# Protein turnover on the scale of the proteome

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**Protein turnover is a neglected dimension in postgenomic studies, defining the dynamics of changes in protein expression and forging a link between transcriptome, proteome and metabolome. Recent advances in postgenomic technologies have led to the development of new proteomic techniques to measure protein turnover on a proteome-wide scale. These methods are driven by stable isotope metabolic labeling of cells in culture or in intact animals. This review considers the merits and difficulties of different methods that allow access to proteome dynamics.**

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One of the goals in large-scale functional genomics and systems biology is to understand the correlation between the concentration of a protein in a cell and the level of its cognate mRNA molecule. In practice, there is often poor correlation between expression of mRNA and the corresponding protein [1,2,3–7]. In systems as diverse as bacteria [3–5], yeast cells in culture [2,6], human liver [7] and lung carcinoma cells [1], the discrepancy between mRNA and protein expression has been noted. Although the concentration of many mRNAs correlated with the corresponding level of protein expression in all of these studies, mRNA levels were not predictive of the cognate protein response for other genes, particularly in systems where there are temporal changes in protein and transcript.

Modulation of protein abundance mediated by post-transcriptional control of translation or protein post-translational modification, including degradation, is not formally or mechanistically linked to the abundance of mRNA. Whilst the correlation between the level of transcript and level of a protein in the cell is imperfect, this should be expected. An mRNA-focused perspective inevitably emphasizes protein synthesis as the main influence on protein content in the cell, but ignores the equally critical contribution made by regulated or unregulated intracellular protein degradation. The multiple parameters that determine the relation between mRNA and protein do not allow straightforward prediction of the protein changes in response to changes in mRNA. Therefore, a true systems biology perspective of transcriptome–proteome relationships will only be feasible when information relating to the dynamics of the macromolecular constituents is available [8,9]. When a protein increases in intracellular concentration, this can be achieved by enhanced synthesis or, equally effectively, diminished degradation (FIGURE 1). Whilst the outcome of these processes is the same (a change in the intracellular concentration of the protein), the mechanism and regulation of the two opposing processes is very different. To fully understand protein expression, it is important to give due cognizance to the flux of individual proteins as well as their concentration. There are strong precedents for monitoring protein synthesis and degradation [10–17]. A range of methodologies have been developed, each with specific benefits or limitations. This review will briefly describe methods for the study of protein turnover and demonstrate how such methods can be reinterpreted and adapted to access proteome turnover on a protein-by-protein basis, both in single-cell organisms and in more complex whole-animal systems.

## Experimental strategies to measure protein dynamics

The measurement of protein dynamics requires methods to determine changes due to replacement of a protein pool, even when the size of


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that protein pool is constant. It follows that the only feasible approach to accessing this dynamic property is to measure the incorporation or loss of a tracer. The tracer can be an unstable (radioactive) isotope of carbon, hydrogen or sulphur or a stable (nonradioactive) isotope of carbon, nitrogen or hydrogen. Radioactive tracers are commonly determined by scintillation counting, whereas stable isotopes are measured by mass spectrometry (MS). A synthesis-based approach measures the incorporation of label into protein, whereas a degradation-based approach measures the loss of label from previously labeled proteins. Further consideration has to be given to the molecule that incorporates the tracer isotopes. It may appear that the most logical molecules to monitor protein dynamics would be amino acids, but other metabolically active precursors of protein synthesis have been used, such as glucose or ammonia.

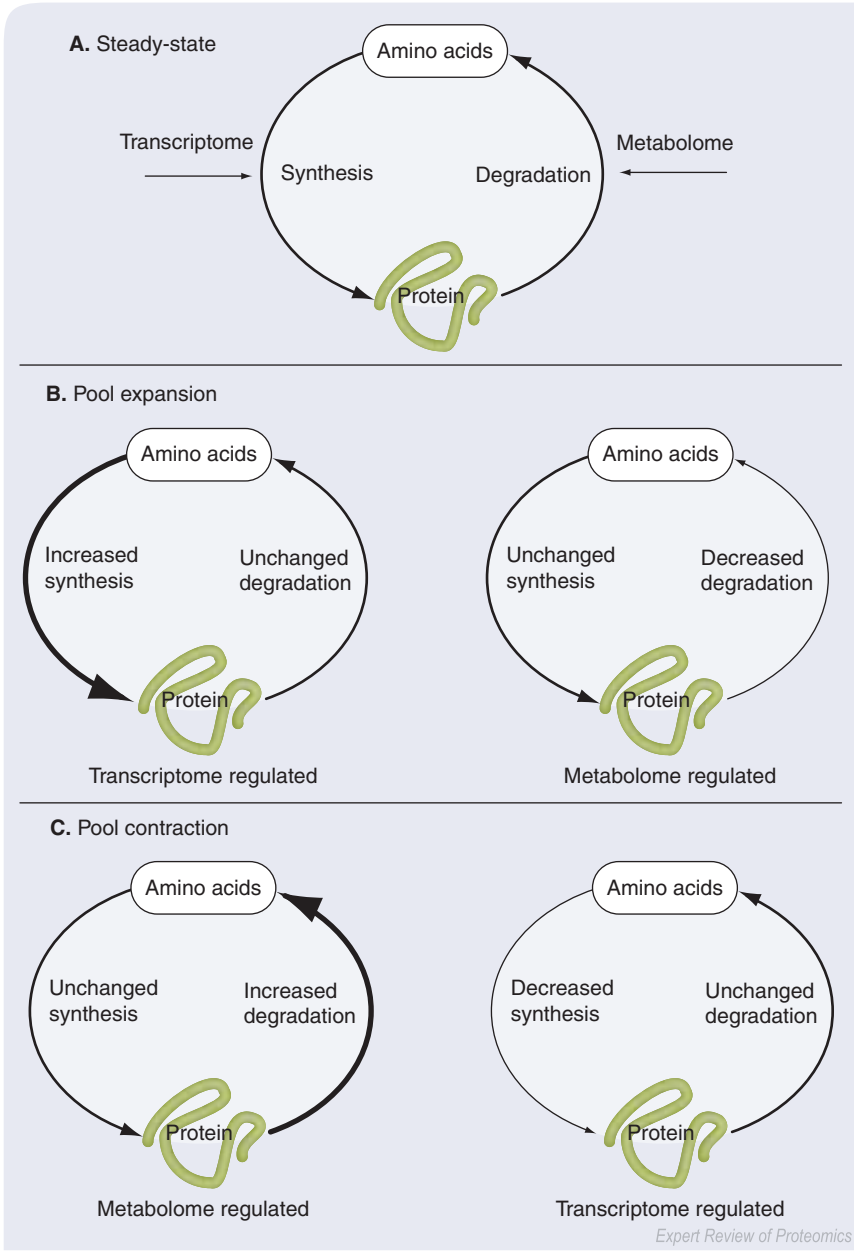


**Figure 1. Protein turnover.** Proteins in a cell are in a dynamic state of flux. In **A**, the steady-state is shown. Although the concentration of the protein pool is not changing, this merely reflects a balance between the two opposing processes of protein synthesis and degradation. Protein synthesis is linked to transcriptional control whereas protein degradation is more closely coupled to the metabolome. When the protein pool must increase in size (**B**), this can be affected by increased synthesis (transcription) or by decreased degradation (metabolism). Conversely, when a protein pool must decrease in size, this can be a result of decreased synthesis or increased degradation (**C**).

