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Mitochondrial protein turnover: methods to measure turnover rates on a large scale

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Abstract

Mitochondrial proteins carry out diverse cellular functions including ATP synthesis, ion homeostasis, cell death signaling, and fatty acid metabolism and biogenesis. Compromised mitochondrial quality control is implicated in various human disorders including cardiac diseases. Recently it has emerged that mitochondrial protein turnover can serve as an informative cellular parameter to characterize mitochondrial quality and uncover disease mechanisms. The turnover rate of a mitochondrial protein reflects its homeostasis and dynamics under the quality control systems acting on mitochondria at a particular cell state. This review article summarizes some recent advances and outstanding challenges for measuring the turnover rates of mitochondrial proteins in health and disease.

Keywords

Mitochondria; protein turnover; proteome dynamics; proteomics; heavy water

1. Introduction

Proteins in the cell exist in a dynamic equilibrium of continual synthesis and degradation. Alterations of this equilibrium are often reflected in changes in static protein abundance, which is commonly measured to determine the involvement of particular proteins in disease mechanisms. Static abundance alone however lacks temporality and gives little insight into the window of impact or homeostasis of a protein. Protein turnover rate is thought to provide this "missing dimension" of protein function [1], and has drawn particular attention in mitochondrial biology due to the importance of mitochondrial dynamics and quality control

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Disclosures

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in human diseases and aging. The proximity of mitochondrial proteins to reactive oxygen species renders them highly susceptible to protein damage. Given the importance of mitochondrial proteins to numerous cardiac processes, it is essential that the renewal, or turnover, of mitochondrial proteins be sustained during elevated stress conditions. Maintenance of mitochondrial quality and integrity requires coordinated turnover of compromised components to preserve cellular functions, whereas the accumulation of protein damage is thought to contribute to disease pathology [2,3].

There has been a longstanding interest in biomedical research to examine the rates and permutations of protein turnover in the past eighty years. Early experiments were pioneered by Rudolf Schoenheimer and David Rittenberg at Columbia University [4–7], who in 1939 used isotope labeling to establish that proteins in the body, and thus life itself, are in a dynamic state of interactions with the environment even in the absence of obvious growth [8]. These tenets ran contrary to popular beliefs of the day, which posited that organic molecules acquired through diet were solely burnt for fuel, but they soon gained wide acceptance and are now held as self-evident. Studies from the 1950's onward coincided with interests in measuring the flux of biochemical pathways, and led to the elucidation of the bulk protein synthesis rate in the human body [9] and of the rate constant of turnover (k) of specific cellular compartments, and a few easily isolatable proteins [10].

In cardiac biology, Murray Rabinowitz at the University of Chicago was among the first to realize important links between mitochondrial protein turnover and hypertrophy [11,12]. In 1973, he observed that following aortic banding, rat heart cytochrome c abundance increased on the first day, then subsequently decreased, whereas its degradation decreased consistently throughout (as measured by pulse-chase of a radioactive heme precursor) [13,14]. He concluded that the decreased degradation of cytochrome c was responsible for its early increase in hypertrophy, and that dissociation in myofibrillar and mitochondrial growth responses ultimately led to energetic decline. Meanwhile, other groups began to explore the turnover of mitochondrial sub-compartments (outer membrane vs. inner membrane) or few individual mitochondrial proteins such as cytochromes [10,15,16]. Two observations from these early studies are of particular interest: **(i)** they showcased the idea that protein turnover is a regulated cellular parameter that responds to disease stimuli; **(ii)** it was realized that individual mitochondrial compartments and proteins exhibit different turnover rates. However, these studies largely lacked the technical prowess to discern individual protein turnover on a large scale, and the ponderous experimental design and large variability of the results both hindered examination of disease models until modern proteomics techniques became available.

2. Methods for proteome-wide turnover studies: from single cells to whole animals

To measure the physiological protein turnover rate constant, i.e., the rate at which existing proteins are replaced, it follows that a method is needed to differentiate a newly synthesized protein from pre-existing proteins after the protein sample is drawn from the living organism (Figure 1A). Since 1935, isotope-labeling methods have been widely employed to label cellular proteins and trace their turnover. Deuterium was among the earliest labels used as an

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analog for biomolecules, when Schoenheimer explored protein metabolism using molecules tagged with ^{2}H and ^{15}N. However, the laborious procedures to discern stable isotope contents (with interferometry or the Kjeldahl method) led to their gradual displacement by the advent of radioisotope analogs such as ^{3}H or ^{14}C- labeled amino acids in the 1940s, which were easy to detect using scintillation counters. With the availability of advanced mass spectrometry techniques in the late 1990s, there has been a marked return to the use of stable isotope labels, which can be easily resolved by their mass. The advantages of stable isotope mass spectrometry over radioisotope approaches include its safety, bio-orthogonality, the ability to quantify multiple labeled proteins without isolation, and the ability to resolve a peptide cluster with multiple numbers of labels, which allows the determination of the fraction of new proteins in a system through precursor-product relationship (see below).

