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Measuring protein breakdown in individual proteins *in vivo*

Lars Holm and Michael Kjaer

Institute of Sports Medicine, Dept. Orthopedic Surgery M, Bispebjerg Hospital, and Center of Healthy Aging, Faculty of Health Sciences, University of Copenhagen, DK.

Abstract

Purpose of the review—To outline different approaches of how protein breakdown can be quantified and present a new approach to determine the fractional breakdown rate of individual slow turnover proteins *in vivo*.

Recent findings—None of the available methods for determining protein breakdown can be used to determine the breakdown rate of specific proteins and therefore do not keep up to the preceding methodological demands in physiological research. A newly developed approach to determine the fractional breakdown rate of single proteins seems promising. Its conceptual advantage is that the proteins of interest are the site of measurement. The application hence initially demands the proteins to be labeled with stable isotopically-labeled amino acids. Subsequently, the loss of label from the proteins will be dependent on the protein breakdown rate when i) no labeled amino acids are re-incorporated into the protein, ii) the protein mass is steady, and iii) when proteins contained in the measured fraction are stochastically selected for degradation.

Summary—While the synthesis rate of specific proteins can be accurately determined, methodological improvements are required to elucidate the physiological role of protein degradation. The novel approach is promising but coming studies are needed to address its wider applicability.

Keywords

Fractional breakdown rate; muscle; nutrition; stable isotope; deuterium

Introduction

Presumable all proteins *in vivo*, irrespective of their function and location, are undergoing renewal through constant degradative and biosynthetic processes. The regulation of these kinetics are finely adjusted in healthy states and accurately determines protein pool size and function. Research fields attempting to link gene expression, molecular signaling, and protein kinetics regulation as the basis for long-term adaptations in health and disease therefore make huge efforts to unwind this complex regulation to improve understanding of basic physiology and develop clinical drug-therapy treatments. It is foreseen that protein-specificity will be an inevitable demand to move this research up a level [1*]. Isolation of

Corresponding author: Lars Holm, Institute of Sports Medicine, Bldg 8, 1st, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark. Tel: +45 3531 6662; fax: +45 3531 2733; L.Holm.isotope@gmail.com.

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the most abundant protein fractions from e.g. muscle homogenates with the purpose to study protein synthesis rates have been done for several years [2,3,4,5*,6*,7*], and the most recent technical improvements have made the mass-spectrometry analyses possible using very small protein quantities [8,9*,10*]. While available approaches to determine protein synthesis rates are available for the future demands, the existing methods to measure protein breakdown markedly lag behind.

Existing methods to determine protein fractional breakdown rate

Conceptually divergent approaches have been employed for determination of the protein breakdown rate. i) Being the most applied of the existing approaches, the tracer-dilution principle assumes that any afferent tracer-enrichment will be diluted at the efferent site only by the release of analyte from the tissue of interest – a causal consequence of protein breakdown. Basically, two approaches exist to determine the fractional breakdown rate of proteins by the tracerdilution technique. The first and the most used is the free amino acid tracer-dilution approach, where the amino acid tracer enrichment at the arterial site is compared to that at the venous [11] or tissue free [12,13]. Despite that sophisticated refinements have simplified [14,15**] and decreased the invasiveness [16] of the method, thereby improving the applicability, the approach cannot determine the breakdown rate of individual or fractions of proteins [17,18]. Also based on the tracer-dilution principle, though with the addition of tracing a certain, protein-specific metabolite, the study of few specific protein fractions; i.e. myocontractile degradation by measuring the 3-methylhistidine (3-MH) release [19*,20,21,22**,23] and collagen degradation by tracing hydroxyproline or –glycine [24]. When applied across a limb and traced with stable isotopically-labeled metabolites the approach is both valid and reliable. Although useful for the study of exact the myocontractile and collagen-derived proteins, the major disadvantage of the protein-specific derived metabolite approach is that the assessment of the breakdown rate is limited only to proteins containing such an identifier, which obviously is reserved for only few proteins and tissues. ii) Alternatively it is well acknowledged that the difference between the fractional synthesis (r_s) and breakdown (r_d) rates equals the net rate of change in the protein mass, i.e. $r_{net} = r_s - r_d$. Thus, by determining protein synthesis rate and changes in protein mass the fractional breakdown rate can be calculated. However, a direct sizable comparison of fractional synthesis rate (FSR) and changes in protein mass appears not to be validly done, since the FSR is determined over short periods (minutes to hours), whereas often several days are necessary to determine changes in protein mass solidly [25,26]. Therefore, the measured r_{net} is the average change in protein mass across a period of time that is several-fold longer than that the synthesis rate is determined across, which makes the latter more dependent on actual behavioral and environmental stimuli. Thus, a direct comparison may be invalid. Furthermore, determining total tissue protein mass or single protein concentration is conceptually connected with problems concerning accuracy and choice of reference. Although applied in physiological trials [27,28] this approach has never really had its breakthrough in *in vivo* studies of larger animals and humans. iii) A direct measure of protein breakdown rate can be determined during protein steady state conditions applying models described more than thirty years ago [29]. By pre-labeling proteins with tracer, the subsequent disappearance of tracer from the media will follow a first-order decay provided that no tracer is re-incorporated [30] and total protein mass is maintained. These required conditions may be readily controlled in cell- and bacteria-cultures to reveal valid results on protein breakdown rates [31,32,33], whereas it may be harder in larger animals *in vivo* [34]. The outlined disadvantages are; long tracer labeling times, problems with tracer re-circulation, and protein mass steady state.

