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Proteome Kinetics: Coupling the Administration of Stable Isotopes with Mass Spectrometry-Based Analyses

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1. Introduction

Proteins serve many purposes by acting as structural supports, receptors, signaling molecules and enzymes, in addition, they facilitate nutrient transport and maintain immunological responses. Although the concentration of a given protein may not change appreciably over a short interval, proteins are continuously remodeled. In this chapter we consider how to study protein kinetics. Attention is directed towards two critical areas which include (i) the logic behind using different tracers and (ii) how to design and execute experiments that are compatible with proteome-based analyses.

A practical illustration may highlight the importance of using isotope tracers to facilitate research in this area. For example, the concentration of circulating albumin provides a measure of protein nutritional status (and is a predictor of a patient's recovery from disease), however, since the fractional turnover of albumin is relatively slow (~ 3 to 5% of the pool is newly made per day) several weeks of an intervention may be required to affect plasma levels. Recognizing that the concentration of albumin is a delayed-onset marker of nutritional status, investigators have used isotope tracers to determine the acute response of plasma albumin synthesis to a dietary manipulation, accordingly, one can make predictions regarding the efficacy of an intervention. Such studies rely on straightforward experimental designs. Namely, an investigator first decides on what amino acid will be used (e.g. $^2\text{H}_3$ -leucine) and how will it be administered (e.g. a primed-constant infusion), samples are then collected for a given amount of time and a protein of interest (e.g. albumin) is isolated. Once isolated, the protein of interest is degraded (typically *via* acid hydrolysis) and the labeling of the free amino acid present in the plasma is compared to that of the amino acid that was bound in the protein, i.e. one determines the precursor:product labeling ratio. Although this scenario is relatively straightforward, our review considers the pros and cons surrounding the use of different tracers. In particular, we discuss recent advances in the use of stable

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isotope protocols that enable more flexible study designs, including the use of ^2H and ^{18}O -labeled water.

The second objective of this chapter aims to consider the utility of modern proteomic methods. For example, in the scenario described above, it is imperative that one purify the protein(s) of interest otherwise a study will reflect the kinetics of a mixture of proteins. Despite the fact that one can extensively purify proteins using immunoprecipitation, gel electrophoresis, etc. those approaches are typically labor intensive. Other methods, e.g. "shotgun" proteomics, can facilitate the resolution of complex mixtures with a minimum of time required for sample preparation, the trade-off is an increase in the amount of time required to process large data sets. It may not be obvious to investigators getting started in this area but the acquisition parameters that are often used in proteome-based studies are not necessarily compatible with the use of stable isotope-based flux protocols. In addition, investigators are often faced with questions such as, is one type of mass spectrometer "better" than another for determining the isotopomer profile? We discuss our experiences in estimating protein flux using proteome-based analyses.

In summary, proteome expression profiles contain information regarding differences between metabolic states yet they are typically of limited value when one aims to explain the nature of those differences. We consider approaches that should allow investigators to perform studies of proteome dynamics and therein move from static expression profiles towards kinetic/mechanistic studies. Where possible, attention is directed towards applications that can be used to advance the study of circulating proteins, especially those that relate to the field of lipoprotein kinetics. We apologize to investigators who do not have their work cited herein, where possible we have tried to identify papers that demonstrate necessary conceptual points and/or represent the initial publications in a given area.

2. Using stable isotope tracers to study protein synthesis and degradation

Rates of protein synthesis can be determined by administering a labeled precursor and then measuring its incorporation into a protein of interest (Figure 1) (Foster et al. 1993; Wolfe and Chinkes 2005). Assuming a simple model, in which there is a well-mixed pool of amino acids and a single product compartment, one can describe the kinetics using equation 1:

$$\text{protein labeling}_{\text{time}} = \text{protein labeling}_{\text{max}} \times (1 - e^{-\text{FSR} \times \text{time}}) \quad (1)$$

where $\text{protein labeling}_{\text{max}}$ represents the asymptotic labeling of a protein and FSR represents its fractional synthetic rate. By measuring the labeling at multiple points in time one can fit the curve and determine FSR. In cases where a steady-state labeling is not reached one typically estimates the kinetics using equation 2:

$$\text{FSR} = \text{pseudo-linear change in protein labeling} / (\text{precursor labeling} \times \text{time}) \quad (2)$$

We consider the following example to demonstrate the effect that timing of sample collection can have on estimating the FSR, in this case we have simulated the labeling of proteins with different FSRs (Figure 1). Panel A demonstrates that fitting an entire data set to Equation 1 yields the expected FSR. Panel B demonstrates a comparable fit of the data using reduced data sets, fitting the points obtained only at 4 hour intervals to Equation 1 yields the expected FSR. Note that it may not be practical to obtain extensive data sets in all cases (Figure 1A vs B), e.g. one may be limited in regards to blood or tissue sampling, as well,

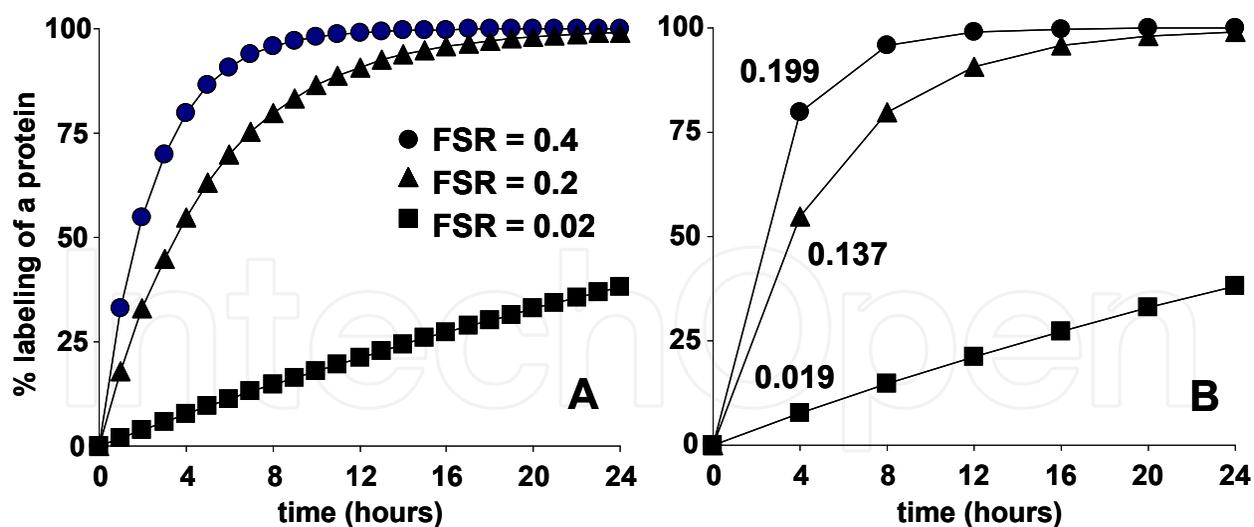


Fig. 1. Effects of modeling on data interpretation. Simulations were run to determine the effect(s) of calculation methods on apparent fractional synthetic rates. Panel A demonstrates a scenario wherein the protein labeling was simulated assuming three rate constants, i.e. FSR = 0.02, 0.2 and 0.4 per hour. Fitting all data points in a given curve using Equation 1 yields the expected rate constants. Panel B demonstrates the effect(s) of using various truncated data sets on apparent FSR. Again, fitting all data in a given curve to Equation 1 yields rate constants that closely agree with the expected values. However, it is possible to substantially underestimate the FSR when using single points, e.g. using Equation 2 and data obtained only at 4 hours leads to estimates of FSR equal to 0.199, 0.137 and 0.019 per hour, as compared to the expected values of 0.4, 0.2 and 0.02 per hour, respectively.

limiting the number of data points increases the throughput since fewer samples need to be analyzed. Panel B also demonstrates the effect of using Equation 2, for example, what would happen if we only obtained data 4 hours after administering a tracer? Clearly there is a reasonable estimate of FSR when the true value is relatively low (~ 0.02) but there is a sizeable underestimate of the FSR in cases where one expects it to equal ~ 0.4 and ~ 0.2 . Although the apparent FSR values reported in Panel B are different from the expected values (i.e. when using Equation 2 and the sample obtained at 4 hours), one can still identify differences between the curves, i.e. the expected FSR of 0.4 yields a value of 0.199 whereas the expected FSR of 0.2 yields a value of 0.137. The effect of this error becomes important in cases where one aims to determine the magnitude of an intervention. For example, there is a 2-fold difference between the true FSR values (i.e. 0.4 vs 0.2) yet the apparent FSR values only differ by ~ 1.5 -fold (i.e. 0.199 vs 0.137). Therefore, the timing of sample collection has important consequences on the interpretation of the data and the conclusions one may reach regarding physiological homeostasis.