As protein analytical techniques mature to enable the analysis of minute amounts of individual protein species, the focus of protein turnover studies has changed in parallel and has steadily gained in finesse and sophistication. Whilst early studies focused on the measurements of bulk body protein turnover, scientists eventually began to measure to the turnover of whole mitochondria, mitochondrial sub-compartments [17], or the turnover of highly abundant proteins that were relatively easy to isolate in bulk [18,19]. The development of modern proteomics techniques has spurred a leap in capability, allowing investigators to go from monitoring a few proteins in one study to measuring the individual turnover rates of hundreds or thousands of proteins simultaneously.

Proteome-wide investigations of turnover rates were first demonstrated in single cell systems in vitro. A number of mature techniques current exist that allows protein turnover or stability to be measured in cultured cells, including both isotope-based as well as some non-isotope-based approaches. Non-isotope-based approaches include the use of immunobiological assays to measure protein abundance following synthesis inhibition (e.g., by cyclohexamide), and the measurement of the decay of protein fluorescence signals from fluorescent protein timers [20,21]. A common stable isotope approach suitable for in vitro studies is the use of dynamic or non-saturating stable isotope labeling by amino acids in cell culture (SILAC) [22,23]. SILAC was originally devised as a means to quantify relative protein abundance, by introducing fully heavy-labeled amino acids, typically arginine and lysine, in the culturing medium such that the cells produce isotope-labeled protein standards for comparison with cells grown in light medium. By measuring the rate of incorporation of the labels into proteins in the heavy-medium grown cells (or removal of labels in chase experiments), the method has been adapted to measure protein turnover. Dynamic SILAC-based experiments have been applied to different cultured cells including in bacteria [24,25], yeast [1] and multiple mammalian cell lines including HeLa, A549 human adenocarcinoma, and C2C12 mouse myoblasts [26–28]. These large-scale experiments have been transformative in defining the basic cell biological principles of protein turnover; e.g., the data revealed that protein complexes such as proteasomes often exhibit synchronized turnover [27]; that housekeeping proteins generally have slow turnover, whereas stress responsive elements such as heat shock proteins have faster turnover; and that protein turnover in part reconciles the discrepancies between mRNA and protein levels in a system [25].

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Nevertheless, beginning with the first proteome wide investigation in animals in 2005 [29], interests are gradually building towards understanding in vivo protein turnover in animal disease models as opposed to that in individual cells. Just as many physiological processes are not easily reproduced in single cell systems, there are reasons to believe that the proteome dynamics of exponentially growing cells in vitro does not recapitulate the physiological regulations that occur in multicellular organisms. Direct comparisons between in vitro and in vivo turnover rates are challenging, in part because most in vitro experiments have been performed on transformed cell lines that do not have direct in vivo equivalents. What is known, however is that protein turnover rates are highly dependent on cell types, and likewise identical proteins from the mitochondria of different tissues of origin can have different turnover rates (see below). More importantly, cultured cells are not under the same protein degradation stress as cells in multicellular organisms, as cultured cells may turn over unwanted proteins both via degradation or dilution into daughter cells, which clearly would confound inference of physiological turnover rates. The minimum turnover rates of proteins are further limited by the doubling time of cells, in vitro experiments typically report mammalian protein half-life of hours (as opposed to days or weeks in whole organisms).

3. Stable isotope labels for proteome-wide turnover studies in animals

Hence, recent studies on mitochondrial turnover are turning toward proteome-wide analyses of individual turnover in whole animals to describe systems-level regulatory behaviors of the entire mitochondria in vivo. A challenge of deducing turnover in complex organisms is that the isotopes need to be effectively delivered into the organisms via infusion or by oral intake via food or water. In cell systems, the heavy isotopes can be expected to enrich to unity, such that no light precursor exists and all newly synthesized proteins carry only the heavy signature. In whole animals, however, the protein precursor cannot in practice be enriched fully within the experimental timeframe, due to dilution by abundant unlabeled precursors in the animal's biomass, compartmentalization, and potential toxicity. Three major types of isotope tracers are available for large-scale animal studies:

3.1. Stable isotope-labeling of essential amino acids

A popular method is to introduce of amino acids labeled with heavy isotopes (^{13}C, ^{2}H, or ^{15}N), such as [$^{2}H_3$]-leucine, into the animal, either through injection or through an enriched synthetic diet. A major advantage of labeled amino acids is the simplicity in data analysis workflow. Since these heavy amino acids have fixed number of labels, no intermediates in the amount of labeling per newly synthesized protein exist. Secondly, precursors with +6 or +8 Da (e.g., [$^{13}C_6$]-arginine [$^{2}H_8$]-valine) are available, which are sufficiently different in mass from the light variant such that isotope clusters do not overlap (Figure 1B). A drawback is the requirement for a synthetic diet supplemented with the labeled amino acid, which may unbalance metabolism or spur protein synthesis. Labeled amino acids were first exploited by Beynon and colleagues to deduce the first proteome-wide protein turnover study in higher organisms [29], and have been employed in a number of studies in diverse animals including mouse [30,31], chicken [29], zebrafish [32], and carp [33]. Using [$^{2}H_3$]-leucine, the Rabinovitch group recently determined the in vivo turnover rates of ~400 mouse liver and heart mitochondrial proteins [31].

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3.2. Metabolic ^{15}N or ^{13}C labeling via diet

Alternatively, proteins can be enriched via metabolic precursors. Enrichment of ^{15}N atoms in an animal has been achieved for SILAC mice (SILAM), a method for creating >95% ^{15}N-labeled mice to be used as internal standards for protein quantitation, where the isotopically pure foodstuff for animals labels all mouse amino acids metabolically. Similar to dynamic SILAC experiments, a non-saturated labeling curve can be used to measure protein turnover in live animals. This method is compatible with proteome-wide turnover inquiry [34,35], and has recently been demonstrated for the measurement of turnover rates of over 1,700 proteins in the brain, liver, and blood of mice fed with the ^{15}N-enriched *spirulina* algae for up to 32 days [35]. Besides ^{15}N, metabolic ^{13}C labeling has also been demonstrated by enriching mouse diets with ^{13}C-labeled glucose, which is metabolized into amino acid precursors [36].

One drawback of metabolic ^{15}N or ^{13}C labeling is the complex isotope patterns generated as compared to SILAC approaches. As the mice gradually acquires excess heavy atoms, the labeled peptide peaks will shift gradually both in relative abundance of heavy isotopes (due to protein turnover) and in the amount of horizontal mass shifts exhibited by the heavy labeled peptides (due to increasing numbers of heavy atoms in newly synthesized proteins), creating a complex pattern of isotopic shifts [37,38] which demands more complicated data processing to deconvolute the spectra into component peptide ions with different numbers of incorporated heavy atoms.

3.3 Metabolic labeling with heavy water

^2H_2O (deuterated heavy water) is gaining in popularity as a protein label for animal studies [39–41]. One primary advantage of ^2H_2O is that it can be straightforwardly introduced into the animal by free intake from the drinking water supply, which avoids potential physiological impacts of dietary modifications or amino acid infusion. The ingested ^2H_2O molecules quickly equilibrate with body water, thus precursor isotope enrichment can be measured accurately from any biofluid. Deuterium atoms from body water is conferred to the carbon-hydrogen bonds of free non-essential amino acids during their enzymatic biosynthesis or via transaminases [42], which is shown to complete within 30 minutes [43]. Unlike in deuterium exchange experiments where solvent-exchangeable amide bonds are labeled, the enzymatically labeled sites are primarily chemically stable C-H bonds and do not back-exchange during sample processing. ^2H_2O labeling does not create separate peptide clusters (which essentially doubles the complexity of the proteomics sample), and thus is very amenable to large-scale analysis. Recently ^2H_2O labeling has been demonstrated to quantify the turnover rates of over 2,900 proteins in the mouse heart. The method has been used for proteome-wide turnover measurements in multiple organisms including the mouse [44,45], rat [46,47], and human [41,48]. A potential drawback is that deuterated peptides elute slightly earlier in liquid chromatography, which may introduce errors in peak area quantification. Secondly, the number of ^2H accessible labeling sites may be uncertain in some scenarios, such as in systems with different biochemistry of amino acid utilization than mammals, or in peptides with post-translational modifications.

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As another isotope analog of water, $H_2{}^{18}O$ shares many similarities with ^2H_2O in labeling characteristics and operational ease. Unlike ^2H_2O, $H_2{}^{18}O$ labels the carboxyl oxygen atoms of amino acids, thus allowing easy ascertainment of the number of labeling sites on a peptide. It is also thought to circumvent the label reutilization. Since labeling occurs upon the cleavage of a peptide bond or from the amino acyl t-RNA linkage [49], any proteolytic amino acids will subsequently become labeled with ^{18}O when they are re-incorporated into peptide bonds. Stephen Previs and colleagues compared the protein turnover rate of serum albumin in mice using both ^2H_2O and $H_2{}^{18}O$ methods [50], and found that $H_2{}^{18}O$ labeling returned faster turnover rates, although the difference did not reach significance (0.325 ± 0.046 d^{-1} for ^2H_2O labeling versus 0.301 ± 0.039 d^{-1} for $H^2{}_{18}O$ labeling, $P = 0.17$). Since $H^2{}_{18}O$ labels fewer atoms than ^2H_2O per peptide, newly synthesized proteins are more difficult to discern and at present it is not commonly used for large-scale studies.