### Protein turnover before proteomics

All approaches to the determination of protein synthesis are based on the same principle: the incorporation of a labeled precursor into protein. In most variants of this approach, it is necessary to know the extent to which the precursor pool has been labeled, either having been expressed as the specific radioactivity (SA) or relative isotope abundance (RIA), depending on whether the precursor is radiolabeled or stable isotope labeled. The precursor pool for protein synthesis is often defined as the amino acid pool even though the true precursor is the aminoacyl-tRNA pool [11,18]. Aminoacyl-tRNAs are in low abundance in cells and are difficult to quantify with precision [19,20]. Therefore, it is common to see amino acids used as a surrogate for the aminoacyl-tRNA pool.

In single cells maintained in culture, it is relatively easy to manipulate the tracer through supplementation of the external medium, since the mass excess of amino acid external to the cells can be designed to be substantial, and the extent of labeling of the intracellular pool rapidly reaches that of the medium. In many circumstances, it is then unnecessary to determine the precursor SA or RIA. Sampling of the labeled proteins then permits the assessment of the rate of turnover of the proteins. Previously, most studies on protein dynamics have used radiolabeled precursors, notably, $^{35}$S-labeled methionine and cysteine [21–28]. In pulse-chase experiments, the sample is subjected to a short pulse exposure to the radiolabel, followed by a chase period where the labeled isotope is removed (by washing) and replaced by an unlabeled media. The loss of previously labeled proteins is then monitored, usually by autoradiography of 1D and 2D gels. This approach has been taken with protein turnover in skeletal muscle-derived cells in culture [29], articular cartilage proteins [30], adipocyte-derived cell cultures [28] and in the analysis of differentiation

synthesis of trypsinogen isoforms in rat pancreas [26]. In the rat pancreas study, for example, $^{35}$S-L-methionine was administered to live rats and the animals sacrificed 30 min post injection. The use of substantial amounts of radiation has obvious health and safety implications (15 MBq/kg body weight was injected), and this technique is not routinely used for whole-animal studies. A further limitation of this method is that it relies on gel-based separations of the proteins. Protein losses of between 20–54% have recently been reported for a single step in the 2D protocol, and the reproducibility between samples was also poor [31]. This means that while gel-based technologies are useful for the separation of proteins, they cannot be used for reliable quantification. Moreover, a comparative study of gene expression profiles of cells exposed to tracer levels of $^{35}$S-radiolabel revealed that substantial changes in gene expression were induced following 2-h labeling [32].

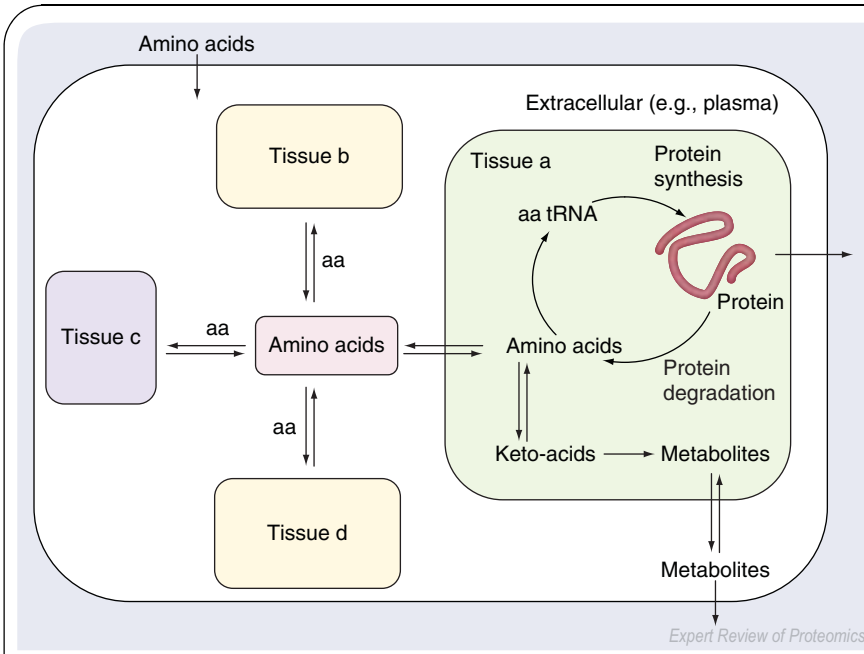


**Figure 2. Protein synthesis and degradation is multifactorial.** The mechanisms of protein turnover are complex and the processes dynamic. Proteins are synthesized from amino acids; however, the immediate precursor of protein is aminoacyl-tRNA. It is technically difficult to determine the concentration and composition of the aminoacyl-tRNA pool and models have been derived that allow this parameter to be derived from analysis of the amino acid pools. Amino acids are present in all tissues and can be derived from diet, *de novo* synthesis or from protein degradation. There is exchange between amino acid pools and a reversible conversion to the reciprocal keto-acid. This keto-acid can undergo further irreversible reactions to form metabolites that are removed from the tissue. Such complexity has led to debate over the correct model for determination of the precursor pool.
aa: Amino acid.

In complex multicellular species, and particularly in animals, interactions between the different tissue pools (FIGURE 2), and the inability to control the precursor RIA or SA to the same extent brings additional challenges for analysis of tracer incorporation, release and exchange. In such systems, the precursor pool is labeled by either continuous infusion of labeled isotopes, which achieves a limited duration steady state of labeling [13,33,34], or through the flooding dose method, in which the precursor pool is labeled to a constant degree by transient expansion and flooding of the precursor pool with a labeled precursor [10,11,14,20,35–37]. Several basic assumptions are made when estimating protein turnover or, more specifically, fractional synthesis rates (FSRs) in complex organisms [10,11]. It is assumed that the specific activity of the amino acid pools approximates the specific activity of the aminoacyl-tRNA pool, and that the amino acids from protein degradation mix rapidly with the intracellular pool of amino acids and are not preferentially reused for, or selectively excluded from, protein synthesis. Continuous intravenous tracer infusion of either stable isotope or radioisotope can be used to estimate the rates of appearance of the tracer in the free amino acid and protein pools [33]. Key to this type of experiment is the length of time required for adequate enrichment of the precursor pool [20,38], which generally requires extended labeling times (up to several hours) and leads to technical difficulties in maintaining steady-state conditions, particularly in animals [20,37,39]. In addition, this method is thought to underestimate the total turnover of tissue proteins due to a delay in the equilibration of the precursor pools [16,20,40]. The main alternative, based on flooding dose methods, overcomes the problems of estimating the precursor tRNA pool available for protein synthesis by swamping the equilibrium of labeling between the intra- and extracellular pools [41,42]. This method involves the relatively rapid administration of a large dose of unlabeled amino acid along with the amino acid tracer, and assumes that the expansion of the amino acid pool results in equilibration of the specific activities among the aminoacyl-tRNA and the blood and tissue free amino acids. Equilibration between the different amino acid pools has been demonstrated in some (but not all) studies. In these cases, a high concentration of the labeled amino acid was present in the medium. Limited studies have been performed *in vivo* that indicate that the aminoacyl-tRNA specific radioactivity equilibrates with that of the intra- and extracellular amino acid pools when a flooding dose of leucine, proline or phenylalanine is used [20]. Rather than measuring the tissue precursor labeling after administration of tracer amounts of labeled amino acid, flooding all of the free amino acid pools to a common value facilitates measurement, after sampling, of the plasma-free pool. The rate of protein synthesis can then be calculated by comparison of the label incorporation throughout the time course of the labeling experiment with the average plasma amino acid labeling. A major criticism of this method is that the rapid injection of the large excess of tracer amino acid may stimulate protein synthesis, and thus influence the rate calculations (although this is not universally accepted) [16,18,43].