Although the mathematics surrounding tracer kinetics have been described in detail (Foster et al. 1993; Wolfe and Chinkes 2005), there are certain caveats that apply in different fields. For example, investigators working in the area of lipoprotein kinetics have recognized the need to add delays in the modeling (Barrett, Chan, and Watts 2006; Foster et al. 1993; Patterson et al. 2002). Namely, although proteins such as apolipoprotein B are continuously synthesized within liver and/or intestine, they are not immediately secreted into the circulation. Consequently there is a lag time between the administration of a tracer and the appearance of labeled apolipoprotein B in the plasma. Foster *et al.* (Foster et al. 1993)

have elegantly outlined the rationale behind different mathematical treatments of a given data set, they demonstrate the impact of various assumptions in regards to the modeling of data on the apparent FSR. It is also important to note the differences when modeling data that are expressed as a tracer-to-tracee ratio vs isotopic enrichment, the former is commonly reported but the latter may be preferred in many instances (Cobelli, Toffolo, and Foster 1992; Ramakrishnan 2006; Toffolo, Foster, and Cobelli 1993).

A second major factor to consider regarding the logic that is applied in kinetic studies centers on heterogeneity in the product pool (note that there are concerns regarding heterogeneity in labeling of the precursor pool, those will be considered in more detail in Section 3) (Foster et al. 1993). To this point we have assumed a simple model in which there is a single pool of product molecules, however, investigators working in the area of lipoprotein kinetics readily recognize the existence of at least two pools of circulating apoB100, one that is associated with VLDL particles and another that is associated with LDL particles (Lichtenstein et al. 1990). While there has been some debate regarding whether or not LDL-apoB100 is made *de novo* or whether it is derived from the delipidation of VLDL it is clear that the labeling curves are dramatically different (Lichtenstein et al. 1990; Shames and Havel 1991). In a classical study, Lichtenstein *et al.* (Lichtenstein et al. 1990) demonstrated that the labeling of VLDL-apoB100 approaches a steady-state in ~ 15 hours whereas the labeling of LDL-apoB100 is still in the pseudo-linear range during the same interval; those studies also demonstrated that there are sizeable differences in the abundance of VLDL-apoB100 vs LDL-apoB100 (Figure 2).

What are the consequences of estimating the FSR of apoB100 from the total labeling, i.e. if one ignores the fact that a small amount of the protein is typically labeled much faster than the bulk pool of apoB? Consider the scenario outlined in Figure 2, the lumped fractional rate constant does not reflect either of the individual fractional rate constants. In addition, although directional changes in the lumped fractional rate constants reflect true changes, the magnitude is underestimated. On the contrary, the ability to measure the absolute flux rate (i.e. the mass of protein made per unit of time) allows one to draw conclusions regarding true changes in the flux, however, one is not able to determine the site of those changes (e.g. Was a single pool affected? If so, which one?). We consider how to estimate protein concentration later.

A final question to consider regarding protein kinetics is centered on quantifying protein breakdown (Figure 3). As noted above, the incorporation of a tracer into a protein of interest can be used to estimate the rate of synthesis, can one estimate the rate of protein breakdown by measuring the elimination of a tracer from a protein of interest? We believe that the answer is "no", or at the very least it is not as straightforward as reports in the literature (Bateman et al. 2006; Bateman et al. 2007). Readers should consider how measurements of isotopic labeling are typically performed and how data are expressed. For example, investigators often use a mass spectrometer to determine isotopic labeling and express data as the ratio of labeled to unlabeled molecules (or the percentage of labeling, i.e. the labeled molecules divided by the sum of labeled and unlabeled molecules) (Dwyer et al. 2002; Lichtenstein et al. 1990; Magkos, Patterson, and Mittendorfer 2007). We agree that in cases where one infuses a labeled amino acid for a given time and then stops the infusion of the tracer that there will be a decrease in the labeling of a given protein over time (Figure 3) (Bateman et al. 2006; Bateman et al. 2007). However, assuming that protein breakdown is a random process, i.e. protein breakdown does not discriminate between labeled and unlabeled molecules, the ratio of labeled to unlabeled protein molecules will not change as

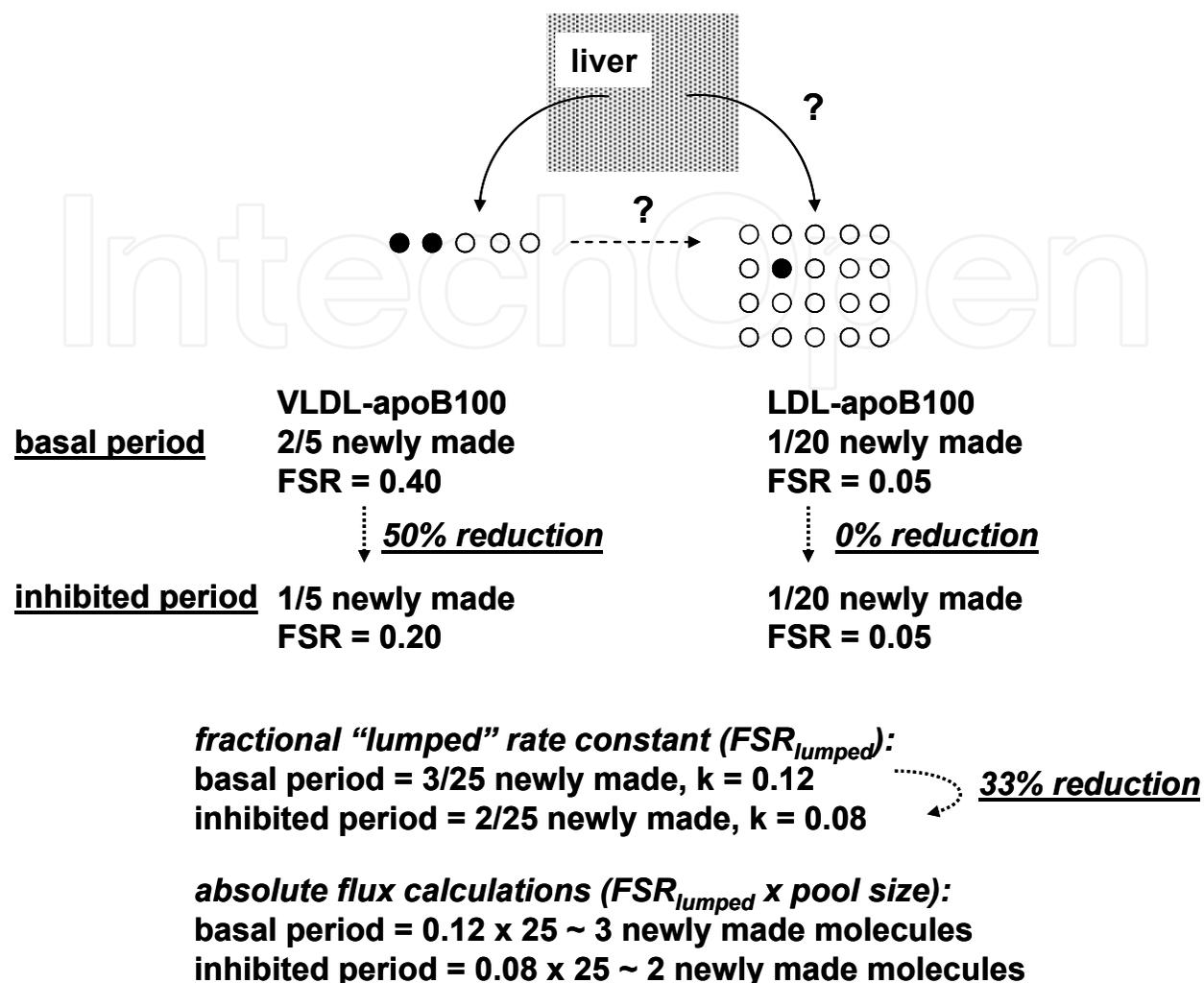


Fig. 2. Effect(s) of lumping pools. Simulations were run to determine the impact of treating a mixed pool as single compartments (open circles represent unlabeled proteins and solid circles represent labeled proteins). For example, apoprotein-B100 is found in VLDL and LDL particles in the plasma. The mass of apoB100 is ~ 5 to 40 times different between these compartments, as well the FSR is considerably different. Assume that VLDL-apoB100 has an FSR of ~ 0.4 and a pool size of ~ 5 molecules whereas LDL-apoB100 has an FSR of ~ 0.05 and a pool size of ~ 20 molecules. If one isolated the individual apolipoprotein pools the aforementioned values would be obtained, however, if one isolated total apoB100 from the plasma the fractional "lumped" synthesis rate would equal ~ 0.12 (3 out of 25 molecules). Now, assume an inhibitor of VLDL-apoB100 is added such that the FSR of VLDL-apoB100 decreases to ~ 0.2 (for simplicity, assume a parallel change occurs in protein degradation so that the pool size remains constant). If one isolates total apoB100 from plasma the fractional "lumped" synthesis rate would equal ~ 0.08 (2 out of 25 molecules). Clearly one would observe a decrease in synthesis but the true effect is substantially underestimated (i.e. the true reduction is 50% in VLDL-apoB100 vs 33% reduction detected in total apoB100). Accounting for the pool size, however, allows one to reliably determine the true change in apoB100 synthesis, i.e. a total 3 molecules are newly made during the basal period vs 2 during the inhibited period.