Protein-specific fractional breakdown rate of pre-labeled proteins

With reference to the strength of the direct incorporation approach for the determination of protein synthesis, it is our impression that the proteins must be the final end-point for the measurement forming the basis for the calculation of the fractional protein breakdown rate. Thus, despite the mentioned challenges and limitations with the latter method mentioned above, we here present a newly developed approach to determine the fractional breakdown rate of individual proteins applicable in larger animals *in vivo*. The principle is similar to the earlier described that is based on the disappearance of a protein-bound stable isotopically-labeled amino acid that prior to the actual measurements was incorporated into the protein of interest [35**].

Pre-labeling of proteins

A sufficient labeling of proteins via incorporation of stable isotopically-labeled amino acids is easily obtained by oral administration of deuterium labeled water, $^2\text{H}_2\text{O}$. Labeling with any other atom pre-bound to an amino acid (either ^2H , ^{13}C , or ^{15}N) can be done sufficiently only by prolonged continuous infusion or multiple floodings that would become too complicated and expensive to perform. Water is by many means very applicable for this purpose and has previously been shown reliably and quickly to label amino acids [34,36,37,38] (see Figure 1). Water serves as an intermediate in the metabolic equilibration between amino acids and their ketoacids and additionally for some amino acids similarly for other reversible reactions exchanging hydrogens covalently bound to carbons on the R-group. The amino acid precursor pool can thereby be maintained at an adequate enrichment by oral administration of one or more boluses of deuterium oxide or by replacing a fraction of the drinking water with deuterium oxide (Figure 1). Additionally, due to the relatively low turnover rate of water and thus the slow disappearance rate as compared to that of free amino acids, the labeled amino acids remain in the protein-precursor pool for an extended period of time after withdrawal of the labeled water (Figure 1 and 2). Therefore, labeling of even slow turnover proteins with ^2H -labeled amino acids can be obtained by administration of deuterium oxide. Alanine can exchange up to four deuteriums with water *in vivo*, which improves the analytical sensitivity of alanine as compared to other amino acids (see Isotope ratio, IR, at Figure 1).

Principle in approach to measure FBR by a novel method

Briefly, the practical approach is a multi-step setup that is illustrated in Figure 1 and 2: Step 1) Pre-labeling of proteins of interest via oral administration of deuterium labeled water. Based on existing values on protein synthesis rates the amount and necessary time for deuterium oxide labeling can be estimated. Step 2) Withdrawal of deuterium oxide and return to plain tap water to allow washout of label from the precursor pool. Step 3) After a sufficient period (>3 half-lives of water, i.e. $<10\%$ of peak enrichment) on unlabeled water, the deuterium has disappeared from water, tightly followed by the free amino acids – and thus the protein precursor pool. For this purpose alanine is especially suitable because it follows the drop in water enrichment more tight than other amino acids (Figure 1). When this condition is established, no re-incorporation of deuterium-labeled amino acids into proteins takes place. Step 4) At this point in time, the proteins will loose the deuterium-labeled amino acids at a rate dependent only on the breakdown rate of the proteins. The mathematical derivation of the relationship is briefly outlined below, where P and P^* are the total proteins and labeled proteins, respectively. The dot ($\dot{}$) denotes differentiation with respect to time. k_d is the rate constant of degradation (d) and r_{net} is the net rate of tissue protein accretion/loss (i.e. the difference between synthesis and breakdown rates).

