are converted to an ASCII file, and the relative intensities are determined by fitting the data to a series of Gaussian peaks centered at 1-Da intervals using Origin software. Parameters of the fit are constrained to (i) the center of the monoisotopic peak and (ii) a common peak width. The background is allowed to vary during the iterations. The "best fit" areas are used to calculate the abundance of different isotopomer peaks.

Calculations

In mice given either ^2H_2O or $H_2{}^{18}O$, excess labeling was calculated by normalizing the M1 or M2 isotopomer of a peptide against the M0 isotopomer, *i.e.* the isotopically substituted species (singly or doubly, respectively) *versus* the monoisotopic species, and then subtracting the mean background ratio(s) observed in control mice (*i.e.* mice that did not receive any isotope) (6). In long term studies, the fractional synthesis rate (FSR) was calculated from the exponential increase in protein labeling using the equation

$$\text{FSR} = \text{final labeling} \times (1 - e^{-(\text{FSR} \times t)}) \qquad \text{(Eq. 1)}$$

where "*t*" is the time after exposure to ^2H_2O and "final labeling" equals the total labeling of a proteolytic peptide (which is influenced by the amino acid composition of the peptide and the equilibration of isotope in the respective amino acids) (6). When ^2H_2O was used, we modeled the change in the M1/M0 ratio, whereas when $H_2{}^{18}O$ was used, we modeled the change in the M2/M0 ratio.

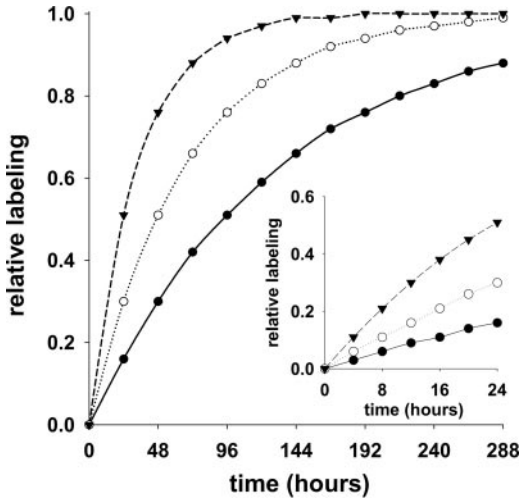

FIG. 1. **Time-dependent changes in protein labeling.** A simulation of labeling profiles for given turnover constants was performed; a range of values was used including 0.0075/h (*solid circles*), 0.015/h (*open circles*)/ and 0.03/h (*solid triangles*). The *main panel* demonstrates the effect of the turnover constant on the time required to reach maximal (or steady-state) labeling. The *inset* demonstrates that the change in labeling is (pseudo)linear during the initial 24 h at all of the simulated fractional rate constants.

In short term studies (*e.g.* following consumption of a meal), the FSR was calculated using the equation

$$\text{FSR} = \text{product labeling}_{8\,h}/(\text{precursor labeling} \times 8\ \text{h}) \qquad \text{(Eq. 2)}$$

where "product labeling$_{8\,h}$" represents the total labeling of a proteolytic peptide 8 h postinjection of the respective tracers (6). As noted earlier, the "precursor labeling" is influenced by the amino acid composition of the peptide and the equilibration of isotope in the respective amino acids. Although one can estimate the precursor labeling from the asymptotic (or steady-state) labeling of albumin that is obtained from a long term study, in cases where ^2H_2O is used acutely (especially in the fed state), it is difficult to predict the maximum labeling because ^2H is incorporated into various carbon-bound positions of amino acids. Therefore, one requires knowledge regarding the equilibration constants between hydrogen in water and peptide-bound amino acids. We estimated the precursor labeling for ^2H by integrating the labeling of the free amino acids that make up the peptide LGEYGFQNAILVR. However, when $H_2{}^{18}O$ is used, we expect one ^{18}O atom to be stably bound per peptide bond and an additional one or two ^{18}O atoms for certain amino acid side chains (*e.g.* glutamine or glutamate). We estimated the precursor labeling for ^{18}O by counting the number of peptide bond oxygens and the side chain oxygens, *i.e.* 12 + 4 = 16.

Note that to define a long *versus* a short term experiment one should consider the half-life of the protein relative to the experimental window (Fig. 1) (30). In the case of albumin, which has a half-life of ~1.7–2.3 days in a mouse, the fractional synthesis rate (or the rate of change of the proportion of labeled protein) is expected to be ~0.40–0.30/day (10, 15), *i.e.* the $t^1/_2 = \ln 2/k$ where *k* is the fractional rate constant. Given these values, the maximum proportion of labeled protein (*i.e.* the steady-state or asymptotic labeling) will be reached after ~9–12 days of exposure to the tracer. Thus, in our experiments,