4. Analytical and computational approaches to deduce turnover rates

The optimal isotope label for an experiment depends greatly on the available analytical instrumentation and computational workflows. Economy, physiological impacts, and ease of data analysis are all valid concerns that may influence decision as has been reviewed elsewhere [51]. Experiments with different labels have generally returned comparable turnover rates of mitochondrial proteins (See Table 1). In all of the above stable labeling experiments, the incorporated labels may be detected by mass spectrometry. Most modern proteomics platforms have been successfully employed for protein turnover studies, including Orbitrap [39,40], Q-ToF [41], MALDI-ToF [29], and LTQ-FT [31,35] instruments.

The major analytical challenge in a protein turnover experiment is to determine the amount of labels in unlabeled, partially labeled, and fully labeled samples in order to deduce the rate of label incorporation. As discussed above, in animal studies a low and uncertain level of enrichment is typically achieved. Converting the amount of labels present to the fraction of new proteins thus presents a challenge when the true precursor isotope enrichment is difficult to ascertain, since it creates an uncertainty in how the fully labeled molecule may resemble in terms of relative isotope abundance (analogous to specific activity in radioisotope labeling), which is needed to deduce the fraction of newly-synthesized product. A general solution to deduce protein synthesis from isotope labels is to assume a combinatorial probability model, which dictates that a biological polymer of more than one monomer (such as a protein) will exhibit a statistical distribution of labels that follow the polynomial expansion of the proportion of labeled and unlabeled precursors. This was exploited in 1986 by Kalderon and colleagues to measure glycogen synthesis from ^{13}C-labeled glucose [52], and by Marc Hellerstein and colleagues in 1991, who put forth an application of the technique to mass spectrometry data, named Mass Isotopomer Distribution Analysis (MIDA) (Reviewed in [53]). Mass isotopomers are isomers containing different isotopes that become resolved by a mass analyzer. Thus as a protein is synthesized by a population of monomers (protein precursor) containing a fixed proportion of heavy isotopes, the resulting statistical distribution of protein isotopomers containing zero, one, two, or more heavier isotopes will follow a mathematically deducible polynomial distributions determined by three MIDA parameters: the number of monomer units in the

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protein, the proportion of labels in the precursor, and the fraction of newly made polymers (whose amounts of labels are diluted by the presence of unlabeled polymers in the pool). The feasibility of this method for calculating protein turnover was first demonstrated on serum albumin in [$^2H_3$]-leucine labeled rats [54]. Variations of the combinatorial probability model have since been widely used to analyze MS data in protein turnover experiments. For instance, Robert Beynon and colleagues devised a method to identify the precursor relative isotope abundance in single amino acid labels (e.g., [$^2H_8$]-valine) that uses the combinatorial probability model to compare the isotopomer distributions of peptide clusters containing two instances of the labeled amino acid, in order to deduce the true precursor enrichment, which is then used to calculate the fraction of newly synthesized peptides for all peptides containing at least one instance of the labeled amino acid. In combination with high-throughput proteomics techniques, this approach led to the first large-scale measurements of individual protein half-life in vivo in higher organisms in 2005 [29].

Protein turnover is assumed to be a first-order kinetic process. As the heavy labels are integrated into the newly synthesized proteins, with eventual turnover of the protein pool the amount of label will reach steady-state equilibrium with the surrounding. As the fraction of newly synthesized proteins are experimentally determined at one or more time point, the data could be fitted to a kinetic model. Multiple models have been proposed. In most cases, simple rise-to-pleateau kinetics is assumed, and the isotope distribution before labeling and at steady-state can be calculated as above determine the remaining unknown (the protein turnover rate) [51], or alternatively all three parameters can be found using multi-parameter fitting [44]. Variations to this first-order kinetics model were described. Takhar Kasumov and colleagues have used the quasi-linear region of the first-order kinetic curve to calculate k for long-half-life proteins [55]. Our group has described a model that assumes separate protein and precursor rate constants with detailed methods in Lam et al. [40]. More complex multi-compartment modeling has also been formulated [56].

