Technique review

The dynamics of the proteome: Strategies for measuring protein turnover on a proteome-wide scale

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Abstract

Quantitative proteomics captures the steady-state amount of a protein in a cell but does not explain how a change in protein amount is manifest — whether through a change in synthesis or a change in degradation. If we are to understand the changes in the proteome, we will need to define such processes. In this brief review, strategies for the determination of intracellular protein dynamics on a proteome-wide scale are discussed.

INTRODUCTION

It is now well accepted that the correlation between the abundance of proteins in a proteome and their cognate mRNAs (transcriptomes) is not strong.[1–3] Since both parameters reflect the outcome of complex process that control the cellular concentrations of mRNAs and proteins, and their activities, however, we should be surprised that a correlation exists at all. Both mRNA and protein molecules are dynamic entities, and their presence in the cell is the outcome of opposing processes that bring about their biosynthesis or destruction. Space does not permit a detailed discussion of the processes that regulate mRNA or protein concentrations in the cell, but the proteome, defined here as the abundance-weighted total of all proteins in a cell or subcellular space, is also dynamic. The dynamic nature of the proteome has important consequences, and requires that we evolve our perspective of proteomics in general, and in comparative proteomics in particular.

Other than 'identification proteomics', in which the goal is to identify the constituent proteins of a proteome sample, most studies are of 'comparative proteomics' and usually have the objective of a pairwise comparison of two proteomes. Whether by two-dimensional gels or liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods, the result is the same — a dataset that defines the relative abundance of specific peptides derived from the two proteomes. Few studies are able to define the proteome in terms of the absolute amounts of each protein, and it might be argued that a full understanding of the proteome requires that we also shift from relative to absolute quantification. In response, it could be countered that the goal is identification of proteins that change, from which the mechanisms of change, and the biological drivers, can be discerned. Many proteomics studies therefore yield collations of proteins that change in relative expression levels. The value of such studies is immense, provided that their limitations are also recognised. The greatest of these limitations is that we

Article number = K120

cannot assume that a change in protein expression is the direct outcome of a change in the rate of transcription of the cognate gene. This assumption is untenable, and, indeed, it is not possible to reconstruct the transcriptome: proteome relationship without detailed knowledge of the dynamics of both processes.

With a specific relationship to the proteome, the amount of a true intracellular protein in a cell is the result of the opposing processes of protein synthesis and protein degradation — for extracellular proteins, we also have to factor in the irreversible loss due to secretion. If a protein decreases in amount in a cell, this can be a consequence of a diminution of mRNA, or of a decrease in ribosomal activity or translation initiation. Equally likely, it could be the result of enhanced degradation of the protein (Figure 1). It follows that the measurement of protein abundance (absolute or relative) does not implicitly define mechanism, and that the condensation of two separate rate processes into a single measured parameter renders the solution indeterminate. Only by direct assessment of the dynamics, as well as the quantity, of the proteome, is it possible to unravel the response.

This brief article addresses the challenges attendant upon determination of proteome dynamics (synonymous with protein turnover), and discusses some experimental strategies that can provide at least some of the data that are required. Methodologies for the measurement of rates of turnover are distinguished from those targeted at mechanistic aspects of protein breakdown, with a particular emphasis on the former.

PROTEIN TURNOVER: CONCEPTS, MECHANISMS AND CHALLENGES

Our overall view on the kinetics of protein metabolism has not evolved a great deal in recent years, and there are some excellent reference works from several decades ago that still serve as useful primers, especially in animal systems;[4] however, there have been some noteworthy recent reviews on protein turnover in microbial systems, as cited here.[5–7] In general, it is assumed that the rate of protein synthesis in the cell is a function of the mRNA concentration, the rate of initiation of translation and an overall term describing ribosomal activity. Most importantly, the rate of protein synthesis is not thought to be directly coupled to the concentration of protein in the cell — in other words, translation is