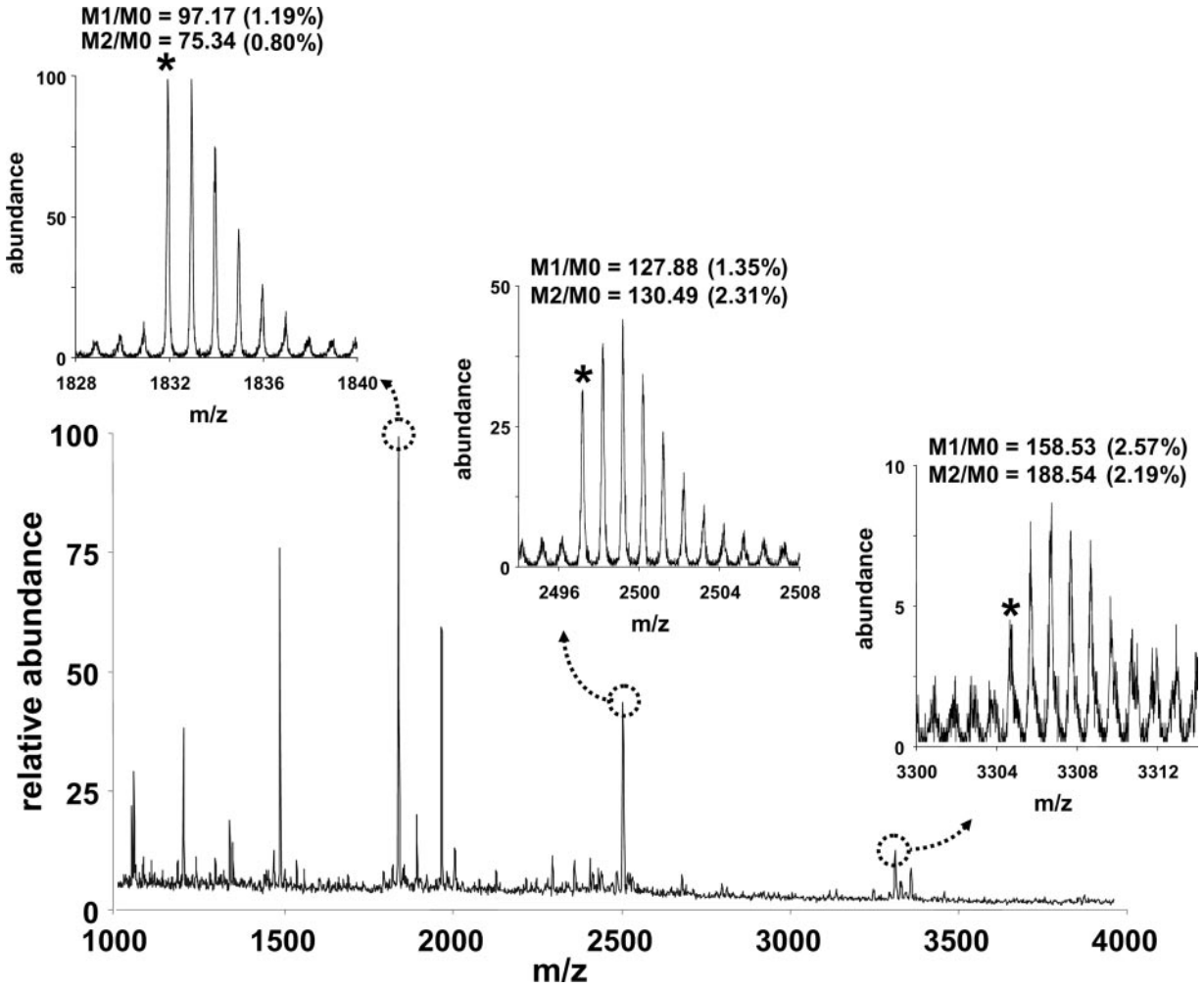

FIG. 2. **MALDI-TOF analyses of trypsin-digested albumin.** Albumin was purified from plasma and digested using trypsin. MALDI-TOF spectra were acquired in the reflectron mode (positive ions), and replicate analyses were performed to determine the reproducibility of the peptide isotopomer profiles. The *insets* demonstrate examples of the isotope profiles of different peptides at different signal/noise ratios. The reproducibility of the measured isotope ratios is shown; the mean was determined for four replicate analyses (the coefficient of variation is included in *parentheses*). The M0 isotopomer is the monoisotopic mass and is marked with an *asterisk*; M1 and M2 refer to isotopomers with one and two substituted heavy atoms, respectively.

which rely on Equation 2, "short term" is defined as those conditions in which we expect the change in albumin labeling to be (pseudo)linear, *e.g.* during the initial 8 h following administration of the tracer.

Statistics

Two-tailed *t* tests were performed in which we assumed equal variance. Unless noted, data are shown as mean ± S.E.

RESULTS

We recently demonstrated how to obtain relatively precise measurements of isotopic profiles of relatively large molecules (*e.g.* peptides) (16, 17). We considered whether the ionization mode(s) and mass analyzer(s) might affect the reproducibility of the measured isotope ratios. Although MALDI-TOF showed the greatest coefficient of variation, the error was still within an acceptable range for our application; *i.e.* we could quantify temporal changes in the isotopic distribution profiles of tryptic peptides from albumin using samples that were obtained from mice given ^2H_2O (17, 32). Fig. 2 demonstrates an example of the MALDI-TOF spectra of plasma albumin following tryptic digestion from a control mouse (which did not receive labeled water). Replicate analyses were performed to determine the precision of natural isotopic abundance distributions using peptides at different signal/noise ratios. Using peptides at *m/z* 1831.8, 2497.2, and 3304.4, we observed M1/M0 and M2/M0 ratios with coefficients of variation ≤2.6%. Because the peptide at *m/z* 1479.5 (*i.e.* LGEYGFQNAILVR (33)) was observed in all samples with precision comparable with that of the other peptides, we used its labeling to calculate rates of albumin synthesis. Although other approaches may offer still better precision of measured isotope ratios (17, 34), the level of reproducibility reported herein is sufficient for general studies of protein dynamics.