$$\frac{dP^*}{dt} = \dot{P}^* = -k_d P^*$$

$$\frac{dP}{dt} = \dot{P} = r_{\text{net}}$$

$$\begin{aligned} \frac{d\left(\frac{P^*}{P}\right)}{dt} &= \frac{\dot{P}^* P - P^* \dot{P}}{P^2} = \frac{(-k_d P^*) P - P^* r_{\text{net}}}{P^2} = -k_d \left(\frac{P^*}{P}\right) - \frac{r_{\text{net}}}{P} \left(\frac{P^*}{P}\right) \quad \text{where } E = \frac{P^*}{P} \\ \Downarrow \\ \frac{dE}{dt} &= -\left(k_d + \frac{r_{\text{net}}}{P}\right) E \end{aligned}$$

As it is the case for determination of the fractional synthesis rate [39], a steady protein mass is required during these experiments as well [29,31,40]. When the protein mass rightly can be assumed to be unchanged over the course of the FBR-assessment ($r_{\text{net}} = 0$), the change in protein enrichment is described by the mono-exponential decay with the FBR, k_d , as the disappearance rate:

$$\frac{dE}{dt} = -k_d E \iff E(t) = e^{-k_d t}$$

Assumptions and considerations for the FBR approach

Presently, only data obtained in rats have been reported using this approach [34,35]. For human application it cannot be ethically approved to enrich body water to the same extent as in animals [34,36,41] and additionally due to the applicability of the approach the labeling period will probably be inadequate to allow precursor and slow turnover protein enrichments to equilibrate. Taken together, human protein labeling is very limited compared to existing animal data. However, due to the relatively slow turnover rate of water the deuterium remains in the *in vivo* system for some time after return to tap water. Therefore, slow turnover proteins will be labeled over many days after just a single loading dose of deuterium oxide (Figure 2). Giving only one bolus of deuterium oxide, enriching body water and thus free amino acids with 0.5-1.0% deuterium, the slowest turnover body proteins ($\sim 1\% \cdot \text{d}^{-1}$, such as muscle contractile and connective tissue collagen proteins) will be enriched up till about one-tenth of that of the precursor pool (Figure 2). Subsequently, when the labeling has been lost from the precursor pool, it will demand several days to detect a difference in protein labeling due to breakdown of $\sim 1\% \cdot \text{d}^{-1}$ with the sensitivity of the available equipment (GC/MS/MS EI-mode and GC/P/IRMS). Thus, the FBR can only be determined as a mean over several days, whereas the FSR determination in comparison can be completed within hours. Therefore, a direct comparison of FSR and FBR in absolute values should be avoided. However, the values are assessed independently and thus can be discussed as individual measures. They are not in conflict with one another as was the case in the previously mentioned approach solving $r_d = r_s - r_{\text{net}}$. Independently of experimental setups, the designs should be set up so that the FBR reflect a chronic, weighted average value across the intervention-period. In comparison, the FSR values may reflect more acute conditions and its measurement should preferably be repeated at various time-points throughout the FBR-intervention-period to generate a similar 'weighted mean' comparable to the FBR-value.

Although a steady protein mass is fundamental for both the FSR and FBR approaches, it is more important to consider in the FBR approach as the intervention-period is several days as compared to hours for the FSR. Thus, future interventions must be adjusted accordingly not to violate this assumption. Also, as it is the case for the FSR measurement, no tracer re-circulation must take place during the FBR intervention-period. Although this assumption is met in terms of no label in the precursor pool, proteins containing labeled amino acids will continuously release those into the free intracellular space. Therefore, it is of most importance that the label is removed from the amino acid before it may be reutilized to charge another tRNA and thereby re-circle (illustrated in Figure 3). Alanine has been shown to equilibrate with water deuterium enrichment within minutes [36] and faster than other amino acids (Figure 1), why especially alanine is suitable as a tracer for this FBR approach.

Maturity of proteins – important for both synthesis and breakdown

In approaches where the proteins are the site of measurement, the complexity of the protein kinetic processes should be acknowledged (Figure 3). During production of a new and functionally active protein, the first step is the translation of the mRNA into a peptide-chain. Already at this step the stable isotopically-labeled amino acid tracer appears in the pre-protein. However, multiple post-translational events often occur to form a stable and functional protein. The amount of labeled amino acids at a certain point in time will depend on the synthesis rate of any specific sub-protein-pool. It is well-known that the synthesis rate of different pools of the same proteins differ several-fold [32,42]. When isolating the protein (pool) of interest for assessment of the fractional *synthesis* rate, it is therefore of most importance that the protein fraction is homogenous in terms of the protein maturity, i.e. state of post-translational modification. Of similar importance is it though that the proteins for determination of the fractional *breakdown* rate originates from the same sub-pool. Further, the basis for the breakdown approach is that the protein, selected for degradation, is targeted randomly. Such stochastic selection however can only be true within each maturity state if they inhere different turnover rates. Therefore, cross-contaminations between protein-pools at different maturity-states will invalidate the assessed enrichment irrespective of *synthesis* or *breakdown* rates.