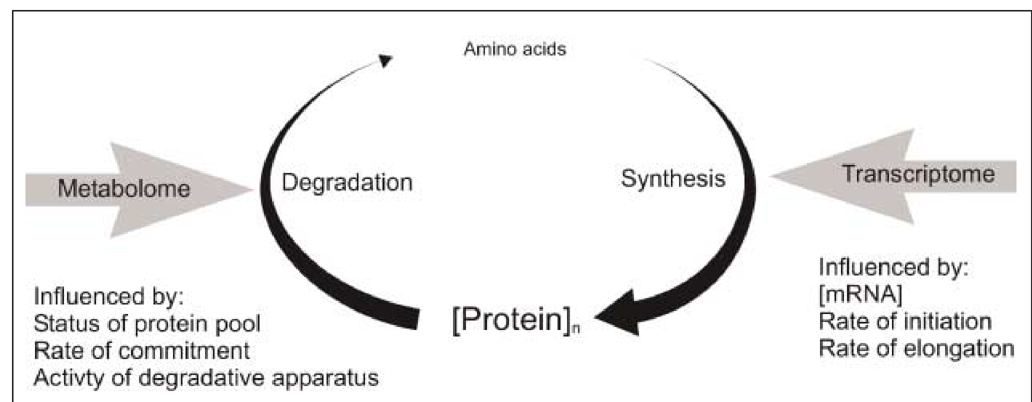

Figure 1: A simple model of protein turnover. The dynamic state of proteins in the cell is often overlooked. Each protein has a defined intracellular stability that is an evolved property, and the amount of any protein in the cell is the outcome of the opposing processes of synthesis and destruction. The rate of synthesis is most closely linked to the transcriptome, and the rate of degradation is linked to the metabolome, emphasising the central role of proteome dynamics in the definition of the cellular state.

not sensitive to the protein concentration. Protein degradation is, to a first approximation, a first-order process. The first-order rate constant, often expressed as a half-life, defines the fractional rate of degradation of a protein, for example, a value of 0.1 h^{-1} is synonymous with degradation of one-tenth, or 10 per cent of the protein pool each hour. Flux through the pathway is therefore also dictated by the size of the protein pool, which is then in the steady state, balanced by the flux through the synthetic route. This gives us a simple turnover equation:

$$\frac{dP}{dt} = ks - kd \cdot [P] \quad (1)$$

which links the rate change of size of the protein pool to synthesis (k_s) and degradation (k_d) and the protein pool [P]. Of course, if the protein pool is in steady state, then dP/dt is zero and the equation simplifies even further to:

$$[P] = \frac{ks}{kd} \quad (2)$$

Taking the steady-state situation (Equation 2), we therefore have a three-term relationship, linking the rates of synthesis and breakdown to the protein concentration. It follows that if we can measure two of these parameters, we are able to calculate the third. Assuming that the simplest of the three terms to measure is protein concentration [P] (which, incidentally, emphasises the challenge of absolute quantification in proteomics), it follows that we must devise methods to measure protein synthesis or protein degradation rates, and to be able to deliver these measurements over a large subset of the proteome, on a protein by protein basis. This might seem obvious, but it is worth recalling that most studies on protein turnover have either focused on single proteins or have measured the turnover of total protein, which obscures the behaviour of different proteins within the proteome.

The determination of protein turnover rates is of particular significance when two proteomes are compared. Most proteomics investigations are comparisons between two different physiological or pathophysiological conditions, and differences in protein abundance that are discovered if placed in mechanistic terms must then be defined as changes in input to the protein pool (synthesis) or changes in removal (degradation). It follows that we will need to develop methodologies to measure the rates of flux through the pool of a particular protein.

METHODS FOR MEASUREMENT OF PROTEIN TURNOVER

As might be anticipated, measurement of flux through a protein pool, which might in itself be unchanging in size, requires labelled metabolic tracers. Previous studies have either used radioactive isotopes ([^{14}C], [^{3}H], [^{35}S]) to label 'total protein' or as an adjunct to immunoprecipitation, or gel separation/autoradiography to monitor individual proteins. Apart from the hazards associated with the use of radioisotopes, it can be challenging to obtain adequate incorporation to label low abundance or low turnover proteins. The advantage of radioisotopes is that the degree of incorporation does not need to be high in order to generate a measurable extent of labelling, provided that the specific radioactivity of the precursor pool is high enough. Scintillation counting or autoradiography is capable of measuring a very small extent of incorporation. In proteomics studies, however, we are more comfortable with the use of stable isotopes for comparative proteomics. The stable isotopes are either introduced chemically (for example, isotope-coded affinity tags and isotope tags for relative and absolute quantification),[8,9] enzymatically ([^{18}O] labelling during proteolysis,[10] or metabolically. In this last category, the emergent approach of stable isotope labelling with amino acids in cell culture (SILAC) has enormous potential (refs). In SILAC experiments, cells are fully labelled with stable isotope-labelled amino acids, and the labelled cells are