Although the isotope labeled spectra can be manually analyzed to yield turnover rates, high-throughput studies demand the aid of computational software automation. Many existing tools can integrate the peak areas of native or isotope-labeled peptide ions for protein quantification process. Since these software applications must add together the relative peak areas of each isotope, they may be coopted for protein turnover studies when a custom kinetic model is supplied to handle the integration data.

Several software applications have been written explicitly for protein turnover analysis, each of which is targeted for specific applications. **(i)** SILACtor: Developed by James Bruce and colleagues at the University of Washington, it is a command line software tool written to facilitate the analysis of dynamic SILAC experiments in cell culture. An advantage of SILACtor lies in its simplicity, as the software assumes that the heavy precursor is enriched to unity, such that protein labels go from all light peptides to all heavy peptides and vice versa, as is attainable in in vitro experiments. The relative isotope abundance of a peptide time series at each time point is collated and fitted to a kinetic curve to output. The software additionally has the capability to quantify peptide ions that do not have specific database identifications through an accurate mass retention time inclusion list to increase data depth. SILACtor is not compatible with partial enrichment such as in larger animals. **(ii)**

Topograph: Topograph is a Windows application developed by Michael MacCoss and colleagues at the University of Washington. Topograph (version 2) is compatible with ^{15}N and isotope-labeled amino acids, and is suitable for both in vitro and in vivo animal studies [31], by automating the method of using the spectra of multiply-labeled peptides to deduce precursor RIA first proposed by Beynon. Topograph also contains a user interface that can generate visual output, as well as advanced options including to account for earlier elution of deuterated peptides. **(iii)** ProTurn: ProTurn is a cross-platform software application specifically written by our group specifically for the analysis $^{2}H_2O$ labeling experiment data. ProTurn (version 2.0.5) features a user interface which allows the users to specify protein identification output from a number of search engines (e.g., ProLuCID [57], Thermo Proteome Discoverer, and COPaKB [58]), options for peak integration mass window and multiple data smoothing types. Users can also visualize and compare results in the interface. A general limitation of the above tools is that at present they remain standalone specialty applications, requiring users to adapt to their data analysis workflows and perform multiple file format conversions to the specifications of software input/output. It is thought that with improvements to data sharing policy and software crosstalk and federation, future analytical tools will more tightly integrate with existing workflows on data acquisition and protein identification.

5. General features of mitochondrial proteome dynamics

The scope and coverage of protein turnover experiments are fast approaching that of quantitative protein expression profiling. In a recent experimental dataset in mouse heart, liver, and kidney by our group (Peipei Ping laboratory), we measured the turnover rates of 694 out of the total of 1,088 (64%) mouse proteins that are annotated as mitochondrial proteins on SwissProt/PIR. Several properties of mitochondrial proteome dynamics can be inferred from these large-scale studies. For instance, mitochondrial proteins exhibit a dynamic range of turnover rates even at basal level, which covers up to three orders of magnitude. Subunits of multi-protein complexes have more similar turnover rates, but individual protein variability exists that may be explained by sub-complex association [44]. There does not appear to be a significant difference in mitochondrial protein turnover when measured from purified mitochondrial samples and in whole-cell lysate, indicating import is probably not a bottleneck to the measured turnover rates [40]. The turnover rates of mitochondrial proteins differ by the sampled tissue, with the approximate order being that of blood > liver ~ kidney > heart > brain ~ skeletal muscle among the investigated tissues in mice (Table 1). Mitochondrial subpopulations in the heart (interfibrillar and subsarcolemmal mitochondria) can show different turnover rates, with subsarcolemmal proteins turning over faster [47]. Furthermore, identical protein species can exhibit very different turnover behavior relative to the whole mitochondrial sub-proteome (Figure 2). Differences in individual protein turnover do not appear to be governed by simple biophysical parameters (protein hydrophobicity, molecular weight, or isoelectric point) or sequence features. PEST motifs [59] and sequence disorders [60] have both been proposed to govern protein stability, but their presence has generally not been found to correlate with protein turnover rates in large-scale analyses.

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Large-scale studies of protein turnover in animals are also enabling the examination of changing protein dynamics under various physiological and pathological processes. Our group measured the proteome-wide turnover of an animal heart disease model via isoproterenol challenge in mice, whereas William Stanley and colleagues examined the changes in interfibrillar and subsarcolemmal mitochondrial protein turnover in a transverse aortic constriction model. Hellerstein and colleagues found slower turnover liver of mitochondrial proteins in long-term caloric restriction in mice [45]; Peter Rabinovitch and colleagues examined the effect of aging and short-term caloric restriction [61]. A common feature of many such investigations is the complex, bidirectional changes in mitochondrial components that cannot be captured from the median of all mitochondrial proteins. The turnover rates of subsarcolemmal and interfibrillar mitochondrial proteins appear to permute independently in disease [46]. Protein subunits within a stable complex such as respiratory complex I can likewise respond to the same stimulus differently [40].