Total protein turnover has been measured in a variety of organisms and tissues (TABLES 1 & 2). These rapid methods for bulk protein turnover analyses are valuable, particularly in whole-animal metabolism/energetics and in clinical research, but can only inform on the weight-biased behavior of the total protein pool, at the tissue or whole-organism level. The lack of protein-specific information has arisen, in part, from the historical difficulties of labeling small quantities of individual proteins to a sufficient degree that individual protein turnover can be assessed. Until the emergence of proteomics, there was little imperative to develop a comprehensive, proteome-wide analysis of the role that intracellular protein stability plays in expression. Specifically, techniques for global profiling of synthesis and degradation rates need to be developed. It is timely to recapitulate the methods developed for total protein metabolism, and explore whether any of these methods are applicable to the analysis of single proteins, and with a sufficient degree of multiplexing so that many proteins can be analyzed in the same labeling experiment. This would be a true proteome-wide analysis of protein dynamics.

### Protein turnover in the era of proteomics

The technological developments in MS and refinements in attendant protein and peptide separation technologies mean that it has become feasible to entertain the possibility of simultaneous identification and analysis of large numbers of proteins. The first phase of the emergent science of proteomics was focused on identification of proteins in a mixture, but as our ability to gain such identifications has advanced, so proteomics has evolved to encompass characterization proteomics and quantitative proteomics. The last of these developments has provided new approaches for the determination of the relative [44,45] or absolute [46,47] amount of proteins in cellular systems. However, it will be recognized that the quantity of a protein in a cell, or changes in that parameter, does not define the mechanism by which that outcome is achieved. There is a growing recognition that the previously developed methods of measurement of protein dynamics can be embellished by application of analytical methods in proteomics, to deliver information on proteome dynamics at the level of individual proteins.

The experiments that access proteome turnover will be conceptually the same as those used for total protein turnover studies, with an added step of proteome simplification to the extent that single proteins can be analyzed in subpicogram amounts. However, this simple change in experimental strategy is not without consequences. Since the specific radioactivity of commonly used radiolabeled precursors is so high, it is feasible to measure a low degree of incorporation of radiolabel in total protein, but this would not be sufficient to monitor individual proteins.

### Choice of label

Since changes in protein abundance are the outcome of a change in the balance of protein synthesis and protein degradation, it is necessary to measure any two of these parameters in order to calculate the third. Therefore, labeling strategies have to be developed to measure the synthesis of new protein, or the loss of pre-existing proteins. There have been some studies of proteome changes using radioisotopes, notably $^{35}$S-methionine [48], but the inevitable role of MS in protein identification, characterization and quantification enables labeling strategies that measure the incorporation or loss of stable isotope-labeled amino acids. This has an advantage over the use of radioisotopes since discrete labeling events can be discriminated with stable isotopes. Using radioisotopes, it cannot be determined whether, in a particular protein or peptide, specific amino acids have been labeled or if this labeling is full or partial, neither can the relative amount of radioactivity ascribed to each labeling event be determined. Furthermore, the addition of stable isotopes to a system has, to date, no reported stimulatory effects on specific genes, unlike radiolabels [49].

Table 1. Common methods for the determination of fractional synthesis rates for total protein in intact animals.

| Method | Description | Label | Advantages | Limitations | Ref. |
|---|---|---|---|---|---|
| Continuous infusion | A labeled amino acid tracer is infused at a constant rate over the experimental period. The disappearance of label is monitored over time as it is incorporated into protein | $^{14}$C Leu (R)/$^{15}$N or $^{2}$H Leu (S); $^{13}$C Glu (S); $^{14}$C Lys (R); $^{2}$H Phe (S); $^{2}$H Tyr | Simple technique, no stimulation of protein synthesis | Requires extended labeling time; underestimates turnover; relies on compartmental analysis | [13,16,20,33,34,40] |
| Flooding dose | A bolus of amino acid is added to the system, forcing the equilibrium of labeling between extra- and intracellular free pools. Disappearance of the label is monitored with time | $^{3}$H Phe (R)/$^{2}H_5$ Phe (S); $^{13}$C Leu (S) Choice is critical due to stimulatory effects | Rapid equilibration of the precursor pool; short experimental time; analysis of acute changes in turnover | Possible stimulation of protein synthesis; incomplete equilibration would affect calculations; limited tracers; relies on compartmental analysis | [10,11,14,16,20,35–37] |

R: Radioisotope; S: Stable isotope.

Table 2. Comparison of whole-body protein and proteome turnover.

| Parameter | Whole-body protein turnover | Proteome turnover/mass isotopomer distribution analysis |
|---|---|---|
| Methods | Continuous infusion; flooding dose. Generally use radioisotopes | Proteomic separation of cells labeled with stable isotopes followed by mass spectrometric analyses and mathematical interpretation of spectra |
| Advantages | Rapid results for bulk analysis. Useful in clinical research situations | Information on individual protein turnover on a global scale. Can identify subtle changes in protein turnover. Not reliant on model analysis of the precursor pool |
| Disadvantages | Contradicting data from continuous infusion and flooding dose experiments imply a methodological inadequacy. This method does not return information on individual proteins; therefore, the analysis can obscure subtle (and often unsubtle) changes at the individual protein level | Still limited applications in intact animals. Not routinely used |

The factors governing the choice of stable isotope-labeled precursor have been recently reviewed elsewhere and include the stable isotope (deuterium, $^{13}C$ or $^{15}N$ are common), the mass offset between the labeled and unlabeled amino acid, and the nature of the precursor [50]. The performance of mass spectrometers used for proteomics is such that resolution of two peptides separated by 1 Da is straightforward in a typical tryptic fragment (1000–3000 Da) that typifies most proteomics studies. Therefore, in principle, it should be feasible to measure the incorporation of an amino acid labeled with stable isotope at a single atom center (such as $^{13}C_1$ glycine. However, the heavy variant, generated metabolically, would generate a natural isotope abundance profile that overlapped with the natural isotope abundance profile of the unlabeled light peptide, thus making deconvolution of the overlapping profiles and quantification unnecessarily difficult. There is considerable merit in using a labeled precursor that provides at least a 4-Da separation between the labeled and unlabeled forms.

In principle, any stable isotope-labeled amino acid could be used as a precursor for protein synthesis studies. In practice, there are a number of considerations that conspire to restrict this choice [50]. An ideal labeling precursor would have the following characteristics:

- It would be metabolically isolated, so that the labeled atom centers would not distribute to any other amino acid
- The cell should be auxotrophic for the amino acid, to eliminate dilution of the precursor pool by synthesis *de novo* (in animals, this means the essential amino acids)
- The precursor pool should be metabolically very active, such that rapid changes in precursor isotope abundance are possible
- It should be an abundant amino acid, to increase the probability that a peptide derived from the protein will inform on the rate of turnover, particularly if multiple amino acids are needed for mass isotopomer distribution analysis (MIDA)
- It should be labeled with sufficient heavy atom centers as to achieve a mass offset between labeled and unlabeled peptides of at least 4 Da

The mass offset is readily achieved using deuterium, but there are two minor issues with this label. First, deuterated peptides tend to elute slightly ahead of their unlabeled counterparts on reverse-phase chromatography. Second, a uniformly deuterium labeled peptide can include a deuterium atom at the $\alpha$ carbon atom, which, although chemically stable, is metabolically unstable through transamination (see later). Although more expensive, $^{13}C$- and $^{15}N$-labeled amino acids are preferred. There is some merit in using, for example, either $^{13}C_6$ Arg and/or $^{13}C_6$ Lys, because most tryptic peptides would then contain a single labeled amino acid at a constant mass offset. However, in this instance, MIDA would be challenging, as very few peptides would contain multiple instances of a single amino acid.