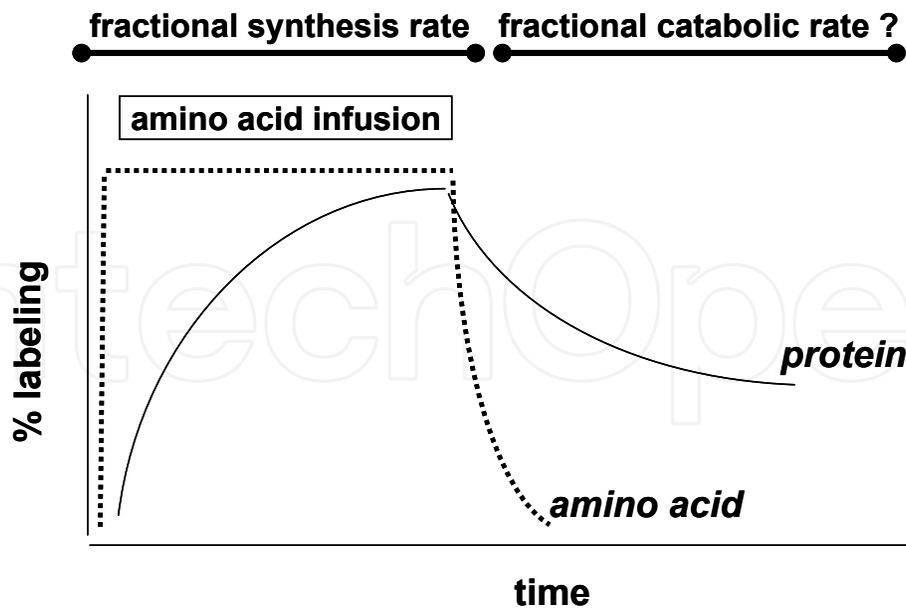


Fig. 3. Tracer-based estimates of protein breakdown. During the infusion of a labeled amino acid (dotted line) one can estimate protein synthesis by determining the change in protein labeling (solid line). Following the infusion of a labeled amino acid one expects a "washout" or a decrease in the labeling. As shown here, however, the rate at which the protein labeling decreases is dependent on the rate of protein synthesis and not protein breakdown. Note that the y-axis is expressed as "% labeling" (consistent with reports in the literature). A major assumption of any tracer method is that the tracer and tracee are indiscriminately metabolized, therefore, after one stops administering a labeled precursor amino acid the labeling in the protein can only decrease if new protein is made in absence of labeled precursor amino acids.

the protein is degraded; the labeling decreases because new proteins are being made from unlabeled precursors (Previs et al. 2004;Waterlow 2006).

We believe that it is possible to estimate protein breakdown using the following logic, changes in the abundance of a protein equal the rate of synthesis minus the rate of breakdown. Protein breakdown can be determined by measuring the abundance of a protein and estimating the rate of synthesis, i.e. one solves the equation for protein breakdown (Bederman et al. 2006). Section 4 considers the merits of different approaches for measuring the abundance of a protein. It should also be emphasized that the ability to measure the abundance of a protein is important in cases where one aims to determine a rate of flux (i.e. the mass of protein that is being renewed per unit of time). For example, to this point we have focused on measuring a fractional rate constant (or a percent of a pool that is turned over per unit of time), one can calculate the absolute amount of newly made protein per unit of time by multiplying the FSR by the pool size (i.e. concentration multiplied by the volume of distribution). In studies of apolipoprotein kinetics, the pool size is typically assumed to equal the plasma volume which is estimated to be 4.5% of body weight (Lichtenstein et al. 1990;Magkos, Patterson, and Mittendorfer 2007). In cases where one aims to study the kinetics of other circulating proteins, for example albumin, it may be necessary to account for distribution between the extravascular and intravascular spaces (Sigurdsson, Shames, and Havel 1981;Wasserman, Joseph, and Mayerson 1955).

3. How can I label the precursor pool?

Our discussion of protein synthesis is entirely focused on the logic of using precursor:product labeling ratios to estimate rates of flux, we are not examining cases in which one injects a pre-labeled protein and then measures its kinetics. Therefore, one should consider how to label the amino acid building blocks used in protein synthesis (Figure 4). Perhaps the most obvious design that comes to mind centers on administering a labeled amino acid (Dudley et al. 1998;Lichtenstein et al. 1990), however, investigators have also administered other labeled precursors (e.g. ^{13}C -glucose, $^2\text{H}_2\text{O}$ and H_2^{18}O) (Bernlohr 1972;Bernlohr and Webster 1958;Borek, Ponticorvo, and Rittenberg 1958;Busch et al. 2006;De Riva et al. 2010;Rachdaoui et al. 2009;Rittenberg, Ponticorvo, and Borek 1961;Vogt et al. 2005;Wykes, Jahoor, and Reeds 1998). Before discussing the merits of specific approaches we briefly consider the mode of administering the labeled precursor, e.g. a labeled amino acid can be administered as a primed-constant infusion or a single bolus injection (Dwyer et al. 2002;Lichtenstein et al. 1990;Wolfe and Chinkes 2005).

The general logic behind the primed-constant infusion is that one can instantaneously achieve and then maintain a steady-state labeling of the precursor pool (Lichtenstein et al. 1990), whereas a single bolus injection is typically associated with a wave (or pulse) of labeling (Dwyer et al. 2002). A concern with using a primed-constant infusion is that one must have catheterized subjects, while certainly feasible in human studies this is not as practical in many pre-clinical models (especially in drug discovery programs where large numbers of compounds are routinely screened). However, a pro of the primed-constant infusion centers on the degree of product labeling that can be achieved, this can be rather dramatic in studies of apolipoprotein kinetics. For example, when investigators have administered $^2\text{H}_3$ -leucine using a primed-constant infusion the plasma pool can be enriched to nearly 10% for several hours (Lichtenstein et al. 1990). Although some proteins have a rapid turnover others are labeled to a much lesser degree, e.g. the FSR of VLDL-apoB100 and HDL-apoA1 are in the range of ~ 5 and ~ 0.2 pools per day and the labeling typically approaches 7% and 0.75%, respectively.

In contrast to a primed-infusion, when administering a single bolus of $^2\text{H}_3$ -leucine the labeling of VLDL-apoB100 and HDL-apoA1 approaches $\sim 2.5\%$ and $\sim 0.25\%$, respectively (Dwyer et al. 2002). These differences in protein flux impact the isotopic labeling and have important implications on the analytical methods that are used to measure the enrichment. One might be able to enhance the use of a bolus injection method by choosing (i) an essential amino acid and/or (ii) an amino acid with a relatively long half-life. For example, one expects less dilution of essential amino acids since they can only be produced by one source (protein breakdown and not *de novo* synthesis), as well, compared to some non-essential amino acids (which participate in rapid inter-organ nitrogen transport) the $t_{1/2}$ of essential amino acids can be relatively slow. It is not surprising that ^{13}C -lysine has been used to make SILAC models (Kruger et al. 2008), since lysine is needed in relatively small amounts complete substitution of unlabeled lysine for ^{13}C -labeled lysine can be managed. The same types of experiments with ^{13}C -alanine would probably be of limited value since alanine is rapidly turned over and it sits at a highly branched point in intermediary metabolism (Wykes, Jahoor, and Reeds 1998). Nevertheless, in limited cases ^{13}C -glucose has been used to quantify protein synthesis (Figure 4). For example, ^{13}C -glucose is converted ^{13}C -pyruvate which readily equilibrates with alanine to yield ^{13}C -alanine, entry of ^{13}C -pyruvate into the citric acid cycle will generate other ^{13}C -labeled amino acids *via* comparable equilibration reactions (Vogt et al. 2005;Wykes, Jahoor, and Reeds 1998).