Conclusion

The range of possible applications of the disappearance of labeled protein-approach to determine the protein fractional breakdown rate is wide. The future challenge will be to apply physiological interventions that on one hand can be assumed not to induce marked changes in protein mass over the course of FBR determination period, but on the other hand apply enough stimulus to study its impact. No natural occurring condition whether it is nutritional, physical activity, or hormonal result in severe changes that affect protein mass significantly within few days. However, detrimental interventions such as administration of superphysiological hormone levels, critical illness, and immobilization may inhere the impact of fast and marked changes in protein mass. Future studies and coming pilot studies must appreciate the efforts to reveal the nature of acute adaptation and correct study designs accordingly.

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Abbreviations

$^2\text{H}_2\text{O}$	deuterium labeled water or deuterium oxide
FSR	fractional synthesis rate
FBR	fractional breakdown rate
GC/MS/MS	gas chromatograph-mass spectrometer-mass spectrometer
GC/P/IRMS	gas chromatograph-pyrolysis-isotope ratio mass spectrometer
EI	electron ionization
3-MH	3-methylhistidine

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Figure 1.

Deuterium isotope ratio (IR) subtracted the background IR and enrichments (E, calculated as $[\text{sample IR} - \text{background IR}] / \text{number of deuterium labels}$) of three serum free amino acids; alanine, valine, and serine and serum water from 4-10 rats administered 8 vol% $^2\text{H}_2\text{O}$ in their drinking water for 42 days from the age of appr. 4 weeks. After 42 days the deuterium labeled water was withdrawn from the drinking water and the rats continued on plain tap water. The amino acids IR-values inhere the likelihood of finding one heavier isotope on the amino acid. I.e. an IR twice the enrichment value (serine) means that two deuteriums are incorporated whereas an IR almost four times the enrichment (alanine) means four possible sites for hydrogens to be exchanged with deuterium *in vivo*. The loss of deuterium label on alanine closely follows the disappearance of deuterium label in water, whereas the label remains longer on valine and serine. This means that alanine turnover is faster than the other two amino acids. Values as mean $\pm$ SD.

Figure 2.

Model of deuterium disappearance from body water after a single oral intake and concomitant appearance of water-derived deuterium labeled amino acids in slow turnover protein. The model is based on human fasting turnover rates of skeletal muscle and connective tissue proteins ($0.7\% \cdot d^{-1}$) and body water ($9\% \cdot d^{-1}$). It is obvious from the illustration that the water and thus free amino acid precursor pool enrichment is higher than the protein-bound amino acid due to the huge differences between turnover rates. Not until after 30 days does the precursor enrichment drop to a level lower than the protein bound amino acids. I.e. first from this point in time does a net loss of deuterium-labeled amino acids take place from the protein-bound pool.

Figure 3.

Fate of amino acids during the intracellular protein kinetic processes. From the capillary bed, the free amino acids move across the interstitial space and are transported into the cytoplasm. Here they undergo enzymatic transamination (TA), phosphoenolpyruvate carboxykinase (PEP-CK) and subsequent conversion in the tricarboxylic acid cycle for alanine, serine tetrahydrofolate methyl transferase (STHM) equilibrating serine and glycine, and other reactions where water is involved as a donor or receiver of hydrogens by which processes the deuteriums are exchanged into the amino acids. A) The amino acids also charge the tRNAs, which subsequently B) deliver the amino acids to the ribosomes synthesizing new peptides. C) After finalizing the peptide-chain that, it is released as a pre-protein D) that must undergo some kind of modification before it obtain full functionality. E) Exocytosis and incorporation of protein to its functional location that may be extracellular. F) Presumable at every stage, the peptide/protein can be targeted for degradation and the likelihood for this to happen and hence the breakdown rate, is illustrated by the number of arrows pointing into the site of degradation (scissor). From here free amino acids are released of which some will remain to be deuterium labeled although the precursor pool is not. The label from these released amino acids will though be diluted from the enzymatic reactions to reflect the presence in water. Therefore, it can be presumed that the deuterium label of free amino acids in the precursor pool at any point in time is similar to the deuterium enrichment of body water.