#### Mass isotopomer distribution analysis

Stable isotope labeling has one notable advantage over radiolabeling, namely the ability to discriminate between variants of analyte that differ in the degree of labeling. For example, a peptide containing one, two or three radiolabeled amino acids cannot be deconvoluted to yield the relative proportion of each. By contrast, the mass difference between the three species is readily discriminated by MS, leading to the possibility of analysis of labeling patterns based on mass isotopomer analysis. MIDA has been pioneered by Hellerstein and coworkers as a strategy to deconvolute complex mass spectra generated by partial stable isotope labeling of polymeric macromolecules, including proteins [19,51–54].

MIDA is based on combinatorial probabilities of precursor incorporation. Protein synthesis is a combinatorial process, with monomeric units (the amino acids) from a precursor pool combining into a polymeric collection (the protein or peptide). If the amino acids are a mixture of labeled and unlabeled, then the assembled proteins or peptides thus derived will not be of a uniform isotopic composition. The proteins and peptides will exist as distinguishable species containing varying numbers of the different types of amino acids (FIGURE 3). Some proteins and peptides will lack labeled amino acids, some will contain a single instance, some two and so on. Using MS, it is possible to quantify the relative proportion of each isotopic species of a peptide. This is defined by the binomial expansion (FIGURE 4).

The main benefit of these data is that the relative proportions of the different species allow the relative isotopic composition (RIA) of the precursor pool at the true site of cellular biosynthesis to be calculated. At the start of the labeling period, only the unlabeled light species would be present (this holds for all values of RIA). Assuming that the precursor RIA is maintained at unity, 50% of the precursor pool will be fully labeled and 50% will be unlabeled after one half life, giving rise to two peak envelopes in the mass spectrum, of equal intensity and separated by the additional mass of the labeled precursor multiplied by the number of occurrences of the precursor in the analyte. After a large number of half lives, all of the peptide will be labeled and only one peak envelope, corresponding to the fully labeled (heavy) peptide, will be observed.

If the RIA is less than unity, more complex mass spectra will be obtained (FIGURE 4). For example, if the precursor RIA were maintained at 0.5, then the protein synthetic apparatus would exert a 50:50 stochastic incorporation of a heavy (H) or light (L) amino acid each time the appropriate codon was encountered. Combinatorially, if a peptide contained two instances of the amino acid, then there are two ways to make a single labeled peptide (HL and LH), and one way to make HH and LL. Thus, at a precursor RIA of 0.5, the labeled material, when fully labeled, will show three peptide ions in the ratio of 1:2:1, separated by the mass difference of the heavy amino acid. This complexity brings a significant advantage. The relative intensities of the peak envelopes arising from a labeled peptide mean that it is possible to calculate the RIA of the precursor pool directly from multiply labeled peptides. MIDA requires that only the peptide itself be analyzed to establish the isotopic enrichment of both the precursor and the product. Functional compartmentalization within the precursor pool does not affect MIDA calculations because the precursor amino acids that actually enter each particular polymer are used for the calculation of precursor enrichment in that protein or peptide, regardless of their relationship to other precursor pools or other proteins. This obviates analysis of the precursor pool. MIDA is extremely compatible with conventional protein mass spectrometric techniques that generally involve the analysis of limit peptides resulting from the proteolytic digest of a protein. However, it should be noted that the level of incorporation of stable isotope required for detection (nominally 5–10% incorporation) can be a limiting factor.

Algorithms have been developed to deconvolute complex samples where stable isotopic envelopes overlap [51–54]. The combination of proteomic separations, stable isotope labeling and modern mass spectrometric techniques have opened a powerful avenue of analysis, which will allow the incorporation of stable isotopes into individual proteins in complex systems to be analyzed rapidly. A schematic of a typical proteome turnover experiment is shown in FIGURE 5. Briefly, limit peptides are obtained by digesting a protein, either extracted from a gel or in solution, with a proteolytic enzyme such as trypsin. The peptide mass fingerprint of the sample is obtained and the protein(s) identified by database searching. Peptides of interest (i.e., those that contain the stable isotope) can be examined more closely and the pattern of incorporation determined. Methods for achieving this will vary depending on the mass spectrometer, but this technique has been successfully used with both matrix-associated laser desorption/ionization (MALDI) time-of-flight (TOF) MS and liquid chromatography (LC) tandem MS (MS/MS) approaches [55–57].

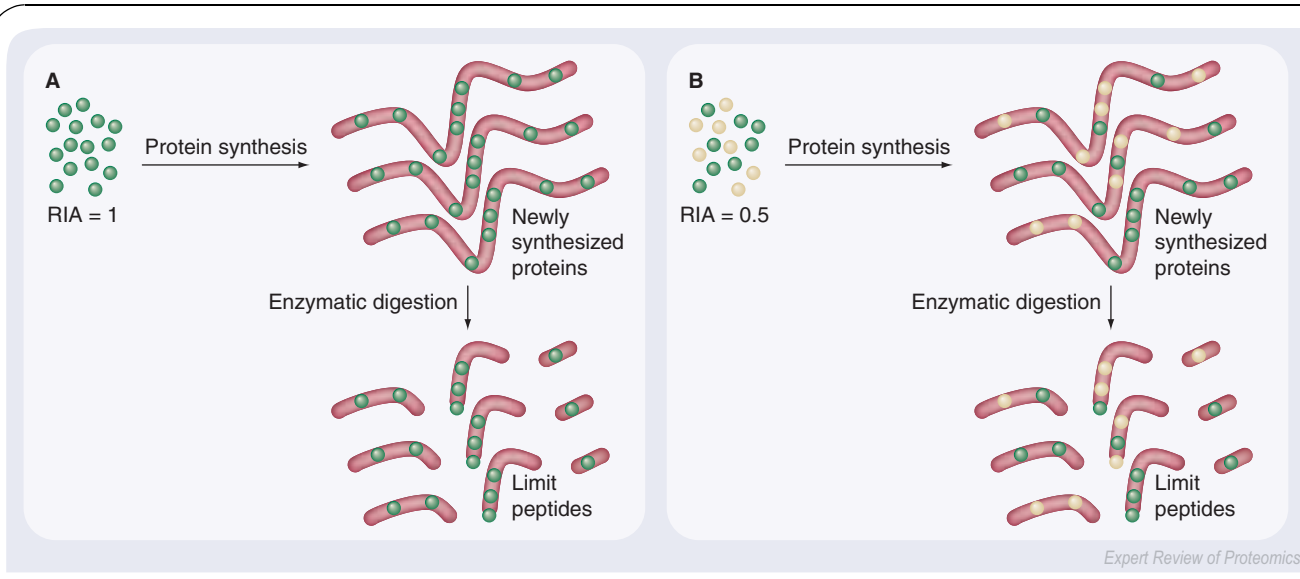


Figure 3. Calculation of the RIA of the precursor pool in proteome turnover experiments. (A) If the RIA of a precursor pool is 1 (i.e., all of that amino acid is available in the labeled form), all of the corresponding amino acid residues in the resultant newly synthesized protein will be labeled. Upon enzymatic digestion this will result in fully labeled limit peptides. (B) If the RIA is 0.5, there will be an equal chance of a protein incorporating either a labeled or unlabeled amino acid in the polypeptide chain. Upon proteolysis, the limit peptides will contain a defined number of labeled or unlabeled amino acids, predicted by binomial equations. RIA: Relative isotope abundance.