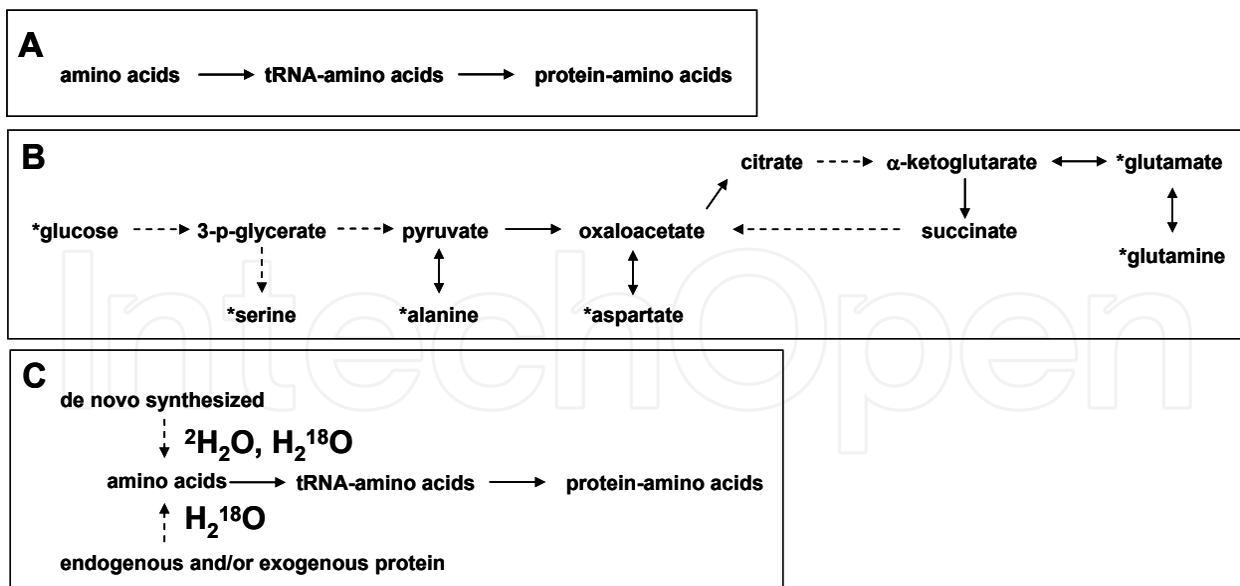


Fig. 4. Approaches to labeling amino acids. Panel A considers a straightforward method in which a labeled amino acid is administered. Panel B considers a scenario in which labeled glucose is administered; glycolytic metabolism will lead to the labeling of several amino acids. Note that an abbreviated metabolic scheme is shown to emphasize certain points of exchange, other amino acids can become labeled as well. Panel C considers the administration of labeled water. In cases where $^2\text{H}_2\text{O}$ is administered it is expected that *de novo* synthesized amino acids will be labeled, as well, amino acids derived from protein breakdown will be labeled provided that amino acid turnover is faster than the rate of amino acid incorporation into newly made protein. In cases where H_2^{18}O is administered one expects “instantaneous” labeling of amino acids regardless of their origin.

Another stable isotope that has seen substantial use is ^{15}N -glycine. Historically, this tracer was administered and the excretion of ^{15}N -urea and/or ^{15}N -ammonia was used to estimate the rate of whole-body nitrogen flux (San Pietro and Rittenberg 1953a; San Pietro and Rittenberg 1953b). Note that although investigators administer ^{15}N -glycine, the isotope rapidly mixes (or equilibrates) with other amino acid bound nitrogens which is the rationale for using it to trace “total” nitrogen flux (Matthews et al. 1981; Stein et al. 1980). More recently investigators have fed ^{15}N -labeled diets to animals in an effort to generate heavily labeled proteins that could then be used as internal standards to quantify protein concentrations in other subjects (MacCoss et al. 2005). In clever studies, Price *et al.* (Price et al. 2010) and Zhang *et al.* (Zhang et al. 2011) fed mice ^{15}N -labeled diets and were then able to estimate proteome turnover. The advantage of feeding ^{15}N -labeled diets as compared to a single labeled amino acid (e.g. ^{13}C -lysine) is that numerous protein-bound nitrogens will be labeled, therein increasing the window when measuring shifts in the isotope distribution of a proteolytic-peptide.

A final approach to label the precursor pool centers on the administration of labeled water, either $^2\text{H}_2\text{O}$ or H_2^{18}O (Figure 4C) (Cabral et al. 2008; De Riva et al. 2010; Kombu et al. 2009; Rachdaoui et al. 2009; Xiao et al. 2008). The rationale is that cells will generate labeled amino acids in the presence of labeled water, e.g. ^2H -labeling can occur *via* transamination and/or *de novo* synthesis. In contrast to the generation of ^{13}C -labeled amino acids from ^{13}C -glucose, which does not label essential amino acids, in the presence of $^2\text{H}_2\text{O}$ one can

observe ^2H -labeling of essential amino acids (Herath et al. 2011a). Namely, although essential amino acids are not made in a net sense (i.e. ^{13}C -glucose does not yield ^{13}C -leucine), transamination of leucine in $^2\text{H}_2\text{O}$ will label the α -hydrogen. Despite the fact that studies based on the use of labeled water revolutionized our understanding of metabolic biochemistry nearly 80 years ago there appears to have been a dramatic shift away from the use of labeled water in the field of protein dynamics for reasons that remain unclear (Borek, Ponticorvo, and Rittenberg 1958; Schoenheimer and Rittenberg 1938; Ussing 1938; Ussing 1941; Ussing 1980).

We, and others, have recently revisited the use of $^2\text{H}_2\text{O}$ in studies of protein synthesis (Busch et al. 2006; Cabral et al. 2008; De Riva et al. 2010; Kombu et al. 2009; Previs et al. 2004; Rachdaoui et al. 2009; Xiao et al. 2008), we also recognized the potential advantage(s) of using H_2^{18}O (Bernlohr 1972; Bernlohr and Webster 1958; Borek, Ponticorvo, and Rittenberg 1958; Rachdaoui et al. 2009; Rittenberg, Ponticorvo, and Borek 1961). Our use of H_2^{18}O was based on a classical study in which Rittenberg and colleagues demonstrated that H_2^{18}O could be used to study protein synthesis (the outstanding contributions of Bernlohr and others further tested the approach and more clearly outlined the logic) (Bernlohr 1972; Bernlohr and Webster 1958; Borek, Ponticorvo, and Rittenberg 1958; Rittenberg, Ponticorvo, and Borek 1961). Unlike $^2\text{H}_2\text{O}$ which labels amino acids in a less uniform manner, H_2^{18}O is expected to label virtually all amino acids to a similar degree. For example, oxygen in the carboxylic group can be labeled during *de novo* production, the degradation of proteins and/or the activation of amino acids (Figure 4C). Indeed, modern quantitative proteomic methods rely on this logic *albeit* for a different purpose, i.e. proteolytic cleavage in the presence of H_2^{18}O leads to the generation of ^{18}O -labeled peptides (Miyagi and Rao 2007; Yao et al. 2001).

One point to consider when thinking about using different tracers, e.g. $^2\text{H}_3$ -leucine vs H_2^{18}O vs $^2\text{H}_2\text{O}$, is the background labeling over which one measures the incorporation. Since these are all stable isotopes one needs to contend with background labeling, e.g. naturally occurring ^{13}C and ^{15}N account for $\sim 1.1\%$ and $\sim 0.4\%$ of all carbon and nitrogen, respectively, and make substantial contributions to the isotope profile over which one measures excess labeling from the administered tracer (note that other isotopes also affect the background labeling but to a lesser degree since they are present at lower abundance (e.g. ^2H , ^{17}O and/or ^{18}O) and/or are less prevalent (e.g. ^{33}S or ^{34}S) in various proteins. The use of heavily substituted precursors, e.g. $^2\text{H}_3$ -leucine, could be advantageous since the background labeling is lower at the $M+3$ isotopomer whereas the use of $^2\text{H}_2\text{O}$ and H_2^{18}O typically requires that one measure shifts in the $M+1/M0$ and $M+2/M0$ ratios, respectively (where the background labeling can be considerably higher). Consequently, the impact of analytical error is expected to be somewhat worse when measuring the $M+1/M0$ ratio vs the $M+3/M0$ ratio since the background is higher. One can minimize the effect of analytical error by administering more tracer and/or relying on the fact that multiple copies of a precursor are incorporated into a given protein (e.g. it is possible to incorporate more copies of ^2H from body water as compared to $^2\text{H}_3$ -leucine). These points are explained below in more detail. Last, in cases where one administers a pre-labeled amino acid (e.g. $^2\text{H}_3$ -leucine) one is immediately limited when quantifying protein synthesis since it is necessary to identify those peptides that contain the designated amino acid (e.g. $^2\text{H}_3$ -leucine). In contrast, when using a more general tracer, e.g. $^2\text{H}_2\text{O}$ or H_2^{18}O , it is possible to quantify protein synthesis *via* the labeling of various proteolytic peptides.

