In vivo protein isoform turnover analysis using $^{15}$N metabolically-labeled mouse tissue

Sharon Liu$^{1,2}$, Christiane Rewerts$^1$, Giuseppina Maccarrone$^1$, Christoph W. Turck$^1$

$^1$Max Planck Institute of Psychiatry, Munich, Germany. $^2$University College London, United Kingdom. Correspondence to: Jiawen.liu.11@ucl.ac.uk

Abstract

Protein turnover, the dynamic process of protein synthesis and degradation, is known to be modulated by structural variations in proteins arising from alternative splicing or post-translational modifications (PTMs). The present study aimed to identify protein isoforms of $^{15}$N metabolically-labeled mouse lung and heart tissue, and to estimate protein turnover with the novel, in-house software Proturnyzer. We extracted and homogenized heart and lung tissue from 3 different mice that had been partially metabolically-labeled with $^{15}$N. Protein isoforms were separated using 2D-gel electrophoresis, subjected to in-gel enzymatic digest followed by mass spectrometry-based identification. We were able to identify 17 and 19 distinct proteins with isoforms in the mouse heart and lung, respectively. PTMs were identified in some isoforms and differences in turnover among isoforms could be determined from tryptic peptide $^{15}$N incorporation rates. Collectively, this platform offers a potential framework for future work using protein turnover data as a novel biomarker to analyze subtle differences between healthy and diseased tissues.

1. Introduction

Protein isoforms can arise from alternative splicing of the same gene or from post-translational modifications (PTMs) such as phosphorylation and acetylation. These structural variations in isoforms are known to alter their biological properties, thereby making it possible to distinguish and characterize them [1]. The dynamic process of protein synthesis and degradation, protein turnover, is one such biological property that has been gained recent interest due to accumulating evidence of its role in neuronal function [2, 3]. Measurements of protein turnover within cells could thus serve as an important biomarker to detect subtle differences between healthy and diseased tissues. However, pioneering work on protein turnover was plagued by a spectrum of difficulties. For example, the instruments available were not sensitive enough to detect the presence of proteins with transient PTMs [4] or the Relative Isotope Abundance (RIA) could not be determined accurately due to low incorporation rates of isotope-labeled amino acids [5]. Fortunately, the advent of cutting-edge, highly sensitive mass spectrometers now facilitates accurate identification of proteins and their modifications and various research groups have fine-tuned algorithms and strategies to quantify turnover rates of proteins within cells.

The present study aimed to identify protein isoforms of $^{15}$N metabolically-labeled mouse lung and heart tissue, and to estimate protein turnover with the novel, in-house software Proturnyzer. [6] Proturnyzer is able to distinguish the relative abundance of labeled and unlabeled peptides and calculates a 'Labeled Peptide Fraction' (LPF) value for each protein. A protein with high turnover will have greater $^{15}$N incorporation rates and thus reflect a larger LPF value than a protein with low turnover. [6]

2. Methodology

2.1 Sample preparation

Three 8-week old mice metabolically-labeled with $^{15}$N (for feeding procedure, refer to [7, 8]) were obtained from Silantes and sacrificed. The heart, cortex and lung tissues were then extracted from each mouse. All tissues were homogenized and Bradford assays were conducted to determine protein concentrations.
2.2 2D-Gel electrophoresis

Samples were loaded onto 17cm immobilized pH gradient (IPG) strips with pH range 4-7 and separated with 2D-Gel electrophoresis. The gels were stained with Coomassie Blue and resultant gel images were scanned with PDQuest. Potential isoforms, represented by gel spots in a cluster (Figure 1), are picked out using self-made pipette tips. These gel pieces were destained and subjected to in-gel trypic digestion.

2.3 Mass Spectrometry analysis

Using LC-ESI-MSMS, the proteins present in the gel spots were identified using peptide mass fingerprinting and we cross-referenced these identities with database searches (Mascot and SwissProt).

2.4 Proturnyzer turnover analysis

LPF values of the identified peptides were acquired using Proturnyzer. For each identified peptide, the spectra was inspected and filtered if it failed to meet any of the following criteria:

1) There must be 2 distinct isotopologue distributions, represented by yellow bars for unlabeled peptides and purple for labeled peptides. These distributions should not overlap.

2) Distribution of labeled peptides must be Gaussian.