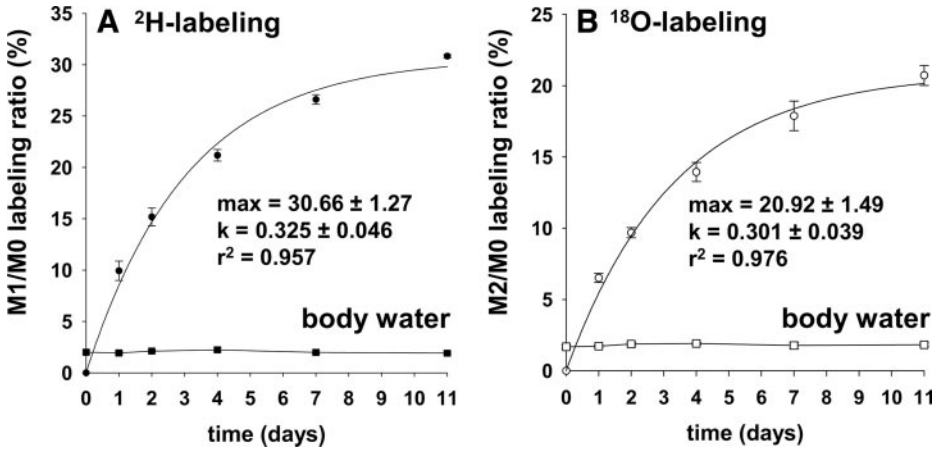

FIG. 3. **Time-dependent labeling of body water and incorporation of ^{2}H or ^{18}O into plasma albumin.** MALDI-TOF analyses of albumin demonstrated an exponential increase in the labeling of peptide LGEYGFQNAILVR. *A* demonstrates the change in the M1/M0 ratio in mice given $^{2}H_2O$, whereas *B* demonstrates the change in the M2/M0 ratio in mice given $H_2{}^{18}O$ for up to 11 days (mean ± S.E., $n = 3$ mice in each group per day). Note that the background (or endogenous) labeling has been subtracted. Data were best fit to a single exponential. In mice given $^{2}H_2O$, the asymptotic labeling ratio of M1/M0 was 30.66 ± 1.27%, and the turnover constant was 0.325 ± 0.046 ($r^2 = 0.957$); in mice given $H_2{}^{18}O$; the asymptotic labeling ratio of M2/M0 was 20.92 ± 1.49%; and the turnover constant was 0.301 ± 0.039 ($r^2 = 0.976$).

Fig. 3 demonstrates that a steady-state labeling of body water was achieved in long term studies, *i.e.* 2.03 ± 0.05% ^{2}H and 1.79 ± 0.04% ^{18}O. In our experience one can maintain these conditions for extended periods; *e.g.* some studies have run for >100 days.[2] We typically found that the labeling of body water is ~50% that of the drinking water because mice produce unlabeled water via endogenous metabolism, etc. The time-dependent labeling of peptide LGEYGFQNAILVR demonstrates comparable fractional rates of albumin synthesis (0.325 ± 0.046 *versus* 0.301 ± 0.039/day) but a greater absolute labeling in mice given ^{2}H- *versus* ^{18}O-labeled water (Fig. 3, *A versus B*, respectively). (Fig. 4 demonstrates the complete time-dependent shifts in the mass isotopomer profiles.) The difference in total peptide labeling was expected and relates in part to the fact that a higher labeling of body water was achieved in mice given $^{2}H_2O$ and in part to the fact that a larger number of ^{2}H atoms are incorporated; *e.g.* several amino acids incorporated multiple copies of the precursor. For example, Table I demonstrates the time-dependent ^{2}H labeling of intrahepatic free amino acids in fasted *versus* fed mice. As expected, we observed greater ^{2}H labeling in most of the non-essential amino acids as compared with the essential amino acids, and although we observed a reasonable steady-state ^{2}H labeling in fasted mice, there was a transient perturbation in the fed mice; *i.e.* the ^{2}H labeling tended to be lower in fed *versus* fasted mice. This was true of virtually all amino acids and is consistent with an influx of unlabeled amino acids from dietary protein.

Table II demonstrates the labeling of peptide LGEYGFQNAILVR in control mice and those given either $^{2}H_2O$ or $H_2{}^{18}O$ for 8 h. We observed 3.00 ± 0.28% excess ^{2}H in M + 1 and 2.61 ± 0.37% excess ^{18}O in M + 2. In mice given $^{2}H_2O$, the precursor labeling was determined by fitting the labeling profiles of the respective amino acids (Table I). In mice given $H_2{}^{18}O$, we assumed that the precursor labeling was equal to the ^{18}O labeling of body water times the number of amino acids in the peptide minus one that is (back-)exchanged from the terminal amino acid during tryptic cleavage plus one additional ^{18}O for asparagine and glutamine and two additional ^{18}O atoms for glutamate. Because there is an amplification of the enrichment as labeled water is incorporated into amino acids, the estimated precursor labeling is ~47.65% $^{2}H_1$ and ~52.29% ^{18}O. We calculated rates of albumin synthesis equal to 6.30 ± 0.59 *versus* 4.96 ± 0.66% newly made albumin over 8 h in mice given $^{2}H_2O$ *versus* $H_2{}^{18}O$, respectively ($p = 0.17$).

[2] V. E. Anderson, T. Kasumov, and S. F. Previs, unpublished observations.