These findings emphasize the importance of finer details in individual protein homeostasis, whether orchestrated by intra-mitochondrial proteases [62], mitochondria-derived vesicles [63], or a combination thereof. Therefore although measurements of bulk mitochondrial turnover continue to be performed, with a number of recent studies of total mitochondrial protein turnover in models of caloric restrictions [64–66], the finding of widely varied turnover of mitochondrial proteins suggests that additional biological details may be revealed in proteome-scale studies. The measurements of proteome-wide dynamic ranges in whole animals will be preferred if finer regulatory details are to be elucidated.

6. Translational potential of proteome dynamics studies

Protein turnover rates can reveal novel protein markers with altered kinetics in human diseases, which may not be discernible from steady-state expression measurements. The potential of this approach has been demonstrated in pilot studies of patients with psoriasis and neurodegenerative diseases [67,68]. Labeling of human subjects is associated with additional challenges, for example: **(i)** The potential physiological impacts and side effects of the label need to be rigorously documented prior to applications to patients. **(ii)** The slower metabolism and protein turnover in human than smaller mammals requires longer labeling period or more stringent accurate measurements of minute degrees of label changes. **(iii)** As the subjects take in labels gradually, the data analytical workflow must be able to account for time-dependent precursor relative abundance in its kinetic models.

Several recent studies described the proteome-wide measurements of plasma turnover rates in human subjects using ^2H_2O labeling [40,41,48]. Hellerstein and colleagues measured the half-life of 100 proteins in four subjects, measuring precursor enrichment at multiple time points to adjust for label intake; whereas our group documented the labeling protocol and post-labeling monitoring in 10 subjects for up to 240 days and found no changes in hemodynamic behaviors and no signs of general discomfort among the subjects [48]. Several advantages of ^2H_2O labeling make it particularly applicable to human investigations; for instance, labeling without dietary modifications alleviates some difficulties in subject recruitment and enforcement of compliance to labeling regimens. ^2H_2O has an extensive and well-documented safety record in humans [41,69], and

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no physiological effects have been reported at experimental dosages (~2%). Label enrichment can be accurately monitored from any body fluid. Lastly, ^2H_2O remains financially feasible within the scale of human consumption. A typical labeling regiment of 2% enrichment over 14 days involves the total intake of 0.75 to 1.5 L of 70% ^2H_2O and costs ~$500 per subject at the time of writing. By contrast, equivalent isotope enrichment with ^{13}C-labeled leucine would be five to ten fold higher based on 3 g of total leucine intake per diem.

Some additional issues remain to be addressed before the measurements of mitochondrial turnover rates in human subjects in vivo can be conducted routinely, not least of all is the logistics of acquiring human mitochondrial samples. Cardiac samples are particularly limited in surgical availability, and repeated biopsy samples at multiple time points are not possible in most scenarios. A viable workflow will need to be carefully designed that acquires labeled samples from specific points such as during ventricular assist device installation or cardiac transplant. As instrument sensitivity increases, deducing mitochondrial protein turnover rates from leakage proteins found in plasma or other biofluids may be a potential, though challenging, alternative.

7. Future outlook

Some ongoing developments in protein turnover measurements include the differentiation of protein synthesis and degradation rates, as well as the integration of protein expression and dynamics for systems level analyses. Under constant homeostasis where a steady-state protein pool is maintained, the synthesis rate and the degradation rate of a protein are identical over time. Thus pulse and chase experiments that measure the enrichment or depletion of heavy isotopes are formally equivalent and should give identical values. This assumption does not hold when the proteome shifts towards another steady state, such as when two physiological states are compared (e.g., disease development or altered environment), which compels distinction between whether it is the change in protein synthesis or degradation that drives protein dynamics. Synthesis rates and degradation rates may be distinguished in experiments where changes in the amount of new proteins created and the amount of old proteins removed are separately quantified (as opposed to only ratio of new proteins from the overall protein pool). This may be achieved in vitro as demonstrated using a triple-label SILAC approach [70,71] (with light, medium, and heavy peptides), where the increased in heavy labeled peptides and the decrease in medium labeled peptides can be independently quantified against the light standard. Such methods may be feasibly extended to animal studies using SILAC labels. Alternatively, other approaches that allow the relative changes of the protein pool and the relative change of isotope composition to be simultaneously quantified may be employed, such as using post-extraction chemical labeling (e.g., iTRAQ) [24], label free quantification [40], or SILAM-^2H_2O double labeling [45]. Such data allow the relative contributions of synthesis to be calculated from the absolute turnover and changes in protein abundance. When further compared to transcript expression, it will be possible to infer from protein abundance and turnover which genes are regulated at transcriptional, post-transcriptional, or post-translational levels, and to identify genes with impaired proteolysis. Modeling from in vitro studies have suggested that protein synthesis may be more dynamically regulated than protein degradation [23,71], albeit not

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without important exceptions that suggest important protein regulations via degradation pathways [71].