3) There should not be any abnormal gaps or spikes in the distributions

After filtering unsatisfactory spectra, the LPF value of an identified protein was obtained by taking the median of all of its peptide LPF values. Proteins with 2 or fewer peptide LPF values were not considered.

3. Results

Due to technical difficulties, we were unable to obtain results for the mouse cortex. Out of the 9 gels that we processed, only 1 proved to be of satisfactory quality (m26, heart). The remaining 8 gels failed to separate the proteins and could not be analysed.
3.1 $^{15}$N metabolically-labeled mouse heart (August 2013)

Figure 2 illustrates our experimental results obtained from the $^{15}$N metabolically-labeled mouse heart. We successfully identified 48 distinct proteins and 18 of them showed isoforms. We discovered a phosphorylated isoform of DHSA_MOUSE in spot 11 and an acetylated isoform of 1433Z_MOUSE in spot 22. A portion of the gel in the bottom right-hand corner failed to run vertically and caused clumping of proteins towards the right side of the gel. Hence, we decided to analyse and report data from another $^{15}$N metabolically-labeled mouse heart. This tissue was processed in June 2013.

3.2 $^{15}$N metabolically-labeled mouse heart (June 2013)

A similar experiment was previously conducted in June 2013. After turnover analysis with Protumyzer, we successfully identified 46 distinct proteins and 17 were represented with isoforms.

As illustrated in Figure 3, we identified phosphorylation in DESM_MOUSE (spot 24) and acetylation in CH60_MOUSE (spot 1), 1433Z_MOUSE (spot 28), MLRA_MOUSE (spot 37), MRLV_MOUSE (spots 37, 39, 40) and ATP5H_MOUSE (spot 46).

Figure 2 Gel image of $^{15}$N metabolically-labeled mouse heart. We identified 48 distinct proteins and 18 of them showed isoforms. Mass spectrometry analysis identified phosphorylation in spot 11 and acetylation in spot 22. We failed to detect modifications in the rest of the spots and as seen in the bottom right-hand corner, a portion of the gel failed to run vertically and resulted in spots clustering towards the right side of the gel.

Figure 3 Gel image of $^{15}$N metabolically-labeled mouse heart from an experiment conducted in June 2013. We identified 46 distinct proteins and 17 of them showed isoforms. Mass spectrometry analysis identified phosphorylation in spot 24 and acetylation in spots 1, 28, 37, 39, 40 and 46.
3.3 $^{15}$N metabolically-labeled mouse lung (June 2013)

Figure 4 illustrates findings from another $^{15}$N metabolically-labeled mouse lung processed in June 2013. Even though the spots were sufficiently separated on the gel, many spots had poor mass spectrometry spectra and Protournyzer was unable to assign an LPF value. Consequently, we could not assign a protein identity to that spot. Additionally, we did not detect any PTMs. Hence, we decided to analyse and report data from another $^{15}$N metabolically-labeled mouse lung. This tissue was processed in April 2013 on a smaller gel (150µg).

3.4 $^{15}$N metabolically-labeled mouse lung (April 2013)

We successfully identified 50 distinct proteins and 19 of them showed isoforms (Figure 5).
3.5 Comparison of LPF values among isoforms

We examined the LPF values of protein isoforms found in the mouse heart (June 2013) and lung (April 2013) and observed that different proteins had different LPF values. If isoforms were present, some proteins had more significant differences in LPF values among isoforms than others. For example, an obvious difference among isoforms is noticed in isoforms of GELS_MOUSE whereas isoforms of CPNS1_MOUSE or ECHA_MOUSE have fairly similar LPF values (Figure 6). Similar observations are made in the mouse heart (Figure 7). Interestingly, we identified 10 isoforms of MYH6_MOUSE and their LPF values were fairly similar to each other.

Figure 6 Schematic illustration of LPF values of 19 protein isoforms detected in the 15N metabolically-labeled mouse lung processed in April 2013. Some isoforms had obvious differences in LPF values while others reflected otherwise.

Figure 7 Schematic illustration of LPF values of 17 protein isoforms detected in the 15N metabolically-labeled mouse heart processed in June 2013. Some isoforms had obvious differences in LPF values while others reflected otherwise. We detected 10 isoforms of MYH6_MOUSE and the majority of them had fairly similar LPF values.
3.6 Classification of identified proteins

Classification of the proteins according to their biological processes revealed that the majority of the proteins were enzymes involved in metabolism. Several proteins were adapter proteins involved in cell signaling whereas others were components of the cytoskeleton. Figure 8 summarizes our findings.