DISCUSSION

To use labeled water (*e.g.* $^{2}H_2O$) to quantify protein synthesis, one assumes that the labeling of free amino acids is faster than their rate of incorporation into newly made proteins and that solvent-exchangeable sites (*e.g.* amino-bound hydrogens) will back-exchange with buffer during sample preparation; therefore, changes in the isotopic labeling profile of a peptide reflect the stable incorporation of ^{2}H in carbon-hydrogen bonds. Note that in the presence of $^{2}H_2O$ both non-essential and essential amino acids will become ^{2}H-labeled; thus, the labeling of a protein (or peptide) will reflect the labeling of virtually all amino acids. To predict the maximum labeling of a peptide one must have knowledge of the amino acid composition of that peptide and the equilibration constants for the respective amino acids. For example, it is expected that the total labeling of a peptide (*i.e.* the product) will be greater than that of body water (*i.e.* the precursor) because the ^{2}H labeling in body water is copied multiple times in a given peptide; *e.g.* the incorporation of one alanine will bring

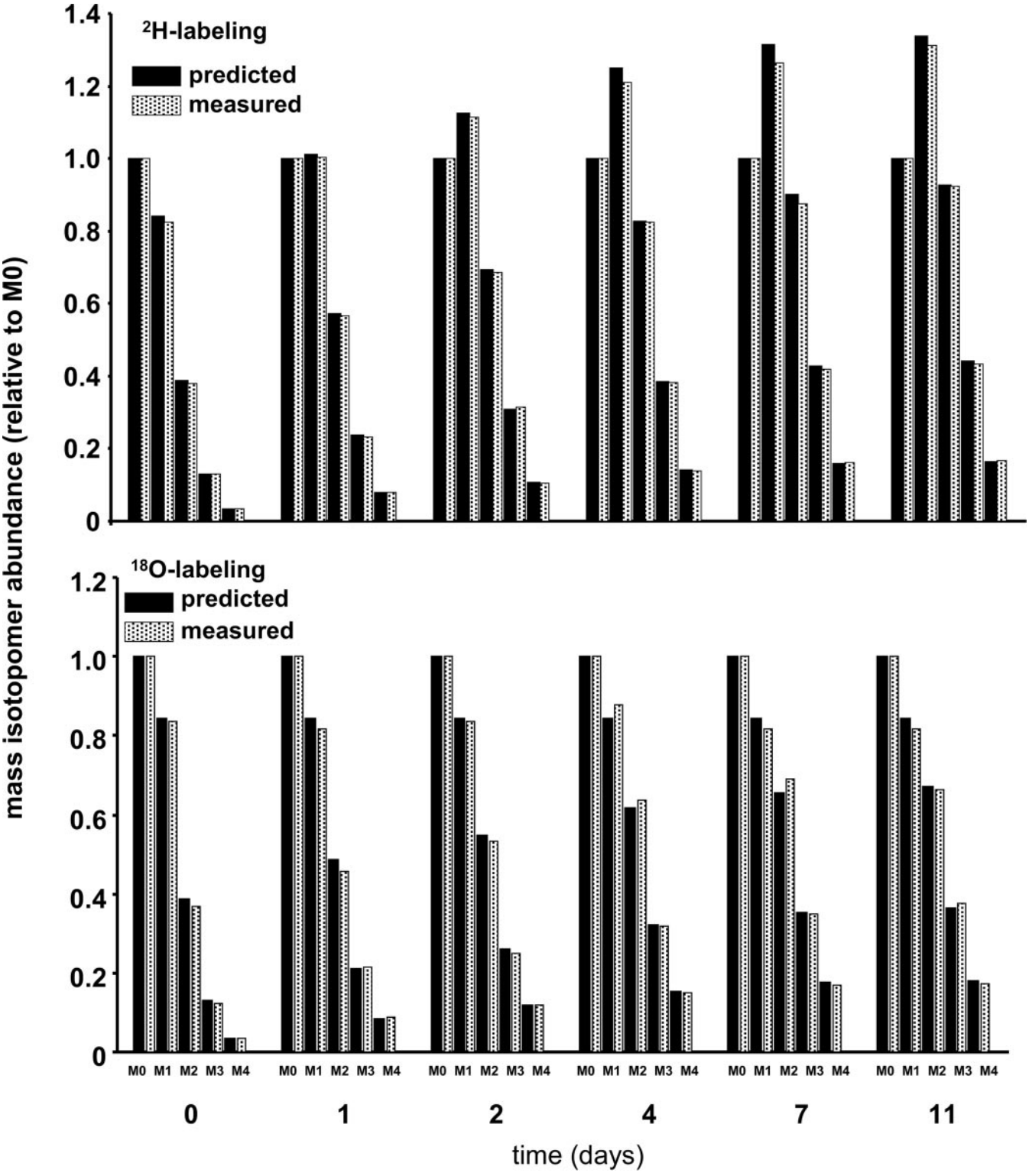

FIG. 4. **Time-dependent labeling of peptide LGEYGFQNAILVR.** MALDI-TOF analyses of albumin peptide LGEYGFQNAILVR were performed. *Top* and *bottom panels* demonstrate the change in the isotopomer distribution profile in mice given ^2H_2O and $H_2{}^{18}O$, respectively. The experimentally measured distributions are shown in *solid bars*. A simulation was run to predict the expected labeling profiles (*open bars*) assuming a fractional rate constant of 0.30/day. The ^2H and ^{18}O labeling in water were 2.03 and 1.79%, respectively. *n* was set to 25 for ^2H and 16 for ^{18}O. Note that the rationale for setting a value for *n* was based on experimental data in Table I when ^2H_2O was given (assuming an equilibration constant for arginine similar to that reported by Commerford *et al.* (37)) and using knowledge of biochemical pathways when $H_2{}^{18}O$ was given.