then compared with the second experimental state, in which unlabelled cells are used. This approach can even be extended to three-label methods, permitting more elegant multiplexed experimental designs.[11–14] A key requirement of the SILAC approach, however, is that the cells are completely labelled. In cell culture, the cells are actively dividing, and the biomass is increasing. After one cell doubling, and assuming that the precursor amino acid pool is fully labelled (a relative isotope abundance of 1), then a minimum of half of the proteins in the cell will be labelled with the stable isotope precursor. Of course, if the protein was subject to a high rate of protein turnover, the extent of labelling will be even higher, and rapid-turnover proteins could be fully labelled in the same time frame. For example, a doubling time of six hours means that all proteins will be minimally 50 per cent labelled during that time. A protein with a half-life of 30 minutes, however, will have undergone substantial replacement during this time and will, to all intents and purposes, be completely labelled. A significant limitation to the use of stable isotopes is the difficulty associated with measurement of trace levels of stable isotope. The types of mass spectrometers used in proteomics would not generally be considered as reliable in the measurement of less than a few per cent of stable isotope-labelled variant, which precludes trace labelling. In turn, this means that a significant degree of incorporation must be factored into the experimental design.

Therefore, the goal in SILAC experiments is to obtain complete labelling, so that the pairwise comparison is not complicated by issues of partial labelling. It follows that if all proteins are fully labelled, it is no longer possible to obtain any information from the extent of labelling about the relative rates of turnover of proteins. Therefore, a properly conducted SILAC experiment provides no information on the mechanism (synthesis or degradation) whereby a differentially expressed protein is expressed at changed levels.

Therefore, the corollary is that to acquire turnover data, the proteins must be partially or incompletely labelled. The precursor can be as simple as [^{15}N] ammonium chloride or [^{13}C] glucose, but, more commonly, is provided in the form of a stable isotope-labelled amino acid. Labelling with [^{15}N] introduces the complication of variable degrees of labelling of different peptides (according to their length and the content of amino acids with N-containing side chains). Labelling with [^{13}C] glucose yields an even more complex labelling pattern, because the carbon atoms will appear in all amino acids (in an organism competent in the biosynthesis of all amino acids), which nonetheless can be used to acquire relative turnover rates.[15] Although such precursors can be used, there is much to commend a strategy based on stable isotope-labelled amino acids, even if only advocated on the basis of simplicity of the subsequent labelling patterns. The choice of amino acid is, in turn, dictated by several factors, the most important of which are abundance in the proteome of interest and metabolic stability.[16] The metabolism of amino acids is a critical feature, especially for particular labelling sites. For example, the guanidino group of arginine is rapidly exchanged through the reactions of the urea cycle in animals, and for deuterated amino acids, an alpha carbon deuteron can be rapidly lost through transamination. Other amino acids are metabolically linked, such that label in one amino acid might also appear in a different amino acid. There are, however, multiple options for amino acid-derived labelling strategies.[16] Irrespective of the precursor amino acid selected, the measurement of protein turnover through stable isotopes is made much simpler if the precursor relative isotope abundance (RIA) can be brought to near unity. In other words, the experiment should be designed such that all of the precursor label is either 'heavy' or 'light', with the further caveat that it is

necessary to effect the change from one to the other rapidly, and with sufficient 'chase' material to eliminate issues of reutilisation.

STRATEGIES WITH CELLS IN CULTURE

There are two strategies for determination of protein dynamics on a proteome-wide scale. Restricting the arguments to single-cell systems to begin with, the first approach starts with cells grown in unlabelled medium, which is then changed to an identical medium in which an amino acid is replaced by its stable isotope counterpart. The second approach is to prelabel cells in medium containing labelled amino acids, and then switch to medium containing unlabelled amino acids. The two experimental approaches are formally equivalent — it does not matter whether one is measuring incorporation of labelled amino acids or incorporation of unlabelled amino acids; however, practical considerations may influence the decision as to which strategy is best. In both approaches, the key requirement is that the precursor RIA changes from zero to unity, or vice versa, very rapidly. This usually requires an excess of the amino acid in the chase phase, and this is most economically attained if the chase amino acid is unlabelled.