### Proteome turnover in single-cell systems

An early application of proteomics to the analysis of protein turnover on a protein-by-protein basis was in the measurement of protein stability in *Saccharomyces cerevisiae* [58]. Cells were grown at a constant rate in a chemostat and proteins were uniformly prelabeled with $^{2}H_{10}$ Leu provided in the growth medium. After proteins were fully labeled, a large excess of unlabeled leucine was added to the culture medium and, at the same time, the medium was replaced by one containing unlabeled amino acid. Since the cells were glucose limited, the addition of a large excess of the unlabeled leucine did not affect the growth rate. The leucine pulse had no effect on $CO_2$ production by the yeast cells, demonstrating that leucine was not being used as an alternative carbon source. The $^{2}H_{10}$ Leu was labeled at all positions other than the α-amino and α-carboxyl groups, and was chosen because leucine is present in the majority of tryptic peptides derived from the yeast proteome [59]. In addition, use of a leucine auxotrophic mutant of yeast ensured that dilution by endogenous *de novo* synthesis of leucine was minimized. The α carbon deuteron is metabolically labile through transamination, and the relative incorporation of $^{2}H_{10}$- or $^{2}H_{9}$-deuterated leucine provided a valuable insight into the metabolic mobility of the precursor pool, which is important in establishing the effectiveness of the unlabeled chase process.

Proteins were labeled for approximately 50 h (over seven doubling times) before the chase period, which continued for a further additional 50 h. 2D gel electrophoresis (2DE) indicated that labeling had no effect on protein expression. The ion profiles for individual peaks in the peptide mass fingerprint tracked the replacement of the labeled protein by unlabeled protein as the cells grew in culture. For peptides containing more than one leucine residue, the lack of peaks of mass values intermediate between the fully labeled and fully unlabeled forms was convincing proof that the RIA of the precursor pool had been reduced to zero, indicating an effective and complete chase period. The abundance of labeled leucine was monitored in tryptic peptides from the 2DE-separated proteins. Peptides

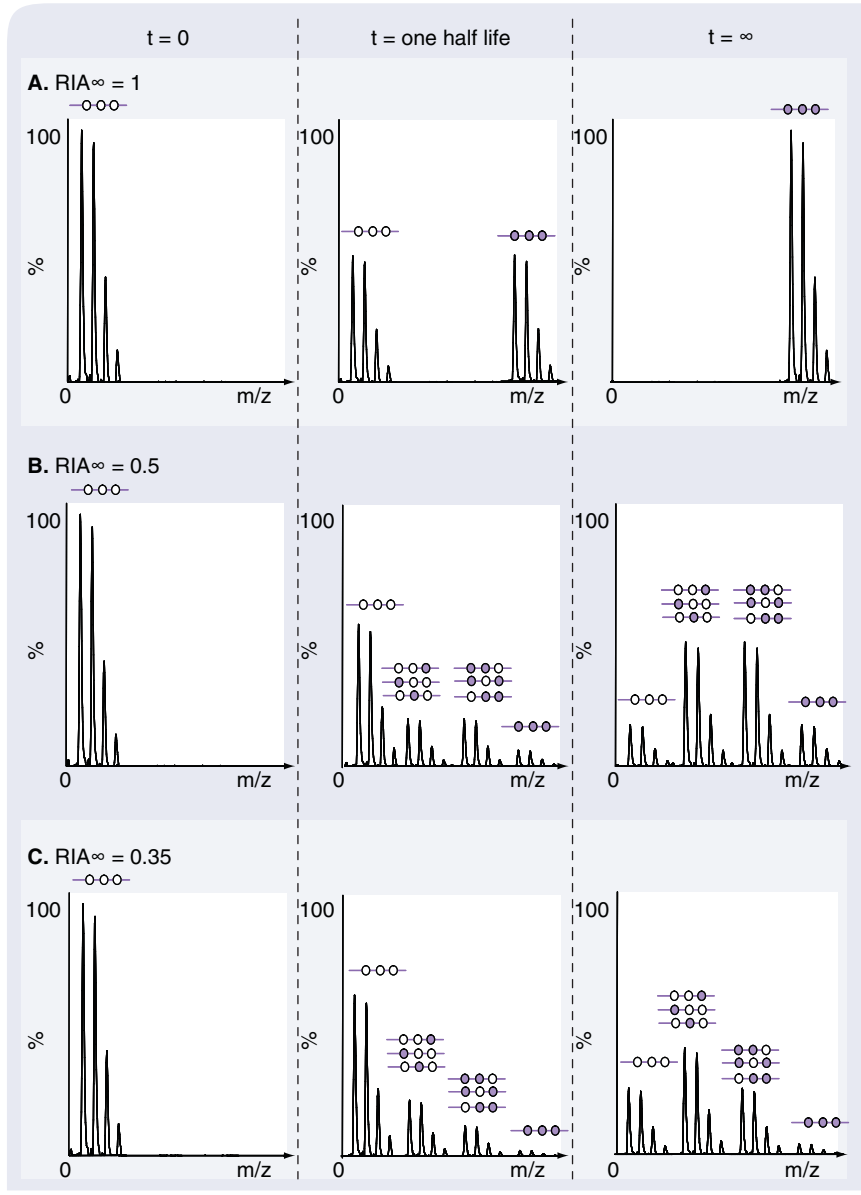


Figure 4. Determination of the precursor RIA from mass spectral data. The predicted mass spectra for three different RIA values for a trivaline peptide are shown at t = 0, after one half life and at infinity. The RIA of the peptide, and hence, the protein and the precursor pool, can be determined by the mathematical analysis of the ratio of these peaks. (A) When RIA = 1 at t = 0, only the light variant of the peptide will be detected. After one half life, there is an equal probability that either the heavy or light variant will be present, which manifests as two peak envelopes of equal intensity, separated in m/z space by the mass of the label (for a trivaline peptide, this would be 3 × m/z offset of the label). At an infinite time, only the heavy variant will be present and so, again, a single peak envelope is observed in the mass spectrum, but shifted according to the mass of the label. (B) If the RIA = 0.5 at t = 0, a single peak envelope is observed, as only light peptide is present. This will hold regardless of the RIA. After one half life, a more complex pattern is observed. Following the binomial expansion, peaks in the ratio of 1:3:3:1 relating to the peptides labeled as $L_3$:$L_2H$:$LH_2$:$H_3$ would be expected (where L = light amino acid and H = heavy amino acid). However, this only accounts for newly synthesized material and does not report on the amount of light material that was already present in the system. This manifests as a $L_3$ peak of increased intensity. At infinity, all pre-existing light material will have been replaced and so the ideal 1:3:3:1 ratio will be attained. Using this rationale, the true precursor RIA can be calculated directly from the peptides of interest using the ratios of the $L_2H$ and $LH_2$ peak intensities. (C) In the case of chicken muscle, although a RIA of 0.5 was presented to the birds, due to dilution and amino acid reutilization, the true RIA was calculated to be 0.35. m/z: Mass-to-charge ratio; RIA: Relative isotope abundance.

containing the amino acid selected for labeling (in this case leucine) will be informative of the rate of loss of material from the system. Multiple peptides from a single protein yield separate estimates of this variable, thereby improving the quality of the calculated first-order rate constant.