TABLE I

Time-dependent ^2H labeling of individual amino acids

Mice were given ^2H_2O and either fasted or fed. Blood and liver samples were collected at various time points ($n = 1$ mouse per time point) to determine the percentage of ^2H labeling of water (in blood) and amino acids (in liver). The ^2H labeling of body water was effectively constant over time, 3.18 ± 0.10% in fasted mice and 3.27 ± 0.08% in fed mice (mean ± S.E.). Data are shown as the mean ± S.E. for the analysis of amino acid standards ($n = 4$ replicate injections). The subscript numbers after an amino acid refer to the *m/z* signals used to quantify labeling (*i.e.* the M1/M0 ratio); these correspond to the $[M - 57]^+$ ion cluster of the *t*-butyldimethylsilyl derivative and therefore contain all carbon-bound hydrogens. Data from individual fasted or fed mice are shown as percent "Excess ^2H Labeling" after subtracting the background labeling. Note that "Arg" was included in the standard mixture but could not be detected, and although "Cys" and "Trp" could be detected in the standard mixture, they were not detected (ND) in the biological samples.

Amino acid background labeling ((M1/M0) × 100)	Excess ^2H labeling							
	$Time_{45\ min}$		$Time_{90\ min}$		$Time_{180\ min}$		$Time_{360\ min}$	
	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed
	%		%		%		%	
$Ala_{261/260}$ (22.94 ± 0.05)	12.21	11.21	12.73	10.77	11.43	12.62	11.16	11.87
$Gly_{247/246}$ (22.59 ± 0.02)	6.15	4.49	6.42	4.62	5.76	5.28	5.63	6.02
$Val_{289/288}$ (25.22 ± 0.03)	1.22	0.68	1.27	0.75	1.14	0.87	1.11	0.99
$Leu_{303/302}$ (26.83 ± 0.05)	1.89	0.52	1.97	1.37	1.77	1.70	1.73	1.49
$Ile_{303/302}$ (26.78 ± 0.02)	2.05	1.58	2.14	1.64	1.92	1.32	1.88	1.55
$Pro_{287/286}$ (25.13 ± 0.05)	2.34	1.44	2.44	2.10	2.19	1.96	2.14	2.43
$Ser_{391/390}$ (34.64 ± 0.09)	2.85	1.65	2.97	1.74	2.67	1.69	2.61	2.27
$Thr_{405/404}$ (25.91 ± 0.07)	0.54	0.18	0.57	0.20	0.51	0.37	0.50	0.38
$Met_{321/320}$ (25.63 ± 0.03)	2.21	1.28	2.31	1.51	2.07	0.98	2.02	1.89
$Asp_{419/418}$ (35.89 ± 0.07)	1.86	0.91	1.94	1.34	1.74	1.66	1.70	2.08
$Phe_{337/336}$ (29.41 ± 0.09)	0.19	0.06	0.20	0.14	0.18	0.14	0.18	0.16
$Cys_{407/406}$ (35.32 ± 0.08)	ND	ND	ND	ND	ND	ND	ND	ND
$Glu_{433/432}$ (37.74 ± 0.05)	11.33	8.01	11.90	10.90	11.48	10.63	11.19	10.32
$Lys_{432/431}$ (39.27 ± 0.11)	0.13	0.02	0.13	0.05	0.12	0.08	0.12	0.05
$Asn_{418/417}$ (36.06 ± 0.09)	1.35	0.41	1.40	0.97	1.26	0.94	1.23	0.87
$Gln_{432/431}$ (37.82 ± 0.12)	10.18	6.52	10.66	7.50	9.47	9.44	9.23	8.44
$Tyr_{467/466}$ (41.21 ± 0.09)	0.54	0.27	0.57	0.29	0.51	0.38	0.50	0.43
$His_{441/440}$ (38.12 ± 0.10)	4.10	2.93	4.06	2.47	3.86	3.08	3.78	2.99
$Trp_{456/455}$ (41.34 ± 0.09)	ND	ND	ND	ND	ND	ND	ND	ND

TABLE II

Isotopic labeling ratios of LGEYGFQNAILVR

MALDI-TOF analyses of albumin were performed using samples from control mice (given an intraperitoneal bolus of saline) and mice given ^2H_2O or $H_2{}^{18}O$, and samples were collected 8 h postinjection. Replicate analyses of each sample ($n = 3$ analyses per mouse) were performed following tryptic digestion, and data from each mouse were averaged and expressed as the mean ± S.E. per group ($n = 5$ mice per group). Note that the body water labeling was 3.11 ± 0.08% ^2H and 3.29 ± 0.11% ^{18}O. M0 refers to the monoisotopic form of the peptide, *i.e.* all ^{12}C, ^1H, ^{16}O, ^{14}N, etc., whereas M1 and M2 refer to peptide containing one or two substituted heavy isotopes, respectively.

Experimental group	Isotopic labeling ratios of "LGEYGFQNAILVR"	
	M1/M0 × 100	M2/M0 × 100
Control	86.79 ± 0.57	41.73 ± 0.42
^2H_2O	89.82 ± 0.28	44.61 ± 0.30
$H_2{}^{18}O$	86.36 ± 0.85	44.35 ± 0.35

approximately four carbon-bound ^2H atoms (10–13). The same logic would apply when $H_2{}^{18}O$ is used; *i.e.* protein synthesis acts as a polymerization reaction, incorporating multiple copies of ^{18}O from body water.