4. What should I consider when measuring the labeling of a protein?

In studies of protein synthesis one needs to compare the labeling of the product with that of the precursor. Although this section is primarily centered on the application of proteomic-based analyses for measuring the former, we will first briefly consider measurements of precursor labeling.

Several methods have been developed to measure the labeling of free amino acids; presumably, GC-quadrupole-MS-based methods are so commonplace since the hardware was readily available during the early 1980s when the use of stable isotopes began to dominate the literature (Matthews et al. 1980). In addition, these instruments have reasonable spectral accuracy therein allowing reliable estimates of isotope distributions. Typical protocols require a purification step (often using ion exchange chromatography) followed by derivatization prior to GCMS analyses. Although there are pros and cons to the generation of different derivatives (e.g. tertbutyldimethylsilyl vs N-acetyl-n-propyl, vs oxazolinone derivatives) (Dwyer et al. 2002;Matthews et al. 1980;Patterson, Carraro, and Wolfe 1993) it is clear that excellent precision of the isotope ratios can be achieved using standard equipment, for example, the coefficient of variation in the measured isotope ratios is often $\leq 1.0\%$, ensuring a certain degree of confidence when measuring the labeling of free amino acids. In cases where one decides to administer either $^2\text{H}_2\text{O}$ or H_2^{18}O (and therein allow the subject to generate labeled amino acids) it is necessary to measure the ^2H - or ^{18}O -labeling of water (Rachdaoui et al. 2009). Historically, IRMS was used to measure water labeling, however, simple and robust GC-quadrupole-MS-based methods are available for measuring the ^2H and ^{18}O -labeling of water (Brunengraber et al. 2002;Shah et al. 2010;Yang et al. 1998).

So then, how can investigators couple isotope tracers with proteomic-based analyses? In our experience we have generally faced two major issues when addressing this question. First, how reproducible are the mass spectrometer-based measurements? Second, what type of instrument is the best? Although the two questions are somewhat related we will consider them separately.

During our earlier work we considered alternative approaches to processing the raw data (Cassano et al. 2007;Wang et al. 2007). For example, our initial studies were conducted with a mostly out-of-date Bruker MALDI-ToF, we devised a strategy in which we would download the raw data and then fit the isotopic distributions to a series of Gaussian peaks (this was done using the commercially available software package "Origin"). One reason for devising this approach centered on the fact that the relatively low resolution achieved on the isotope peaks was not easily integrated using the instrument's software. Please note that the statements made here are not intended to reflect poorly on any vendor, in our previous academic experiences we simply had limited access to state-of-the-art equipment. In developing our earlier work (Cassano et al. 2007;Wang et al. 2007), we performed numerous simulations to ensure the reliability of our approach for integrating the data and therein evaluating how the quality of the primary data would impact the results of the fitting routine, we consider two examples that may be of interest (Figure 5).

Briefly, simulations were run in which 3 Gaussian shaped peaks were generated (e.g. M0, M1 and M2 ions), noise was added using the random number generator in MS Excel; the expected ratios for M1/M0 and M2/M0 were set at 70% and 30%, respectively, and the resolution was set at $\sim 30\%$ valley between peaks (this resolution setting was chosen since it compared with what we had observed on the older Bruker MALDI-ToF, which did not

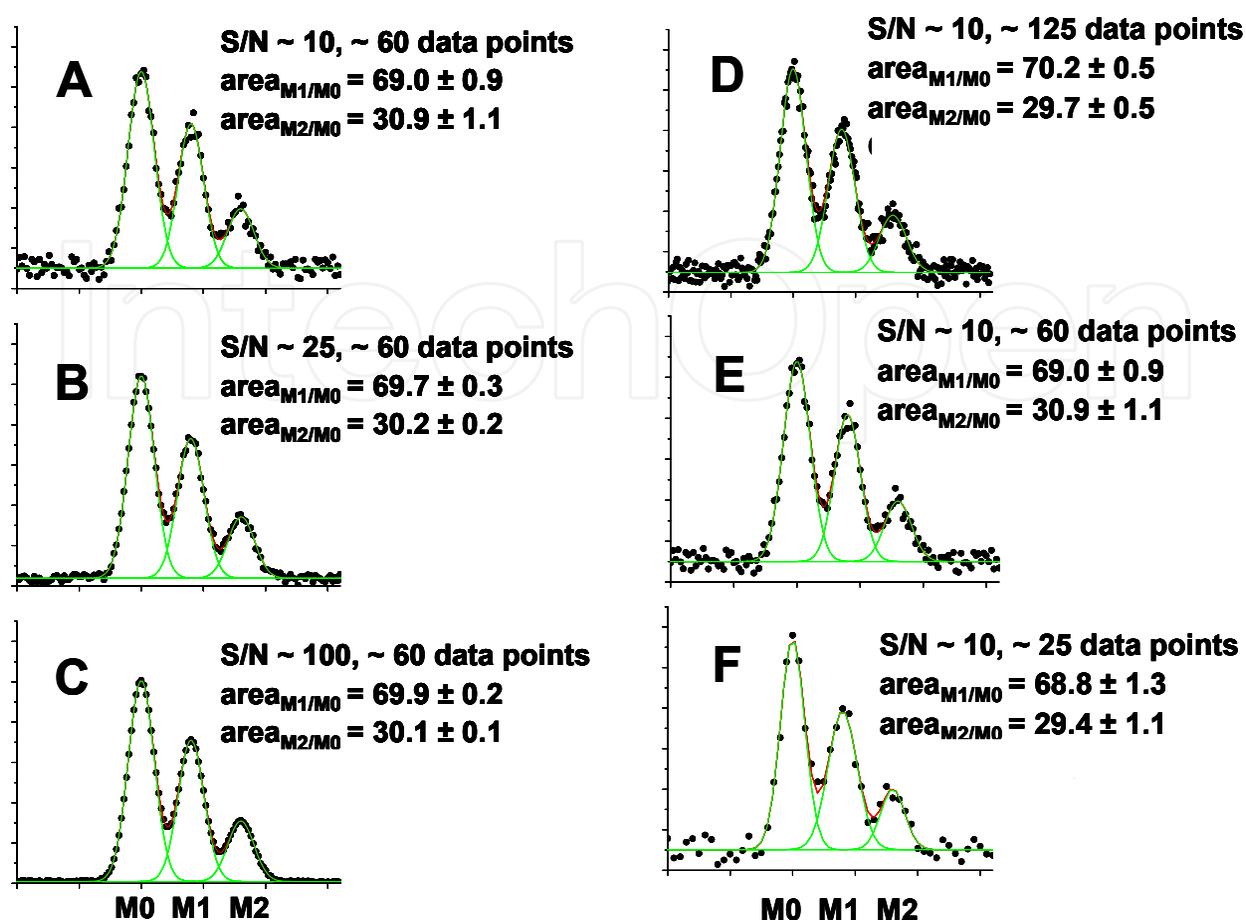


Fig. 5. Determining isotopic distributions. Simulations were run to determine the effect(s) of data quality and fitting on the calculated isotope ratios. In all cases 3 Gaussian shaped peaks were generated (e.g. M0, M1 and M2 ions); noise was added using the random number generator in MS Excel, the expected ratios for M1/M0 and M2/M0 are 70% and 30%, respectively, and the resolution was set at ~ 30% valley between peaks (note that this resolution setting was chosen for our simulations since it corresponded with the data that we were obtaining with an older Bruker MALDI-ToF when run in a linear mode, a somewhat worst-case scenario). The simulated data were exported to Origin and fitted assuming a Gaussian model, each simulation was run 5 times, data are expressed as mean \pm sem of the measured ratios. In Panels A, B and C we maintained a constant number of data points across the M0, M1 and M2 cluster (~ 60 data points) and we varied the S/N. In panels D, E and F, we maintained a constant and relatively low S/N (~ 10) and varied the number of data points. In all cases, there is reasonably good agreement between the measured and expected ratios.

always cooperate when run in the reflectron, or high-resolution, mode). In each example ~ 60 data points were observed across the 3 peaks, each simulation was run 5 times and data are expressed as mean \pm sem of the measured ratios (Figure 5A, B and C). The study demonstrates that our integration method yields a reliable quantification of isotopomer profiles, in all cases there was good agreement between measured:expected ratios. This study is especially useful since protein analyses typically have to contend with peptides at different abundance, e.g. a given digest may contain peptides at S/N ~ 10 whereas others

may be present at $S/N \sim 100$. Thus, we can estimate the level of confidence when determining the isotopic profiles of peptides with low vs high S/N . Although this example implies that a somewhat wide range of abundances can be used to estimate protein labeling, we suggest that it is best to focus quantitations on those peptides that are in greatest abundance since the precision generally improves.