For each protein, the first-order rate constant for any one protein was remarkably consistent, whether derived from peptides with one or more than one leucine residue. Errors in the rate constant were small. In this experiment, prelabeled proteins could have two fates; to be degraded or to be diluted into daughter cells. Therefore, the rate of loss of labeled leucine from any protein reflected the composite term comprising dilution losses (growth) and degradation. As the cells were in true steady state in the chemostat, the rate of loss of label included the irreversible losses by exit of cells from the system at a defined rate. Thus, the intracellular degradation rates were obtained by applying an appropriate correction factor to account for such loss; the first-order constants for dilution and degradation were simply additive to generate the first-order rate constant for loss. While this work was performed in tightly controlled cell culture and involved the relatively low-throughput 2DE methodology, the techniques provide a basis for future studies in more complex systems, in addition to yielding important information about intracellular protein stability in yeast. Whilst high-throughput methodology was not used, the 2DE was used in the preliminary study to provide a proof-of-concept and to generate multiple, rate-informative peptides from each protein. Naturally, the labeling methodology is ultimately amenable to analysis by LC/MS or LC/MS/MS methods, but this would increase reliance of a turnover rate determined by a single peptide.

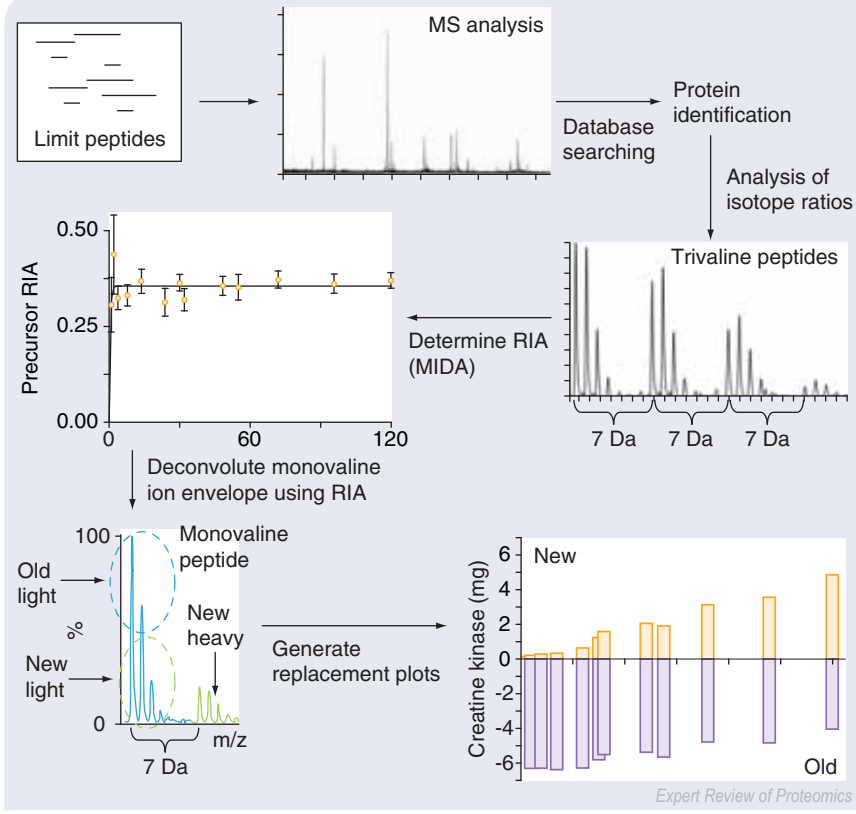


**Figure 5. Proteome turnover: experimental design.** In a typical proteomics experiment, limit peptides are derived from the proteolytic digest of a sample of interest. These are subjected to mass spectrometric analysis and the protein identified either by searching databases with peptide mass fingerprint or sequence information. In proteome turnover, the mass spectrum is manually inspected for peak envelopes attributed to isotopomers of the same peptide. In the example shown, a peptide containing three valine residues has been identified from glyceraldehyde-3-phosphate dehydrogenase. The ratio of the peak intensities can be used to determine the relative isotope abundance of the precursor pool directly from the protein of interest. This can be used to deconstruct the peak pairs derived from a monovaline peptide to allow calculation of the amount of newly synthesized material and the amount of residual material. This is performed for several time points, thereby allowing replacement plots to be determined and, ultimately, synthesis and degradation rates.
MIDA: Mass isotopomer distribution analysis; MS: Mass spectrometry; m/z: Mass-to-charge ratio; RIA: Relative isotope abundance.

In a similar study, Bouwman and colleagues studied mature 3T3-L1 adipocytes from mouse labeled with L-(2,3,4,5,6 [$^2H_5$]) phenylalanine [56]. Differentiated mouse adipocytes were incubated with the labeled amino acid for 0, 6 and 24 h. As with the study of *S. cerevisiae* [58], no differences in protein profile were observed by either 1D or 2D sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), indicating that labeling did not interfere with the pattern of protein expression. Labeling of individual proteins was assessed by random selection of 27 of the most prominent bands by 1DE. Of the 27 excised proteins, 17 had at least one identifiable phenylalanine-containing peptide. A semiquantitative value with respect to labeling efficiency was assigned to the proteins by determining the isotopomer peak area ratios at each time point. Of the 17 proteins, two were significantly labeled after 6 h and an additional nine were labeled after 24 h. No significant labeling was observed for the other proteins. It was possible to discriminate between rapidly synthesized proteins (collagen) and slowly synthesized proteins (cytoskeletal). This indicated an active metabolism of the extracellular matrix. Reported errors in turnover rate were very high for many proteins analyzed by the stable isotope-labeling method, especially in the 1DE analysis. This could be as a result of low signal-to-noise ratio in some spectra. Additionally, in the 1D experiment, over 35% of the proteins could not be analyzed due to the absence of a phenylalanine in the tryptic peptides in the analyzable mass-to-charge ratio range. While this

can have advantages in simplification of the analyte mixture, in MIDA, it is important to balance simplification of the sample with adequate abundance of the labeled amino acid. Also, mass spectra must be of sufficient quality to allow quantification of the ion derived from the labeled peptide.

It is possible to use metabolites other than amino acids to measure protein turnover. Glucose labeled uniformly with $^{13}C$ in combination with MS and MIDA was used as the precursor to monitor the relative synthesis/degradation ratio of proteins from *Escherichia coli* [60]. Cultures were grown to an optical density of 0.4 prior to labeling. $^{13}C$ Glu was added to the cell culture and cells were harvested after 30 min of labeling. Proteins were separated by SDS-PAGE, subjected to enzymatic proteolysis followed by nanobore reverse-phase high-performance (HP)LC and analysis by MALDI-TOF/TOF-MS. Essentially, the method used culture doping to measure the relative ratio of new protein synthesis to protein degradation for each specific protein in a cell. Culture doping requires that the cells have enough time to take up and start incorporating the substrate into proteins. For prokaryotes, this problem is solved by waiting approximately one cell doubling time, which is possible as the measurement is relative, not absolute. Since the proteins (and thus the peptides) were labeled with a $^{13}C$ isotope, the labeled and unlabeled peptides co-eluted chromatographically. This co-elution of peptides has been said to be critical for the calculation of a ratio for a particular peptide pair [60]. However, such ratios can be calculated without co-elution of the peaks (e.g., when a deuterated amino acid is used as the precursor) [61,62]. The mass-to-charge ratio offset observed can be calculated and scanned for, thus facilitating high-throughput methods. This can now be automated using software to generate chromatograms directly from tandem mass spectra [63].