To conclude, protein turnover investigations have been buoyed by the advances in proteomics and bioinformatics technologies, and can now reveal the turnover of hundreds of mitochondrial proteins in a single experiment. The resulting protein dynamics data hold the promise for advancing our understanding on mechanistic details of mitochondrial dynamics. For instance, measurements of protein turnover under perturbation will shed light on the proteolytic mechanisms of candidate disease proteins and suggest potential therapeutic strategies to correct their homeostasis. The ability to measure altering protein half-life will allow new hypothese to be tested. For instance, current investigations on protein degradation pathways rely heavily on pharmacological inhibition of proteases to engender steady-state protein accumulation. Such methods prove limiting in many scenarios such as in defining the repertoire of physiological substrates of the mitochondrial Lon protease, when protein accumulation is not apparent [72]. Finally, one can envision the integration of protein turnover data with other systems-level parameters to enable an integrative description of mitochondrial physiology from molecule to phenotype.

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Highlights

- Protein turnover is an important component of mitochondrial quality control.
- Proteomics techniques now allow protein turnover to be measured on a large scale.
- Individual mitochondrial proteins exhibit very different turnover rates in vivo.
- Protein turnover may alter independently from protein expression during disease.

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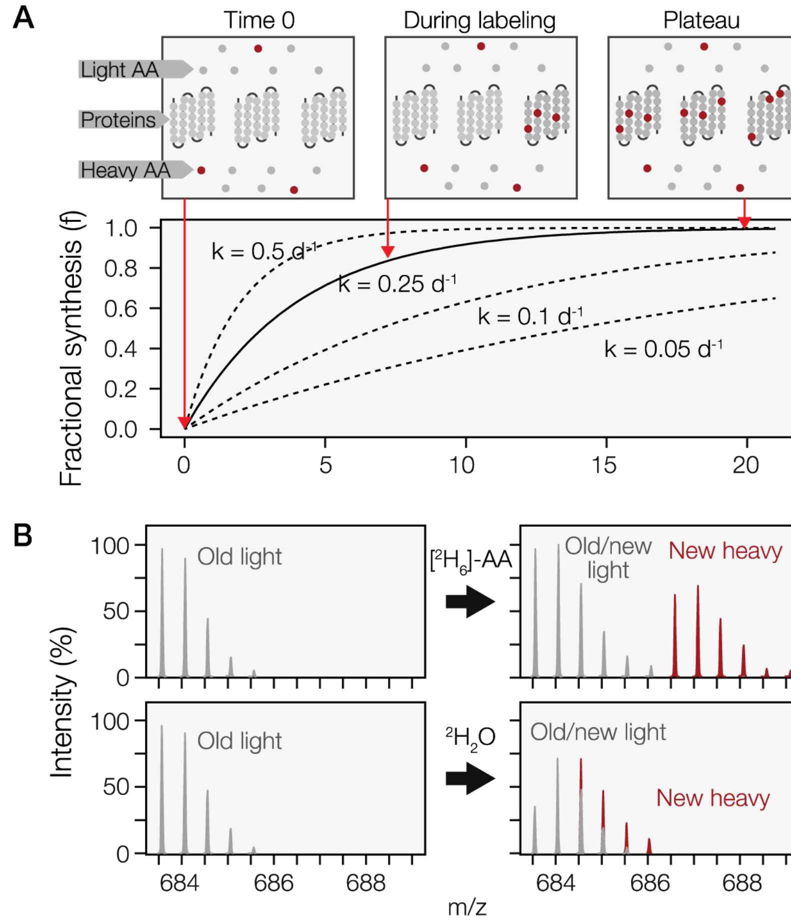

Figure 1.

Determination of protein turnover rates with stable isotope labeling. **A.** (Upper left): At time 0 of the labeling experiment, some cellular amino acids (gray circles) are labeled with heavy isotopes (red circles). (Upper middle): During the course of the labeling experiment, as old proteins are degraded and new proteins are made, a fixed amount of heavy isotopes becomes incorporated into the protein pool. (Upper right): (Lower panel): incorporation rate of stable isotope labels correspond to the portion of new proteins in the biological systems, which can be used to calculate the turnover rate of the proteins. The first-order kinetic curves corresponding to a protein with multiple hypothetical turnover rates (k = 0.05, 0.1, 0.25, or 0.5 d^{-1}) are shown. **B.** The analytical approach to quantify the amount of heavy isotope labels with mass spectrometry at any given time is dependent on the type of labels used. (Upper): Stable isotope labeled amino acid approaches create a new peptide isotopomer envelope in the mass spectrum that corresponds with newly made peptides with heavy labels. (Lower): Incorporation of ^2H labels from ^2H_2O results in a shift in the fractional abundance of isotopomers in the envelope.