A second scenario to consider in regards to data processing centers on the number of points that one observes across a series of peaks, this can be affected by various factors including the amount of sample that is analyzed, the type of mass analyzer and the analog-to-digital conversion rate. Our previous work mostly relied on the analyses of relatively pure samples, consequently, we primarily used MALDI-ToF (Rachdaoui et al. 2009). In our current work we almost exclusively rely on LC-MS since less purification is required prior to analysis (Kasumov et al. 2011; Zhou et al. 2011). Since one expects that coupling LC to a “discriminating” mass analyzer (e.g. a quadrupole) will reduce the number of data points that are used to describe a peptide’s isotopomer profile we ran simulations to determine how the number of data points would affect the fitting/quantitation of the isotopic profile (Figure 5D, E and F). As in the previous example, the expected values of $M1/M0$ and $M2/M0$ are 70% and 30%, respectively, and the resolution was set at $\sim 30\%$ valley between peaks (the simulation was run 5 times so that data could be expressed as mean \pm sem of the measured ratios). Although the simulations were run at a low S/N (~ 10 , a somewhat worst-case scenario), it is possible to reasonably fit the peaks even when as few as ~ 25 points are recorded across the 3 isotopes in the profile (Figure 5F).

The examples described above are less about the type of mass spectrometer and more about the processing of raw data. In our current studies, the commercially available software appears to be generally sufficient for obtaining relatively precise measures of isotope clusters. Thus the need for extra effort in regards to data processing may not be justified in all cases. However, an area where data processing may be worth considering centers on using FT-ICR MS (MacCoss et al. 2005). Reports in the literature have discussed a potential bias against isotope peaks present at low abundance (Bresson et al. 1998; Erve et al. 2009), recent efforts by our colleagues have started to address those apparent limitations (Ilchenko et al. 2011). We suspect that LC-FT-ICR MS analyses may offer another unique advantage when quantifying low levels of 2H -labeling. For example, we have demonstrated the ability to quantify low levels of 2H -labeling by resolving the $M+1$ isotope peak into its ^{13}C and 2H components (Herath et al. 2011b).

To this point we have not considered the acquisition mode under which data would be collected, the examples noted above do not imply MS or MS/MS-based analyses. Indeed, a substantial portion of our previous work was centered around MS-based analyses with less effort towards examining MS/MS-based measurements (Rachdaoui et al. 2009; Wang et al. 2007). Some of the advantages to using MS/MS analyses include (i) enhanced signal:noise, (ii) reduced concerns for overlapping peptides by identifying and characterizing the labeling of numerous fragments and (iii) sequence information on the peptide. The acquisition of MS/MS data to determine isotopic composition on a Q-ToF instrument is demonstrated using an apoA1 derived peptide (Figure 6). The doubly charged parent ion (m/z 520.85) is isolated at low-resolution in the quadrupole and then fragmented, the daughter ions are detected with the ToF analyzer. It is important to note that the relative intensities of the daughter ion profiles are close to the predicted natural abundance and the expected shift in the mass isotopomer distribution to higher isotopic composition with increased mass is

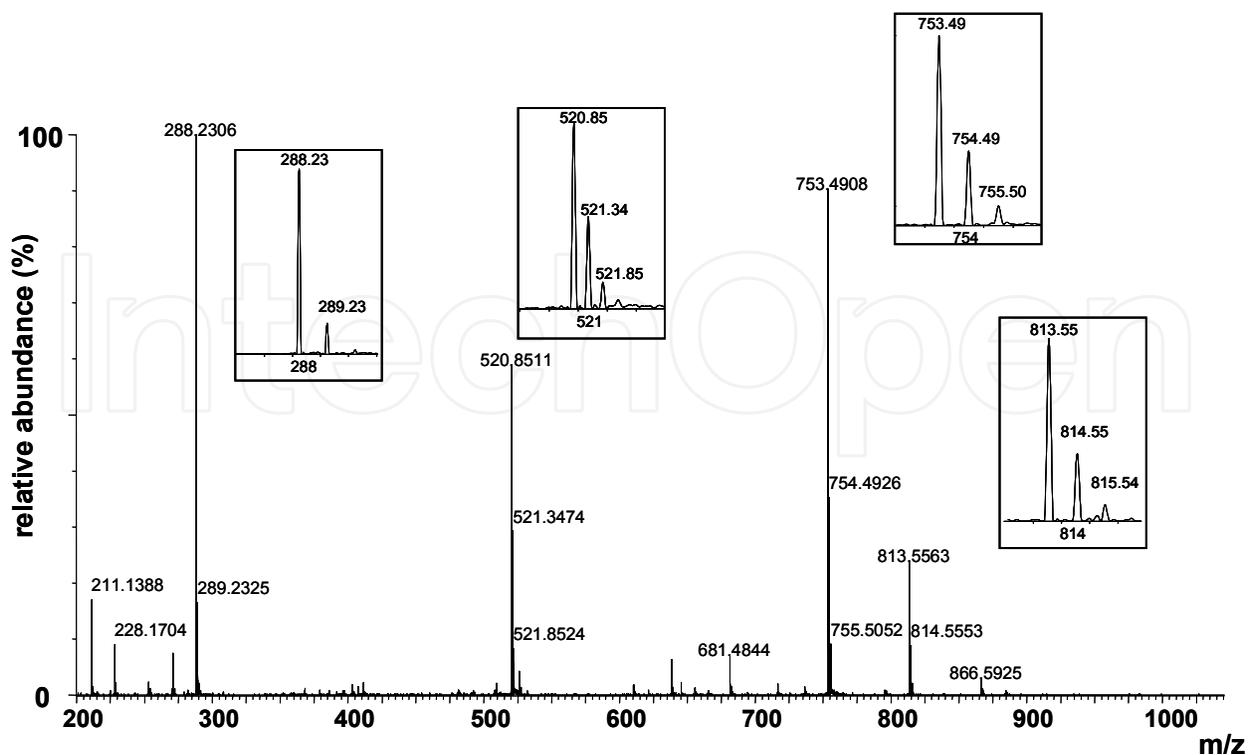


Fig. 6. LC-Q-ToF spectra of ARPALEDLR. The acquisition of MS/MS data to determine isotopic composition on a Q-ToF instrument are demonstrated using the apoA1 derived peptide ARPALEDLR. The doubly charged parent ion (m/z 520.8) is isolated at low-resolution in the quadrupole, fragmented by CID and the daughter ions detected with the ToF analyzer. The relative intensities of the daughter ion profiles are in close agreement with the predicted natural abundance (insets), the expected shift in the mass isotopomer distribution to higher isotopic composition with increased mass is readily apparent by the increase in the $M1/M0$ ratio of the daughter ions. Note that the insets show changes in 1 amu for isotope clusters at 288.23, 753.49 and 813.55 vs a shift of 0.5 amu for the cluster at 520.85 since these correspond with singly vs doubly charge species, respectively.

readily apparent by the increase in the $M1/M0$ ratio. These data are in agreement with a recent study in which we demonstrated the ability to measure the labeling of individual amino acids in tryptic peptides (Kasumov et al. 2011). We suspect that MS/MS-based measurements may need to consider the instrument configuration. For example, triple quadrupole measurements are likely to be good but have an inherent bias since one must decide what transitions to monitor. In contrast, Q-ToF measurements have the potential to capture more data and appear to have good reproducibility in regards to quantifying isotope labeling patterns (Castro-Perez et al. 2010).