Additionally, we found 14 proteins common to the heart and lung (Figure 9) and we identified isoforms among 8 of them. As unequal numbers of isoforms were found in the tissues, we could not compare isoform LPF values to determine if protein turnover rates change in different tissues.

Figure 8 Schematic illustration of classification of biological processes of identified proteins. The majority of the proteins identified were observed to be enzymes involved in metabolism. Other proteins were signalling proteins, associated with the cytoskeleton or chaperones.

<table>
<thead>
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<th>Protein ID</th>
<th>Cell localization</th>
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<th>Isoforms detected</th>
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Figure 9 (right) Schematic illustration of characteristics of 14 proteins common to the mouse lung and heart. The majority of the proteins were identified to be enzymes involved in metabolism. Other proteins were signalling proteins, associated with the cytoskeleton or chaperones.

Figure 10 (below) Summary of isoforms and their LPF values, theoretical PTMs (from SwissProt) and experimentally-detected PTMs.
4. Discussion

This study investigated protein turnover in $^{15}$N metabolically-labeled mouse tissues by calculating the relative abundance of labeled and unlabeled peptides. Through 2D gel electrophoresis, we have demonstrated that protein isoforms exist within tissues and can be separated based on their molecular weights and isoelectric points. After enzyme digestion and peptide mass fingerprinting by mass spectrometry, we identified protein isoforms and in some cases, we were able to pinpoint phosphorylation or acetylation in the isoforms. We could also estimate the turnover of the partially $^{15}$N-labeled proteins and we showed that some protein isoforms had obvious differences in turnover rates whereas others reflected differences subtly.

Previously, various strategies for in vivo turnover analysis have been utilized. For example, Mass Isotopomer Distribution Analysis (MIDA) calculates the precursor RIA after labeling an organism with a single amino acid tracer [9] and in another method, isotope-labeled heavy water is administered to analyze protein dynamics [10]. Yet, complications arise because certain precursor pools have dynamic RIA or turnover rates are so low that the changes in isotopologue distributions cannot be detected. In Stable Isotope Labeling with Amino acids in Cell culture (SILAC), the precursor RIA has to be known or the method cannot be applied to live animals [6].

To analyse turnover rates in our mouse tissues, we utilized Protturnyzer, a novel software that is designed to circumvent the need for a precursor RIA. Although we managed to determine protein turnover in 99 proteins across the mouse heart and lung, there were some proteins that displayed such poor spectra that Protturnyzer could not calculate an LPF value. Moreover, we removed peptides with signal overlaps and these strict filtering criteria could have enhanced accuracy at the expense of coverage. We can possibly reduce signal overlaps by increasing the rigor of protein fractionation before mass spectrometry analysis [6].

Alternatively, we could have missed identification of certain proteins because they did not focus well on the IPG strips or the gels had poor resolution and we did not pick them out. One way to improve the efficiency of gel electrophoresis will be to increase the concentration of proteins extracted from tissues.

We attempted to establish a correlation between isoform LPF values and the proteins' inherent characteristics because it was reported that proteins had differences in turnover rates across tissues—for example liver proteins had on average a ten-fold higher turnover rate than skeletal muscle proteins [10]. Additionally, proteins with structural functions were postulated to have relatively lower turnover rates than proteins with secretory, signaling or developmental functions [6]. After comparing LPF values within and across tissues, we observed that some isoforms reflected differences but we could not form a satisfactory explanation due to the variability of the data. This could be a consequence of homogenizing the tissues during sample preparation and we cannot identify the source of the protein. Certain proteins are known to exist in multiple cell locations and their turnover rates vary depending on whether they are in the location of assembly or location of function [5, 11].

A final point of consideration would be that protein turnover rates could change depending on the age of the tissues used. For example, it is known that certain proteins are highly expressed at early developmental stages and their turnover will gradually decrease as the organism ages [12]. Hence, we could explore turnover rates in mice of various ages to see if we identify more proteins or if the turnover rates of previously-identified proteins are different.
5. Conclusion

Collectively, we have achieved our aims of analyzing turnover in protein isoforms of mouse tissue. Although we did not identify as many proteins as expected, our findings offer a potential framework for future work on using protein turnover data as a novel biomarker to analyze subtle differences between healthy and diseased tissues.

6. Acknowledgements

I would like to thank Amgen Foundation for funding this project and members of AG Turck for their invaluable guidance.

7. References