In one study on protein breakdown in *Saccharomyces cerevisiae*, the authors elected to use the prelabelling strategy.[17] To maintain a constant physiological state, cells were grown in a chemostat at constant growth rate, in order to limit the effects caused by time-dependent changes as the culture moved through lag, exponential and stationary phases. The label was [$^2H_{10}$] leucine. The yeast strain was a leucine auxotroph, which meant that the organism would be incapable of diluting the exogenous pool of label through synthesis *de novo* of the amino acid. After seven doubling times, all of the proteins were fully labelled with [$^2H_9$] leucine (the loss of a single deuteron was due to transamination, and serves to confirm the equilibration of the precursor pool with the intracellular amino acid pool). Parenthetically, there was no influence of the labelling protocol on the growth rate, rate of CO_2 production or pattern of expressed proteins on a two-dimensional gel, when compared with an unlabelled equivalent. To measure the rates of protein degradation, an excess of unlabelled leucine was added to the medium, and the feedstock changed to one lacking labelled leucine. To prevent the change in medium concentration of leucine from affecting the growth rate, the cells were grown under glucose-limiting conditions, which meant that there was no change in biomass or growth rate when leucine was added. At different times, the culture vessel was sampled, and soluble proteins were resolved on two-dimensional gels. The same spot was recovered from each gel, and subjected to in-gel digestion and matrix-assisted laser desorption ionisation–time-of-flight (MALDI-ToF) mass spectrometry. The leucine-containing peptides in the mass spectra showed a gradual transition from all 'heavy' to all 'light' as the protein was turned over and also lost from the culture vessel as cells were eluted (Figure 2). From the ion intensities, it was possible to determine the RIA of the amino acid in the protein. The change in RIA from unity to zero was first order, and the rate constant reflected the sum of $k_{dilution}$ and $k_{degradation}$. Since $k_{dilution}$ was known, this permitted the calculation of $k_{degradation}$ (Figure 3).

MEASUREMENT OF PROTEOME DYNAMICS IN COMPLEX SYSTEMS

The ease with which stable isotope labels can be introduced into single cells grown in culture is not matched by an equivalent ease of experimental design when one wishes to measure proteome stability in, for example, intact animals. Because of the need to incorporate a significant amount of label, it is essential that the exposure of the biosynthetic apparatus to labelled precursors be extended. For

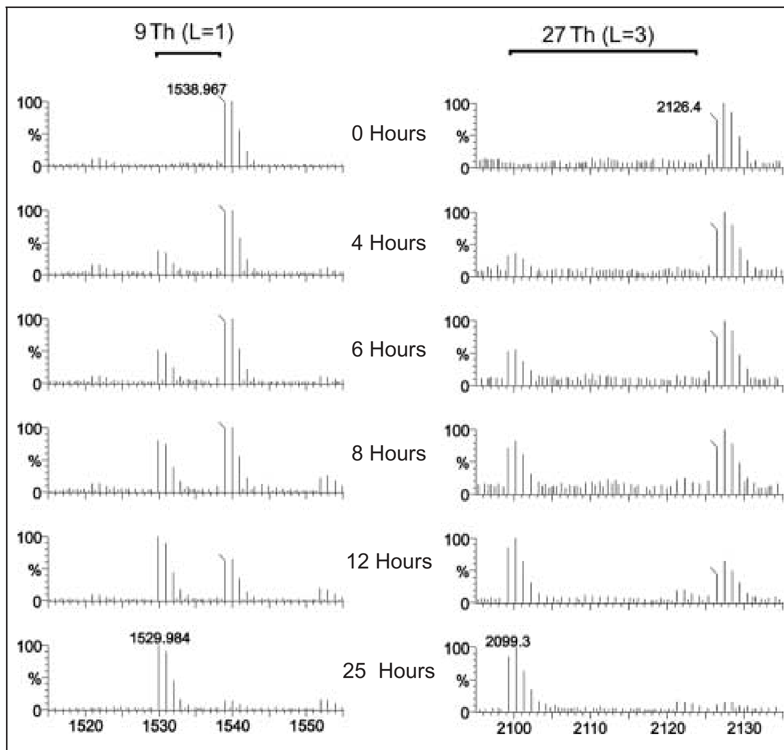

Figure 2: Protein degradation in yeast. *Saccharomyces cerevisiae* was grown in steady state in a chemostat in a medium containing stable isotope-labelled leucine (relative isotope abundance [RIA] = 1) until all proteins were fully labelled. At this point, the medium was switched to one containing unlabelled leucine, and the cells were sampled over the next 25 hours. Proteins were resolved on two-dimensional gel and specific protein spots were excised and subjected to in-gel digestion with trypsin prior to matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry. As the proteins become 'unlabelled', the mass spectrum shows a gradual shift of leucine-containing peptides from fully 'heavy' to fully 'light'. Mass spectra are shown for peptides containing one (L = 1) or three (L = 3) leucine residues. Note the lack of intermediate, partially labelled isotopomers of the trileucine peptide, which is good evidence for the RIA shift from unity to zero being effective and instantaneous (see text). Th: thomson, unit of mass/charge.