The next question to address is, can one perform studies of proteome turnover? We consider what this would require for plasma-based analyses. First, although the concentration range of the plasma proteome varies from $\sim 35 \times 10^9$ pg albumin per ml vs ~ 5 pg interleukin-6 per ml, mass spectrometers are flexible enough to identify and quantify analytes across this range (Anderson et al. 2004; Anderson and Anderson 2002). These seemingly positive statements lead into a consideration of the central problem, i.e. assuming that one can detect a protein can one determine its kinetics? Based on our experience, since the signal:noise can play an important role in affecting the apparent labeling the answer is a clear "maybe". We

believe that the demands of measuring the mass isotopomer profile of a single peptide conflict with the imperative of identifying the largest number of peptides, making LC-MS protocols employed in proteomic studies less than ideal for some tracer-based protein turnover studies. For example, in preliminary work with an ion trap mass spectrometer, we observed that determination of a peptide's mass isotopomer profile with sufficient precision to quantify ^2H -incorporation required that the zoom scan mode be used with multiple scans encompassing an entire peptide chromatographic peak. In principle, this scan sequence (full scan to identify peptides that are present and zoom scan on a desired peptide) conflicts with an emphasis on obtaining data on the largest number of peptides characteristic of proteomic studies. We originally thought that these conflicting demands on the acquisition parameters of the mass spectrometer would limit protein turnover analyses to a smaller number of peptides than are present in the proteome. However, by generating a list of previously identified peptides, from proteins of interest, it should be possible to determine protein turnover rates on 10-100 proteins for a given LC-MS run.

Two recent publications deserve special attention. Namely, Price *et al.* (Price *et al.* 2010) used a hybrid LTQ/FT instrument to measure turnover of ~ 2500 proteins in multiple tissues of mice fed with ^{15}N -labeled algae, their MS/MS method consisted of one survey scan followed by several secondary scans of selected ions. Likewise Zhang *et al.* (Zhang *et al.* 2011) fed mice an *E. coli*-derived ^{15}N -labeled protein mixture. Samples were analyzed using an Orbitrap instrument, full scans at high resolution ($\sim 60,000$ at m/z 400) were used for isotopic distribution analysis; they identified and quantified the kinetics on ~ 700 proteins using a novel software package. It is important to emphasize that in both cases (Price *et al.* 2010; Zhang *et al.* 2011), the investigators observed a substantial mass shift because $\sim 100\%$ of the diet was labeled, the utility of these analytical approaches needs to be examined when the peptide labeling results in more subtle changes in isotopic distribution. In addition, corrections for inherent spectral error are also needed (Erve *et al.* 2009). 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 (Figure 7), e.g. prior to digestion/analyses the samples were subjected to immunodepletion to remove several high abundance proteins.

As discussed earlier, the ability to quantify shifts in the isotopic labeling allow one to estimate the FSR, however, in certain instances it is of interest to determine the absolute rate of synthesis (which requires an estimate of the concentration of a given protein). Numerous techniques can be used to measure the concentration of a protein (or peptides) (Gygi *et al.* 1999; Gygi *et al.* 2000; Jaleel *et al.* 2006; Johnson and Muddiman 2004; van Eijk and Deutz 2003; Yao *et al.* 2001; Zhang *et al.* 2001), however, each requires special considerations when applied in combination with a tracer study. First, in regards to labeling methods such as ICAT, one assumes equal generation and recovery of labeled and unlabeled species before mixing and analyzing. We believe that those techniques are of limited value in some studies. For example, if one administers $^2\text{H}_2\text{O}$ to quantify protein synthesis, some reagents (e.g. ICAT or digestion in H_2^{18}O) may not induce a large enough shift in the peptide mass to allow one to comfortably measure the ^2H -labeling profile and determine protein synthesis. For example, suppose that one aims to determine the synthesis and concentration of apoE, which has a $t_{1/2}$ that is estimated to be < 1 hour in rodents (Figure 7). The rate of synthesis can be determined by measuring the ^2H -labeling of an apoE-derived peptide. The change in concentration can be determined by digesting a 0 min sample in H_2O and digesting a 60 min

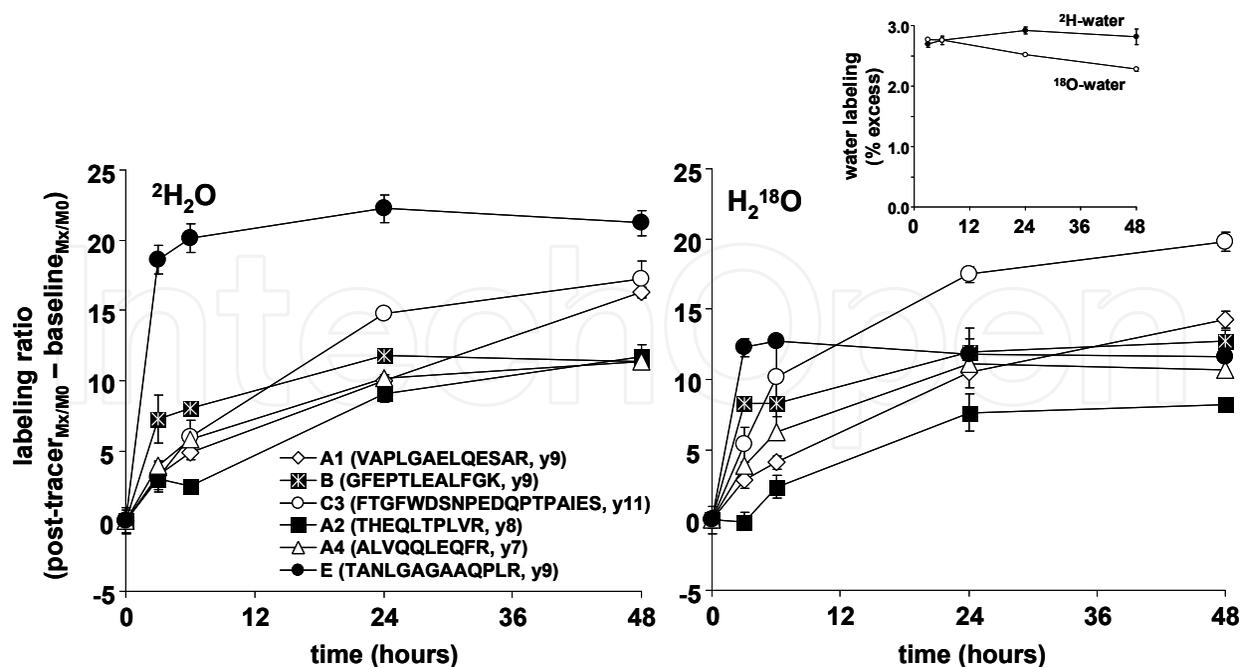


Fig. 7. Labeling of mouse apoproteins. Comparable labeling profiles were observed for several apoproteins in C57BL/6J mice given either $^2\text{H}_2\text{O}$ or H_2^{18}O . Note that animals were given an intraperitoneal bolus of either tracer and then allowed free access to labeled drinking water, as shown in the inset mice exposed to $^2\text{H}_2\text{O}$ reached a steady-state labeling whereas mice exposed to H_2^{18}O demonstrated a slight decrease in the labeling of body water. As expected, there were sizeable differences in the labeling of the various apoproteins, the relative differences are consistent with the literature, e.g. the FSR of apoE ~ apoB > apoA1. The magnitude of the labeling reflects variation in the amino acid composition of the respective peptides and the $t_{1/2}$. Data are shown as the mean \pm standard deviation, $n = 3$ per time point.

sample in H_2^{18}O and then mixing the samples and comparing the relative ^{18}O -labeling in a given peptide. However, the presence of ^{18}O (for quantifying abundance) will likely interfere with measurements of ^2H -labeling (protein synthesis). Therefore, each sample would likely need to be analyzed duplicate (first to determine the ^2H -labeling to estimate the rate of synthesis and second to determine the ^{18}O - or ICAT-labeling to estimate the concentration).

The use of SILAC methods is more likely to be compatible with the use of tracers in flux studies, i.e. one adds a known amount of a heavily labeled protein mixture and then compares the abundance of the cold peptides with that of heavily labeled SILAC peptides (Ong et al. 2002). While it is clear that SILAC methods are well suited for cell-based and rodent studies (Kruger et al. 2008), a potential drawback centers on the fact that it is not possible to fully label many model systems (e.g. humans). Interestingly, recent studies have demonstrated dynamic SILAC (Andersen et al. 2005; Doherty et al. 2009), i.e. investigators used a SILAC approach for administering a tracer but focused their attention on quantifying the change in labeling of numerous proteins in order to determine their flux. It is important to note that the early reports regarding the SILAC approach (for quantitative proteomics) clearly demonstrated the potential for quantifying proteome kinetics (we refer the readers to Figure 3 of (Ong et al. 2002)). Mann and colleagues monitored the temporal changes in

protein labeling to determine when the cells had become fully labeled, from that point they knew that they had generated SILAC cells which could be used to determine the protein expression profiles of other cells (Ong et al. 2002); despite the fact that their major objective was to contrast SILAC and ICAT methods for determining protein expression profiles, they demonstrated the potential for determining proteome turnover.