In the *E. coli* study, a computer program was used to calculate the theoretical distribution based on the estimated percentage incorporated label. This resulted in a 25% error when compared with the experimental data. This suggests that the labeled glucose does not have rapid equilibration in some pools of biological molecules. A Poisson model was used to analyse the data. It was found that, although the Poisson model used was just an approximation of the labeled distribution and did not provide any insight into the biology behind the isotopic distribution, it provided an easy way to automate the data analysis. However, this still resulted in a 15% error compared with experimental data. In addition, there was significant induction of a particular protein (putatively formate acetyltransferase) upon addition of the label. It was stated that this was probably due to the microaerobic environment under which the cells were grown, but this should be verified by relevant control experiments. The relatively large errors encountered and the apparent stimulation of protein synthesis somewhat detracts from this study. The choice of glucose as a carbon source introduces complexity, as it labels different amino acids to different extents. This variation in labeling dynamics between amino acid pools means that it is difficult to define absolute rates of protein labeling and, thus, absolute rates of synthesis, degradation and turnover. The methodology used in the yeast work described earlier overcomes this by using a single amino acid precursor so the source of the label and the position and amount of label in the peptide were clearly defined, thereby facilitating careful manipulation of the RIA of the amino acid precursor pool. Also, control of the growth conditions of the yeast using a chemostat ensured that protein profiles were consistent between labeling experiments and controls.

Proteome dynamics can also be used to probe specific subcellular systems. In a study by Andersen and coworkers, HeLa cell nucleoli were isolated and the response to transcription inhibition determined [57]. A HeLa cell line was metabolically labeled with either unlabeled arginine, $^{14}N_4$ Arg or $^{13}C_6{}^{15}N_4$ Arg, and each sample treated with the transcription inhibitor actinomycin D at different times. Equal amounts of cells from each time point were mixed, the nucleoli isolated and LC/MS/MS performed on the tryptic peptides derived following proteolysis. Different nucleolar factors demonstrated major differences in their kinetics, with proteins involved in biogenesis of the 40S preribosomal subunit displaying some of the most marked changes. The data provided a more detailed and quantitative insight into how environmental stress and growth conditions affect the nucleolus.

### Proteome turnover in multicellular organisms

Whilst the work discussed in the preceding section has advanced our understanding of protein turnover determination, the complex issues encountered with whole-body protein turnover were not addressed. In cell culture, the amount of labeled amino acid that is used by the cell for the synthesis of new proteins can be controlled. Multicellular organisms are more complex in terms of defining label incorporation, compartmentation of amino acids and precursors, and difficulties in determining the true isotope abundance available for incorporation during protein synthesis. These are further compounded by protein degradation from a number of different tissues and subsequent reutilization of the amino acids. When working with animals, additional issues must be considered, including how to administer the label (i.e., by injection or by diet). If choosing to administer the label by diet, the diet must be designed so that the animal receives its full complement of vitamins and minerals while maintaining sufficient palatability, and allowing maximal incorporation of the labeled material. This is not straightforward, and before embarking on such a study, considerations regarding the nutritional needs of the animal subject must be made. The authors' group, and others, have used stable isotope labeling with MIDA to develop methods for determining protein turnover at the proteome level using adaptations of stable isotope labeling techniques in tandem with MIDA analysis [19,56,58,60]. This has proved an additional dimension to both whole-body protein turnover and proteome turnover in unicellular organisms, both in terms of the complexity of the system and experiments and in the potential knowledge gain in the ability, for the first time, to probe the dynamics of protein expression in a whole animal.

Work by Hellerstein and colleagues established and validated methodology for determining protein turnover in complex organisms using a serum extract from a labeled rat to calculate the absolute rate of albumin synthesis [19]. In a subsequent study, they examined the turnover of two muscle proteins (creatine kinase and myosin) in rat using MIDA [64]. The stable isotope-labeled amino acid, in this case 5,5,5-$^{2}H_3$ Leu, was introduced to the rat via a jugular catheter for 24 h. Creatine kinase was isolated by HPLC and subjected to trypsin digestion. A specific, leucine-rich peptide was isolated, again by HPLC, and MIDA was performed. Myosin was also purified from the muscle homogenate and subjected to acid hydrolysis. For creatine kinase, the mass isotopomer abundances of the unlabeled peptide (derived from a control experiment) were close to theoretical values. When the labeled peptide was analyzed, it was found that incorporation of labeled leucine into the peptide was low, and a fractional synthesis rate of 0.12 per day was calculated. Again, with myosin, incorporation of labeled leucine was found to be low, and this was attributed to the fact that both proteins turn over slowly in muscle. Control experiments demonstrated that the aminoacyl-tRNA in the muscle did incorporate labeled amino acid from plasma.

A limitation of the Hellerstein work was that individual peptides were isolated from purified protein preparations. For this methodology to become widely used by the proteomics community, there is a need for adaptation to more high-throughput techniques. An aim of the authors' laboratory is to extend the stable isotope-labeling strategy used by Hellerstein, and that used in the *Saccharomyces* continuous culture experiments, to analyze protein turnover of multiple proteins simultaneously in a complex organism.

The chicken can be regarded as a potent genetic and physiological model system for the study of muscle growth [65–67]. Chickens have been bred for generations for two different traits; layer chicks for their egg production and broilers for meat production. Broilers grow at dramatically higher rates than layers and previous data have indicated that this higher rate of protein accretion is predominantly as a result of decreased rates of intracellular protein degradation [67]. The aim was to determine whether it was possible to calculate rates of protein synthesis and degradation in a growing animal using stable isotope labeling of proteins [65]. The label, $^{2}H_8$ Val, was administered to the birds within their diet. Birds, both broiler and layer, were reared to 5 days post hatching on a semisynthetic diet. The diet was marginally limiting in valine to enhance incorporation into protein. Unlike cells in culture, it is not possible to fully label the proteins in an animal. Chickens will disfavor fully synthetic diets and were supplied a semisynthetic diet in which half of the valine was present in a crystalline form. As the valine could be either deuterated (labeled) or undeuterated (unlabeled), this facilitated easy switching of the diet from the unlabeled to the labeled form. Therefore, it follows that the maximal labeling of protein attainable was 50%. Following the switch to the diet containing $^{2}H_8$ valine, birds were maintained for a 120-h labeling period. Muscle samples were obtained from birds culled at a series of time points through 120 h. The soluble pectoralis muscle proteins were extracted and separated by 1DE. The protein profile was consistent over the labeling period. Proteins were extracted from the gels and the tryptic digests analyzed by MALDI-TOF-MS and MIDA (FIGURE 5). Before turnover rates could be determined, it was essential to determine the RIA of the protein precursor pool and to confirm that this was the same for individual proteins in the system. In this experiment, the RIA is a measure of the degree of labeling of the amino acid that is available for protein synthesis. It can be calculated directly from peptides derived from proteins (FIGURE 3) and, as discussed earlier, obviates the determination of any other precursor pool. This technique is a substantial advance over the methodologies used previously, such as the continuous infusion or flooding dose methods, as it does not require modeling of the precursor pool compartments. Once the RIA has been established, it can be used to determine how much newly synthesized material is present in the tissue at a given time, using the relative intensity of the unlabeled and labeled peptide ions in the mass spectra. In chicken, the RIA was calculated to be 0.35; that is, 35% of the valine available to the cell for protein synthesis was in the deuterated form. This value was essentially constant after approximately 3.5 h of labeling and was the same for all proteins analyzed, with very small biological variation. As the birds were supplied with 50% of their valine in the heavy isotope-labeled form, it may be expected that the observed RIA would approach 0.5. However, the precursor pool is constantly being replenished with amino acids from protein degradation that diluted the labeled amino acid in the precursor pool.

If the exposure to labeled diet had been maintained for long enough, the RIA in the proteins would reach that in the diet, both through protein replacement and expansion of the protein pool, all of which must come from *de novo* synthesis. However, for this type of experiment, it is sufficient that the RIA can be defined and is, to a first approximation, constant over the experimental period. Replacement plots (i.e., the percentage of protein that is newly synthesized compared with that which was pre-existing) were obtained for nine of the major proteins in chicken skeletal muscle, indicating that between 55% (creatine kinase) and 90% (phosphoglycerate mutase) of the protein had been newly synthesized during the 120-h labeling period. Current work will determine turnover rates in broiler chicken and thus provide an insight into the differences in protein dynamics between these two strains, which has such a remarkable effect on muscle growth.