Since our initial reports regarding the use of ^2H_2O in studies of protein synthesis (17, 35), other investigators have also administered ^2H_2O and measured labeling profiles of proteins and/or peptides (33, 36). The work by Cabral *et al.* (36) is particularly elegant in that attention was directed toward determining the rise to steady-state labeling of glutathione; the fractional synthesis rate was determined by fitting the labeling curve. They noted that the asymptotic labeling, which reflects the precursor labeling, only reached ~60% of the maximal (or theoretical) value. Namely, given the structures of the amino acids incorporated into glutathione, they expected that the steady-state labeling could be ~10 times that of body water because there are 10 carbon-bound hydrogens. Cabral *et al.* (36) found that ~6 of the hydrogens are labeled. This incomplete labeling of amino acids is not surprising because all positions may not equilibrate equally with hydrogen (or deuterium) in body water. For example, Commerford *et al.* (37) demonstrated heterogenous labeling of amino acids in rodents given ^3H_2O. In another recent report, Xiao *et al.* (33) measured the ^2H labeling of albumin that was isolated from rats maintained on ^2H_2O for several days. They also concluded that one could use ^2H_2O to estimate protein biosynthesis by measuring changes in the average mass of a peptide. Although Xiao *et al.* (33) utilized a different mathematical approach as compared with Cabral *et al.* (36), their report is particularly interesting in that they observed comparable rates of albumin synthesis using three different tryptic peptides. Xiao *et al.* (33), like Cabral *et al.* (36), demonstrated that the apparent precursor labeling (or "*n*," *i.e.* the number of ex-

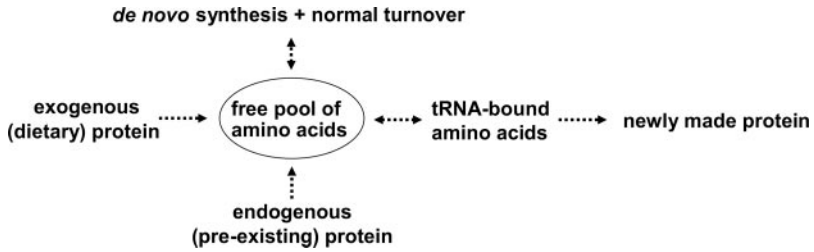

FIG. 5. **Processes that affect amino acid labeling in presence of labeled water.** Amino acids are generated via *de novo* synthesis pathways and/or during the degradation of existing proteins either of dietary origin or that are already present in the organism. In addition, tRNA-bound amino acids are reversibly cleaved back to free amino acids before incorporation into newly made proteins. The hypothesis is that ^{2}H will primarily label amino acids via *de novo* synthesis and complete turnover, whereas ^{18}O will label the oxygen incorporated in the peptide bonds via all pathways before incorporation into new proteins.

changeable hydrogens) is lower than the theoretical maximum. Despite the fact that Xiao *et al.* (33) provide a clear explanation of the calculations, a major assumption with their approach centers on whether the "*n*" remains constant when metabolic and/or physiological conditions change, *e.g.* during feeding (Table I). For example, changes in the average mass of the peptide reflect (i) the labeling of body water, (ii) the number of hydrogens ("*n*") that exchange with ^{2}H, and (iii) the fractional synthesis rate. The approach taken by Xiao *et al.* (33) is valid in cases where "*n*" is constant (and/or known); otherwise, the average mass of the peptide reflects the combined changes caused by variable precursor labeling and protein synthesis. In total, the studies by Cabral *et al.* (36) and Xiao *et al.* (33) and those by our group (17, 32) support the hypothesis that it is possible to measure proteome dynamics *in vivo* using $^{2}H_2O$ (at least in studies that last several days); however, one must account for the variable ^{2}H labeling of the different amino acids.

Contrary to amino acid flux in $^{2}H_2O$, hydrolytic cleavage of peptide (amino acid) bonds and of tRNA-bound amino acids (back to free amino acids) in $H_2{}^{18}O$ results in the immediate ^{18}O labeling of carboxyl oxygen (Fig. 5) (18–21). This is especially important in cases where one expects the rapid entry of unlabeled amino acids, *e.g.* during a meal. If peptides contain amino acids with side chain ^{18}O atoms that are derived from water (*e.g.* aspartate and glutamate), one should consider the potential for non-homogeneous labeling; *e.g.* the oxygen in the peptide bond could be labeled to a greater degree than the oxygen in the amino acid side chain. The latter becomes labeled with the complete turnover of the amino acid and/or via de- and reamination reactions; *e.g.* deamination of glutamine in the presence of $H_2{}^{18}O$ will generate [γ-^{18}O]glutamate, which generates [γ-^{18}O]glutamine during reamination (we expect that asparagine and aspartate would undergo a similar series of reactions). Finally, because one expects that a single ^{18}O atom is incorporated per peptide bond and because ^{18}O should label all proteogenic amino acids equally well, assumptions regarding heterogeneity of the precursor "*n*" and its labeling stability should prove more reliable. In theory, this last point would make $H_2{}^{18}O$ a preferred tracer in short term studies of protein synthesis, *i.e.* in cases where the precursor labeling cannot be determined from the asymptotic labeling of the product (Equation 2 *versus* Equation 1).