example, the average turnover rate of skeletal muscle protein in the mouse is 10 per cent per day. Even if the labelled precursor could be designed to have an RIA of unity, for muscle protein, a labelling period of several days would seem appropriate. This precludes infusion of isotope or the use of osmotic minipumps, as does the sheer quantity of labelled precursor that would be required. An alternative approach is that of oral administration of label in the diet. Since totally synthetic diets are rather unpalatable, the best approach is probably to design a diet in which some of the amino acid is provided in unlabelled form in normal dietary constituents, and some of which is provided as stable isotopes.

This approach has defined the present author's exploratory studies in proteome dynamics in chicken skeletal muscle.[18] The chicken is chosen for two reasons. First, chickens are not meal-feeders, and, under conditions of extended day length, will peck at food and eat throughout the light period. Moreover, the crop and gizzard act as natural integrators of the ingesta, and ensure that the labelled diet is delivered to the digestive/absorptive systems at a constant rate. Secondly, the dramatic difference in growth rate between chickens grown for meat (broilers) and those bred for egg production (layers) provides a naturally selected system to study muscle growth. Birds were fed an artificial, semisynthetic diet containing stable isotope-labelled valine at a calculated RIA of 0.5. The labelled valine was included as a crystalline amino acid, and the unlabelled valine was present in grain proteins. As the birds ingested the diet, the proteins in the tissues, resolved by gel electrophoresis and analysed by MALDI-ToF of tryptic peptides, became progressively and slowly labelled; however, there was a major difference between this study and the previous study of yeast. In this experiment, the RIA of the precursor was less than unity, and this has a considerable effect on the labelling pattern of the peptides (Figure 4). In particular, because the RIA is less than one, the pattern for peptides containing multiple instances of the labelled amino acid is more complex. If, for example, the RIA is 0.5, this means that there is a 50:50 chance of incorporating a 'heavy' valine at each position in the protein. After tryptic proteolysis, this manifests itself in a complex pattern of isotope distribution. For example, a trivaline peptide from the chicken study can therefore exist in four forms: LLL, |LLH|, |LHH| and HHH. Although there is only one LLL and HHH species, there are three positional isomers of the other two variants. Thus,

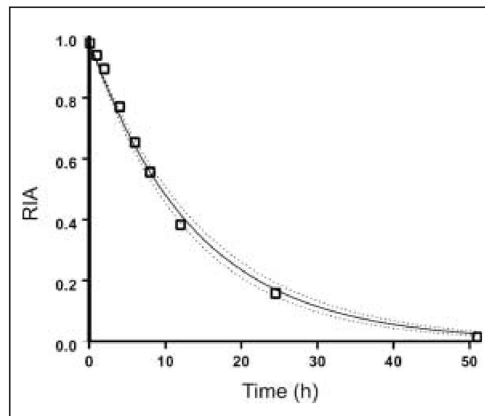

Figure 3: Loss of label in yeast. The loss of label from the 'unlabelling' experiment described in Figure 2 was expressed as a change in relative isotope abundance (RIA) as a function of time. The RIA declines in a strictly first-order process, and from the rate constant and knowledge of the chemostat dilution rate it is possible to calculate the rate of degradation. The solid line is the first-order curve fitted to the data, flanked by the 95 per cent confidence intervals for the fitted curves (dotted lines).

[LLH] should really be defined as [LLH, LHL and HLL] and [LHH] should really be defined as [LHH, HLH and HHL]. In turn, this means that there are three times as many ways to make the two heterogeneous isomers, and that given a precursor RIA of 0.5, the ratio of the ion intensities for the products is 1:3:3:1, a classical binomial expansion. Thus, the labelling pattern is much more complicated than the situation when the RIA is unity. These complex patterns can be turned to advantage, however, as they permit the calculation of the precursor RIA — any asymmetry from a 1:1 ratio of the intensities of the [LLH] and [LHH] ions. Using such approaches, based on the well established precepts of mass isotopomer distribution analysis,[19–21] means that it is feasible to determine proteome dynamics in complex organisms as well.