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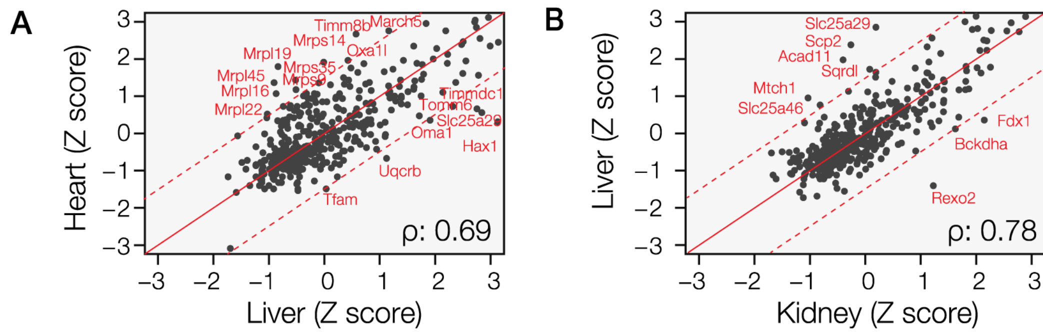

Figure 2. Heterogeneity of mitochondrial protein turnover in multiple tissues

The scatterplots show the standardized log turnover rates (Z score) of 444 mitochondrial proteins commonly measured in mouse heart, liver, and kidney. Comparisons between **A.** heart and liver; and **B.** liver and kidney; are shown. Each data point represents a distinct protein. The red diagonal line represents 1:1 in standardized turnover rates in two respective tissues, whereas dashed lines represent the positions of data points where a protein turns over markedly faster or slower in one tissue (+1.5 or −1.5 standard deviations from average). Proteins that differ markedly in relative turnover rates between two tissues are marked, e.g., a cluster of mitochondrial ribosomal proteins (MRPs) can be seen that turn over relatively faster in the heart than in the liver. Two inner membrane carriers for amino acids/amino acid precursors (SLC25A29 and SLC25A26) turn over relatively faster in the liver than in the kidney. These differences are an indication of the tissue specificity of mitochondrial biology and the accompanying heterogeneity in the regulation of protein turnover rates.

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Table 1

Meta-analysis of mitochondrial turnover rates measured in multiple mouse tissues

The turnover rates of mitochondrial proteins from multiple tissues reported in a number of recent whole-animal labeling experiments are shown.

Tissue	Turnover rates (d^{-1})	Average	Isotope label	Mouse strain	N° mito proteins*	Reference
Blood	0.210 [0.090 – 0.326]	0.210	^{15}N	FVB	22	[35]
Liver	0.149 [0.095 – 0.566]	0.155	$^{2}H_2O$	Hsd:ICR	545	[40]
	0.167 [0.113 – 0.673]		^{15}N	FVB	278	[35]
	0.150 [0.100 – 0.380]		$^{2}H_2O$	C57BL/6J	91	[45]
Kidney	0.144 [0.093 – 0.477]	0.146	$^{2}H_2O$	Hsd:ICR	635	N/A#
	0.147 [0.070 – 0.306]		$[^{2}H_8]$-valine	C57xBALBc	10	[30]
Heart	0.045 [0.021 – 0.198]	0.044	$^{2}H_2O$	Hsd:ICR	537	[40]
	0.039 [0.014 – 0.117]		$[^{2}H_8]$-valine	C57xBALBc	17	[30]
	0.048 [0.024 – 0.285]		$[^{2}H_3]$-leucine	C57BL/6J	258	[61]
Skeletal Muscle	0.034 [0.024 – 0.117]	0.034	$[^{2}H_8]$-valine	C57xBALBc	3	[30]
Brain	0.027 [0.013 – 0.140]	0.019	^{15}N	FVB	153	[35]
	0.011 [0.007 – 0.105]		^{15}N	DBA/2J	17	[34]

Turnover rates are reported as median and [5th to 95th percentile], when available.

*To allow for standard comparison, mitochondrial proteins are here defined as the 1,008 proteins with the "mitochondrial" keyword annotation at SwissProt (release 2014_07, taxonomy: 10090, keyword: KW-0496; reviewed). The table includes studies that specifically enriched for mitochondrial proteins and those that did not.

#Unpublished kidney data were from the same dataset as described in Lam et al. [40].