To examine the utility of using labeled water in studies of protein synthesis *in vivo*, we first initiated a chronic labeling study to measure albumin synthesis. Body water was maintained at steady-state ^{2}H or ^{18}O labeling for 11 days by simply giving a bolus injection of the respective tracer followed by the addition of that tracer to the drinking water. That we observed different asymptotic values for the labeling of peptide LGEYGFQNAILVR (Figs. 3 and 4) is expected because (i) we achieved slightly greater labeling of ^{2}H in body water *versus* ^{18}O and (ii) a larger number of ^{2}H atoms are incorporated when $^{2}H_2O$ is used *versus* ^{18}O when $H_2{}^{18}O$ is used. However, when the rise to steady state was modeled, we observed comparable fractional rate constants, *i.e.* ~0.33 *versus* 0.30/day in $^{2}H_2O$ *versus* in $H_2{}^{18}O$, respectively (Fig. 3). Those values are in agreement with the rate constant that we observed when we measured the incorporation of ^{2}H-labeled alanine into plasma albumin over a similar period using gas chromatography-mass spectrometry methods (35). In addition, the values observed in mice are ~10-fold greater than what we have observed in humans (given $^{2}H_2O$) (11), which is expected because metabolic rates in mice are ~10-fold greater than in humans. Taken together, these chronic labeling studies suggest that either type of labeled water can be used to quantify integrative rates of protein synthesis, *i.e.* in free living subjects over a prolonged period.

The ability to answer a question such as *can one measure protein synthesis several hours after administering labeled water?* requires precise measurements of the product labeling (to differentiate unlabeled from labeled peptides) and knowledge of the precursor labeling (see Equation 2). Because we (16, 17) and others (33, 36, 38) have demonstrated reasonable precision of the mass spectrometry-based measurements of peptides (Fig. 2), we examined the time-dependent changes in the ^{2}H labeling of amino acids in the fasted *versus* the fed state (Table I). Several reactions can affect amino acid labeling, including (i) the *de novo* synthesis of an amino acid, (ii) the digestion of dietary protein, (iii) the degradation of existing endogenous proteins, and (iv) the hydrolysis of tRNA-bound amino acids (Fig. 5). Consistent with previous reports (37), we

found that the number of exchangeable hydrogens varies considerably between the amino acids and that, in general, non-essential amino acids achieve a greater degree of labeling (as expected because ^2H can be incorporated at multiple sites within a given amino acid during *de novo* synthesis and normal turnover). Some ^2H labeling in essential amino acids is expected because they experience reversible metabolism; *e.g.* leucine undergoes transamination with α-ketoisocaproate (39). Finally, although the ^2H labeling of body water is in rapid equilibrium with the carbon-bound hydrogens of free alanine (10, 13), which is consistent with its role as a central metabolic intermediate (40), this is apparently not true for all amino acids (Table I). The observation of a transient perturbation in the ^2H labeling in fed *versus* fasted mice is consistent with what one expects because the digestion of dietary protein, and subsequent absorption of amino acids, will dilute the labeling of free amino acids. Therefore, the ability to measure protein synthesis via ^2H_2O during an acute challenge requires measurements of the time-dependent labeling of individual amino acids in the tissue where the protein of interest is being synthesized.

We suspect that our example represents a somewhat extreme scenario regarding changes in ^2H labeling in the fed state (Table I). Namely, because mice were trained to consume their normal daily ration of food in a 2-h interval, a relatively large bolus of food entered the system. Also, we specifically focused attention on liver amino acids because we were interested in studying albumin synthesis. Because amino acids are absorbed and enter the systemic circulation through the portal vein, the first pass of diet-derived amino acids will primarily affect the ^2H labeling of intrahepatic amino acids. Although knowledge of the equilibration can be obtained from long term studies (*i.e.* the asymptotic labeling of a peptide yields the precursor labeling), transient changes in amino acid labeling that occur with perturbations (*e.g.* eating), if unaccounted for, will lead to an underestimation of protein synthesis. By fitting the amino acid labeling profiles, it is possible to quantify albumin (protein) synthesis; *i.e.* by integrating the labeling curves for each amino acid, we could account for the non-steady-state amino acid labeling (6). Using those data we determined that 6.30 $\pm$ 0.59% of the plasma albumin was newly made within the 8 h during the acute perturbation in mice given ^2H_2O (Table II).

We hypothesized that although ^2H labeling may be relatively slow for some amino acids ^{18}O labeling of amino acids should be relatively fast for virtually all amino acids (18–21); thus, $H_2{}^{18}O$ might hold advantages over ^2H_2O. Namely, although the *de novo* synthesis of an amino acid should result in maximal ^2H labeling, the generation of amino acids during protein breakdown (derived from endogenous or exogenous sources) requires extensive turnover of the unlabeled amino acids to achieve maximal ^2H labeling; *i.e.* the release of unlabeled amino acids during protein breakdown will dilute the ^2H labeling of newly made proteins if those amino acids do not rapidly turn over in the pool of intracellular amino acids. However, one expects immediate ^{18}O labeling of free amino acids (Fig. 5). We did not attempt to measure the ^{18}O labeling of the individual amino acids because some back-exchange of the label would likely occur during the sample preparation and therein confound the interpretation of the measurements (41, 42). Thus, to explain the protein synthesis data, we relied on knowledge of known pathways and enzymatic reaction mechanisms. Based on the chronic labeling study where we observed extensive incorporation of ^{18}O into albumin (Figs. 3 and 4) and based on the logic outlined in Fig. 5, we expect that $H_2{}^{18}O$ should equilibrate more rapidly with amino acids as compared with ^2H_2O, and in most cases we expect that one ^{18}O atom is incorporated per peptide bond (the exceptions being those amino acids that contain additional side chain oxygen atoms that are derived from water, *i.e.* asparagine, glutamine, and glutamate in peptide LGEYGFQNAILVR). As with mice given ^2H_2O, we detected the incorporation of ^{18}O into LGEYGFQNAILVR after 8 h of exposure to $H_2{}^{18}O$ in the fed state (Table II); rates of albumin synthesis were estimated by comparing the total labeling in LGEYGFQNAILVR with the theoretical expected labeling ($n = 16$) and were found to be 4.96 $\pm$ 0.66%, effectively the same as in mice given ^2H_2O.