CONCLUSIONS

Proteomics is still in its infancy, and the 'rules of engagement' for comparative

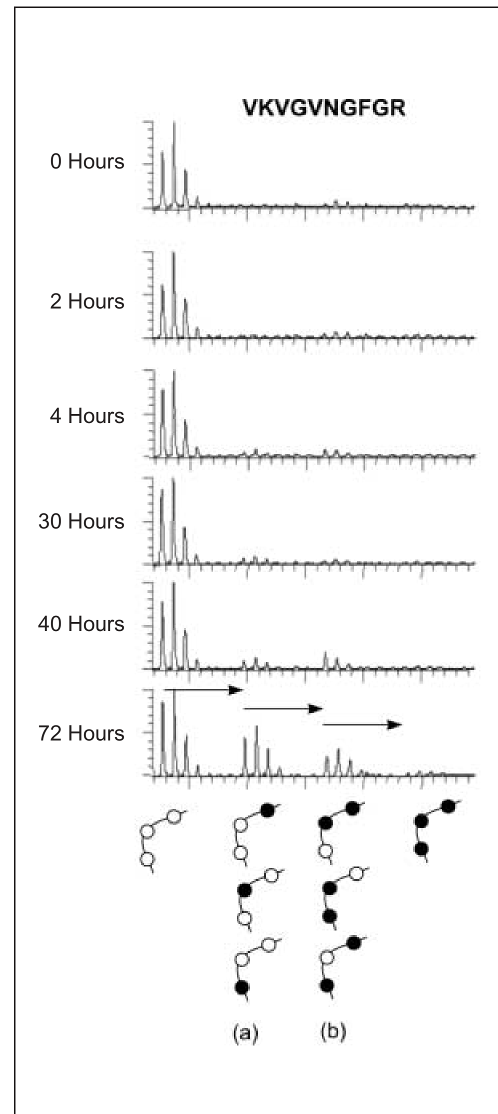

Figure 4: Protein turnover studies in chicken. Chickens were fed a diet containing 50 per cent stable isotope-labelled valine. As the birds consumed the diet, the amino acid was incorporated into proteins, which were then isolated by gel electrophoresis and analysed by matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry. The data shown are for a trivaline peptide, and in this labelling experiment, the intermediate isotopomers are clearly labelled. Unlike the yeast study shown in Figure 2, the intermediate forms become labelled because the relative isotope abundance (RIA) of the precursor pool is less than unity. The ion intensities of the two intermediate isotopomers (labelled a and b in the Figure) can be used to calculate the true RIA of the precursor pool, however, and from this information the rate of synthesis of the protein can be measured.

proteomics are still the subject of debate. Therefore, it might be argued that it is premature to move the argument forward to encompass the measurement of proteome dynamics. Yet, as we use proteomics to deliver data on the behaviour of the protein pools for systems biology, the time is approaching when we must include dynamics in the description. The methodologies outlined here will provide one such route to parameterisation of proteome dynamics. These methods, however, are driven by development of the technological approach, and are not yet optimised for high-throughput proteomics. In particular, labelled peptides derived from metabolic labelling through turnover can cause problems of inclusion and quantification, especially at low levels relative to the unlabelled variant. There is currently no filter that could be applied to a MALDI-ToF spectrum or LC-MS/MS dataset to identify metabolically labelled peptides, identify the protein and calculate the RIA (and hence turnover parameters) of the label. These are often manual tasks. Perhaps it is enough in the first instance to acquire a parameter such as the synthesis/degradation ratio,[15] which can indicate relative turnover rates, although, ultimately, it will be essential to turn those data into absolute degradation rates. Nonetheless, careful consideration of the metabolic and kinetic factors should lead to approaches that are based on single-point sampling along the labelling curve. With appropriate bioinformatics tools for analyses of complex labelling patterns, we might then be in a position to combine identification and turnover analyses — indeed, stable isotope labelling with amino acids can be used to 'count' the occurrence of that amino acid in peptides, which gives approx a tenfold reduction in search space in peptide mass fingerprinting.[22] We can anticipate a time when protein stability is deemed as important as protein abundance, and when both parameters are equally readily assessed, ideally in a single experiment.

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