Vogt and coworkers have taken a different strategy to determine turnover in intact animals [68]. A single mouse was labeled with a constant infusion of $^{13}C$ Glu via a central venous catheter inserted into the right jugular vein. The mouse was exposed to a 10-h labeling regime, sacrificed and the liver retained for analysis. An unlabeled control mouse was similarly sacrificed. Proteins were separated by 2D-PAGE, subjected to in-gel trypsin digestion and the resultant peptides analyzed by MALDI-TOF-MS. Since glucose was used as the labeled precursor, complex

spectral patterns were obtained and mathematical deconvolution was required prior to the derivation of fractional synthesis rates of individual proteins. To consider the overall efficiency of labeling, free metabolites (alanine, glutamate and glucose) in the liver were analyzed using gas chromatography (GC) MS as a preliminary step. These metabolites have high tissue concentrations and reflect the branch points for the transfer of label from glucose to the amino acids. The average mass shifts (unlabeled to labeled) deduced from the GC/MS analysis were 3.4 Da for glucose, 1.2 Da for alanine and 1 Da for glutamate. The carbon skeleton of one half of glucose is converted into pyruvate, which is in close exchange with alanine. This should give a mass shift of 1.7 Da. The observed mass shift of 1.2 Da for alanine implies that almost 60% of the alanine–pyruvate moiety is derived from glucose and the other part is derived from unlabeled sources such as amino acids released by unlabeled protein. Glutamate undergoes a further dilution by unlabeled material. The mass spectra of the proteins derived from labeled and unlabeled mice were analyzed to determine the mass shifts of their peptides. The largest mass shifts were seen for major urinary proteins (MUPs). The labeling of MUPs results in a strong enrichment of the heavy mass peptides in the mass distributions, and is most pronounced for MUP1. This was therefore chosen as the reference protein. The FSR of MUP1 was estimated by calculating the RIA values of the monoisotopic peak, which declines from 0.41 for the peptides with natural isotopic composition to 0.03 for the labeled peptides. The FSR was calculated to be 95% per 8 h and used thereafter as the reference protein. The FSR of more than 40 protein spots was subsequently determined with reference to MUP1. This study is an interesting adaptation of the work of Hellerstein and the authors' laboratory, but is somewhat complicated by the choice of glucose as precursor, as discussed earlier in this review. The mass spectra recorded are complex, and the need to tether all calculations of FSR to an experimentally derived denominator has obvious drawbacks. Moreover, in this study, only one labeled and one control mouse were used, which limits the conclusions that can be made. However, the work presents an encouraging advance towards high-throughput proteome dynamic determination, essential for the full integration required for systems biology.

A number of issues must be addressed when using analytical methods. These include the sensitivity of the technique, the dynamic range and the reproducibility between both sample and experiments. Ultimately, in MS, the sensitivity, and hence the amount of label required to produce a signal distinct from background, is dependent on the mass spectrometer to be used. However, in general, it is advisable that the heavy labeled stable isotope accounts for 5–10% of the intensity of the combined light plus heavy peptide ion intensity. The issue of dynamic range is common to all proteomic experiments. Without some form of fractionation, subsequent to labeling but prior to analysis, abundant proteins will dominate. However, the labeling methodologies discussed do not preclude fractionation and even isolation and enrichment of subcellular complexes or organelle-specific proteins. Whilst many of the methods discussed have involved gel electrophoresis steps for simplification of the sample, this is not an essential step. LC/MS methods are equally applicable, although they may result in more complex mass spectra that will require automated methods for chromatogram extraction. The methods described are highly reproducible with errors typically below 5–10%. As multiple peptides are used for each determination at each time point, confidence in the measurement is typically high.

### Expert commentary & five-year view

Until recently, the ability to determine protein turnover has been limited due to technical limitations in the accurate analysis of subtle changes in protein expression levels. Traditionally, protein turnover has been determined at the whole-tissue or organism level using radioisotope tracers that, while giving a relatively rapid indication of changes in bulk protein turnover, are unable to identify the turnover rates of multiple individual proteins. It has been a useful tool in the monitoring of disease states clinically, but suffers from a number of problems that have been outlined in this review. In contrast, proteomics-based methodologies facilitate the analysis of individual proteins, but on a global scale. These methods generally involve administration of a stable isotope to the organism of interest, fractionation of the protein sample, either by gel electrophoresis or chromatography, followed by mass spectrometric analysis. Proteomic methods are developing at a rapid pace and the ability to combine dynamic data with absolute quantification of individual proteins at a proteome-wide level will allow researchers to probe mechanisms of protein turnover and integrate these data with information from genome, transcriptome and metabolome studies, thereby providing a truly global perspective of a particular biological system or disease state. One limiting factor is the current ability of bioinformatics tools to cope with the volume and scope of data produced by such studies. Currently, data analysis and mass scanning for isotopic variants invariably involves manual inspection and manipulation of mass spectra, which is laborious. Dedicated bioinformatic tools for the analyses of complex labeling patterns are required before stable isotope-labeling strategies for the determination of protein turnover can be truly high throughput. In addition, it is important that the data obtained from proteome turnover studies can be integrated with transcriptomic data. Whilst a large body of data exists that describes changes in transcript levels under different physiological conditions, in common with conventional proteomics, these data represent a snapshot. For a full understanding of the complex interactions, an equivalently complete description of mRNA turnover must be obtained and correlated with proteome dynamics.

As proteomics develops into a strictly quantitative discipline, the challenge will be to acquire multiple parameters in a single analysis, demanding convergent high-efficiency methodologies. A time can be anticipated when the absolute amount of a protein and its rate of turnover may be determined by a single analysis. Since this would be maximally efficient if a single peptide could

be analyzed as a surrogate for each protein, the acquisition of these parameters would be further aided by strategies for selective isolation and recovery of targeted peptides. Recent work from the authors' group has provided two novel strategies, one for multiplexed absolute quantification using concatenated standard peptides encoded into synthetic genes [47], and the second for a highly selective isolation of recovery of the N-terminal peptide from each protein (positional proteomics) [69]. A strategy can be envisaged where convergence of quantitative and positional proteomics, coupled with intelligently designed metabolic labeling, will lead to a complete picture of gene expression in protein space.

### *Conclusions*

Protein turnover is an important link between the transcriptome, proteome and metabolome, without which it is difficult to gain a true systems biology integration of a system. The challenge is to track changes in gene expression to the end point, whether protein or metabolite, and to pursue interactions between biomolecules that act together to dictate phenotypes of cells in their physiological or pathophysiological state. The ability to measure flux and relate it to changes at other levels of a system is key to understanding the complex interactions of genes, proteins and metabolites. This review has detailed different methods of monitoring protein turnover and has discussed the advantages and limitations of each method. The aim has been to provide an overview of both traditional methods used to analyze whole-body protein turnover and the exciting new advances in proteome turnover.

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Key issues

- As functional genomics evolves into systems biology, new approaches are required to link transcriptome, proteome and metabolome, particularly in respect of the dynamics of the different levels.
- The rates of protein synthesis and protein degradation can be determined via stable isotope labeling in conjunction with proteomic technologies, permitting the global analysis of individual protein turnover. This has been successfully applied to both eukaryotic and prokaryotic systems.
- There is a pressing need for new bioinformatic tools to allow high-throughput analyses of proteome turnover, irrespective of experimental model and protocols.

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