Calculated rates of protein synthesis are ultimately sensitive to the relative turnover time of the protein of interest and the time course of amino acid labeling. Our observations of comparable rates of protein synthesis in the presence of ^2H_2O or $H_2{}^{18}O$ are consistent with the classic study of Rittenberg and co-workers (18). Although we could quantify the incorporation of ^2H and ^{18}O into albumin within several hours, we suspect that $H_2{}^{18}O$ offers advantages over ^2H_2O, especially when considering equilibration time and copy number. To circumvent potential problems regarding slow equilibration of amino acid labeling, we expect that one can either measure the time-dependent labeling of all amino acids (as we did in the mice given ^2H_2O) or use tandem mass spectrometry. For example, in our previous studies we determined the labeling of specific peptide-bound amino acids by fragmenting peptides (17). Therefore, tandem mass spectrometry can be used to quantify the labeling of peptide-bound amino acids with rapid/known labeling constants, and/or one can quantify the labeling in peptide fragment(s) with minimal side chain oxygen; *e.g.* because the amino acid sequence AILVR of LGEYGFQNAILVR contains only peptide bond oxygen, the precursor/product labeling ratio can be estimated with a high degree of confidence by simply measuring the ^{18}O labeling of body water. Finally, we expect that tandem mass spectrometry offers additional advantages in that one can better "purify" co-eluting peptides and improve the signal/noise ratio (17).

In summary, we demonstrated the ability to measure protein dynamics *in vivo* following the administration of either ^2H_2O or $H_2{}^{18}O$ (safe, non-radioactive isotopes), and we expect that it is possible to determine the rate of synthesis of

virtually any protein using this approach. Because these tracers are relatively easy to administer and the sensitivity of the proteomics assays allows measurements using small samples, our method should be applicable over a wide range of conditions. Future studies that rely on the use of ^2H_2O should presumably determine (i) the time-dependent labeling of amino acids in other tissues, (ii) the impact of protein intake on rates of equilibration, and (iii) the impact of health *versus* disease on amino acid labeling, whereas studies that use $H_2{}^{18}O$ should be less influenced by the concerns noted above. Finally, we consider the application of labeled water relative to SILAC methods (4, 43, 44). For example, one advantage of SILAC methods is that they allow investigators to obtain higher levels of product labeling because it is possible to "completely" label an amino acid substrate (using a highly substituted stable isotope of an amino acid, *e.g.* [$^{13}C_6$]lysine or [$^{13}C_6$]arginine) with the end result being that investigators can achieve a relatively high signal over a relatively low background labeling. However, there are several points worth noting regarding the widespread use of the SILAC method for *in vivo* studies. First, it is not possible to contrast protein dynamics in fed *versus* fasted animals; in fact, because the tracer is administered via the diet, all studies must be done in fed animals. Second, it is not possible to readily modify the diet as an experimental variable; *i.e.* each diet needs to be customized by adding the labeled amino acid. Third, the SILAC methods do not achieve a steady-state labeling in the precursor pool; therefore, the obvious problem that arises is that estimates of protein synthesis will be lower than the true value as noted by Krüger *et al.* (43).

We conclude with a few general remarks regarding the possible widespread application of our ^2H_2O or $H_2{}^{18}O$ approaches in true proteomics investigations. First, we assume that the method(s) reported herein can easily be implemented at most research centers. For example, one simply needs to capitalize on existing mass spectrometry infrastructure. Second, we recognize that the demands of measuring the isotopic distribution profile of a single peptide conflict with the imperative of identifying the largest number of peptides, making general liquid chromatography-mass spectrometry protocols somewhat unsatisfactory for determining rates of protein synthesis. However, we have found that it is possible to determine the mass isotopomer profile of a peptide with sufficient precision using the "zoom scan mode" (on a Finnigan ion trap) with multiple scans encompassing the chromatographic peak of an entire peptide.[2] Therefore, one might anticipate that the conflicting demands on the mass spectrometer acquisition parameters (*i.e.* full scan to identify peptides that are present and "zoom scan" on a desired peptide to precisely determine its labeling pattern) will limit protein turnover analyses to relatively smaller numbers of peptides/proteins than are present in the proteome. However, by generating a list of candidate peptides, it should be possible to determine water-based rates of protein turnover on multiple proteins for a typical liquid chromatography-mass spectrometry run. Alternatively, in cases where a complex matrix is obtained, the fractionation of protein classes or the isolation of targeted analytes can be used to enhance the application of this method (as demonstrated herein with a quick preparation of albumin). Last, we expect that it is possible to calculate rates of protein breakdown by coupling water-based rates of protein synthesis with existing strategies for quantifying protein abundance (31, 45–48). Namely, changes in protein content (which can be measured by a variety of techniques (31)) reflect the balance between protein synthesis and protein breakdown; as we have shown here, because it is possible to measure protein synthesis, investigators should be able to obtain a functional image of gene expression *in vivo*.

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§§ To whom correspondence should be addressed: Exploratory Biomarkers, Atherosclerosis, Merck, RY80Y-2D-03, 126 E. Lincoln Ave., Rahway, NJ 07065. Tel.: 732-594-3364; E-mail: stephen_previs@merck.com.

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