We believe that a simple and reasonable approach for determining protein abundance, which is compatible with the administration of a tracer for determining proteome kinetics, centers on the use of label-free methods. For example, Wang *et al.* (Wang et al. 2003) reliably quantified numerous peptides by measuring their relative abundance during a given run. Although this approach requires attention to detail during the sample processing and a stable operating system, it is immediately compatible with tracer-based studies since the isotopic labeling patterns are not altered. Clearly, there are factors that may skew the data resulting in estimates of concentrations that are far from the correct value (e.g. ion suppression effects), nevertheless, label-free methods can be used infer relative concentrations and differences between groups (Wang et al. 2003;Wiener et al. 2004). We should note that in cases where one aims to determine the kinetics of a single protein and/or a select group of proteins it is possible to use custom synthesized standards, e.g. this strategy has been used for measuring insulin concentration (Kippen et al. 1997;Stocklin et al. 1997). A related approach would be to use an “isomer dilution” strategy (Thevis et al. 2005), e.g. when studying the kinetics of albumin and/or insulin in rodents one could spike samples with known amounts of human albumin and insulin before processing and analyses.

5. Interpretation of the precursor: Product labeling ratio

Assuming that one has devised a strategy to administer a precursor and one has found a suitable way to measure its incorporation into a protein, there is a final question that must be addressed, how do I interpret the precursor:product labeling ratio? We first consider the scenario in which an investigator has administered a pre-labeled amino acid(s) and later consider the novelty of administering either $^2\text{H}_2\text{O}$ or H_2^{18}O .

As noted earlier, the goal of a primed-infusion is that one will instantaneously achieve and then maintain a steady-state labeling of a given amino acid tracer. Indeed, this was clearly demonstrated by Lichtenstein and colleagues, they simultaneously administered multiple labeled amino acids and observed the incorporation of each into various apoproteins (Lichtenstein et al. 1990). However, although the labeling of VLDL-apoB100 approaches a steady-state by the end of the infusion protocol the enrichment of amino acids in VLDL-apoB100 is substantially lower than the enrichment of those free amino acids in plasma. Although it is not possible to state with certainty the source of this discrepancy, it is clear that the transport of free amino acids into the cell (and/or mixing with the endogenous pool) must be slower than the rate of intracellular protein breakdown (Khairallah and Mortimore 1976) which likely results in marked compartmentation. What are the consequences of this on estimates of protein synthesis? One does not expect problems when the aim is to fit the exponential labeling curve (e.g. collect multiple time points and use Equation 1), in those cases the FSR is estimated from the time it takes to reach steady-state and it does not necessarily matter how labeled the protein is at steady-state (the caveat, however, is that one expects a better fit in cases where the asymptotic value is greatest since there is a large change in labeling over the natural background) (Figure 1A) (Foster et al.

1993). In cases where one aims to determine the synthesis of protein with a small FSR it may be necessary to use Equation 2, therefore, any error in the apparent precursor labeling will have an immediate impact on the estimated FSR. Based on data in the literature, if one assumes that the intracellular labeling equals the plasma labeling one will likely underestimate the FSR of LDL-apoB100 by nearly 2-fold since the labeling of amino acids in plasma is ~ 2 times greater than the estimated intracellular amino acid labeling (Lichtenstein et al. 1990). Note that in many studies, the production of VLDL-apoB100 is not only a parameter of interest but it serves a critical function in estimating LDL-apoB100 production, HDL-apoA1 production, etc. As discussed, the asymptotic labeling of VLDL-apoB100 may be used as a surrogate to estimate the precursor labeling that is needed to calculate LDL-apoB100 and HDL-apoA1 production (Lichtenstein et al. 1990). For example, LDL-apoB100 and HDL-apoA1 have relatively slow rates of synthesis and therefore show pseudo-linear increases in labeling over a short term infusion. As such, it is not practical to model the data and estimate FSR using Eq 1; to estimate the FSR of LDL-apoB100 and/or HDL-apoA1 investigators often use Eq 2 and substitute the asymptotic labeling of VLDL-apoB100 as the precursor labeling (Lichtenstein et al. 1990). The scenario discussed here applies to most cases in which cells are labeled from the outside, e.g. the administration of a pre-labeled amino acid.

One expects more reliable estimates of flux in cases where cells are labeled from the inside provided that one can determine the intracellular precursor labeling. For example, the administration of ^{13}C -glucose leads to the generation of ^{13}C -amino acids (Figure 4) but the labeling of those amino acids is likely to be diluted by carbon exchange (Wykes, Jahoor, and Reeds 1998). In cases where labeled water is used one expects comparable labeling between intracellular and extracellular pools. Dietschy and colleagues clearly demonstrated that water readily distributes in the plasma and that plasma labeling reflects tissue-specific labeling almost instantly (Dietschy and Spady 1984; Jeske and Dietschy 1980). As we have described previously, it is possible to then estimate protein flux by comparing the change in the labeling of proteolytic-peptides with that of body water (Rachdaoui et al. 2009). The caveat is that one must account for the number of copies of the precursor that are incorporated, referred to as n (Cabral et al. 2008; Kasumov et al. 2011; Rachdaoui et al. 2009; Xiao et al. 2008). For example, in cases where H_2^{18}O is administered, the labeling of the protein will exceed that of the precursor since one expects that each peptide bond will incorporate ^{18}O . Note that in the example shown in the inset for Figure 7 the labeling of water is ~ 2.5 to 3.0% yet the labeling of the various proteins greatly exceeds those levels, therefore one needs to correct the precursor:product labeling ratio by including a constant for n (Herath et al. 2011a; Rachdaoui et al. 2009).

6. Summary and conclusions

We believe that it is possible to readily convert static protein expression profiles into dynamic images. Numerous approaches are available for tracing protein synthesis and various strategies have been implemented for measuring the labeling of peptides in complex mixtures. We believe that there is no single best method but certain fundamental points should be recognized. For example, the administration of a labeled precursor can present a challenge for *in vivo* studies. The administration of labeled water may be advantageous in these settings, the tracer can be given orally, it is relatively inexpensive and can be used to study multiple parameters simultaneously (this is especially important in studies of

lipoprotein kinetics since questions regarding protein and lipid flux are often of equal importance) (Castro-Perez et al. 2010;Castro-Perez et al. 2011;Dufner and Previs 2003). In contrast, although we have demonstrated the ability to study protein synthesis in cell culture using labeled water (Dufner et al. 2005), we believe that SILAC methods are generally superior for *in vitro* studies since it is trivial to completely substitute fully labeled amino acids for unlabeled amino acids in that setting.

In regards to the analyses of protein mixtures, we believe that there is no single best MS approach. Although our applications have been focused on small groups of proteins, it is clear that the labeling profiles of analytes present in complex mixtures can be sorted out; again, the SILAC literature strongly supports these conclusions. We believe that an area which will likely have an important impact on future studies centers on data processing; in our experience the MS hardware may be limited by the software. As we have demonstrated it is possible to obtain reliable isotopic ratios using commercially available software, however, in some cases alternative methods have been of great value.

7. Acknowledgments

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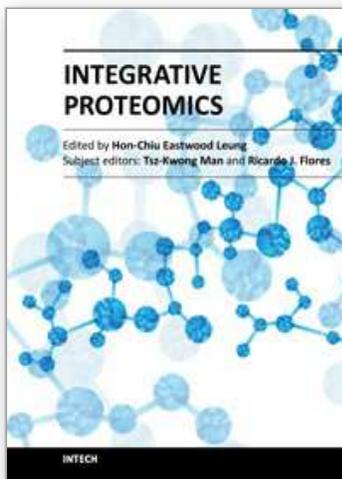
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Proteomics was thought to be a natural extension after the field of genomics has deposited significant amount of data. However, simply taking a straight verbatim approach to catalog all proteins in all tissues of different organisms is not viable. Researchers may need to focus on the perspectives of proteomics that are essential to the functional outcome of the cells. In Integrative Proteomics, expert researchers contribute both historical perspectives, new developments in sample preparation, gel-based and non-gel-based protein separation and identification using mass spectrometry. Substantial chapters are describing studies of the sub-proteomes such as phosphoproteome or glycoproteomes which are directly related to functional outcomes of the cells. Structural proteomics related to pharmaceuticals development is also a perspective of the essence. Bioinformatics tools that can mine proteomics data and lead to pathway analyses become an integral part of proteomics. Integrative proteomics covers both look-backs and look-outs of proteomics. It is an ideal reference for students, new researchers, and experienced scientists who want to get an overview or insights into new development of the proteomics field.

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