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Metabolically stable isotope labeling prior to electrophoretic protein separation reveals differences in fractional synthesis rates between mitochondrial aldehyde dehydrogenase isoforms

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Abstract

Living mice were subjected to whole body labeling by intravenous infusion of [^{13}C]glucose as the sole carbon source. After 10 h infusion the mice were sacrificed, and liver proteins were separated by two-dimensional polyacrylamide gel electrophoresis. Five spots were found to contain mitochondrial aldehyde dehydrogenase (ALDH2) by matrix assisted time of flight mass spectrometry protein identification. By measuring the isotopologue mass distributions of peptide ions, and modeling the ^{13}C content of the precursor amino acid pool, the fractional synthesis rate of ALDH2 molecules synthesized during the labeling period was determined. One of the five spots was observed to have a five-fold higher fraction of ^{13}C-containing newly synthesized ALDH2 than the spot with the highest ALDH2 content, and contained more than 60% of newly synthesized ALDH2 although it accounted for less than 20% of the total ALDH2 detected. The total range in the fraction of ^{13}C-containing proteins between different ALDH2 spots approached 50-fold. The ability to quantitatively characterize different protein isoforms of biological origin for ALDH2 and other proteins from living animals provides new avenues for the exploration of protein function.

Keywords: Fractional synthesis rate; ALDH2; Gel-electrophoresis; MALDI-TOF; Stable isotope; Protein isoform turnover

1. Introduction

With the increasingly refined availability of genomic and proteomic data, the most important avenues of enquiry are emerging as the characterization of protein interactions, their changing subcellular locations, and the temporal dynamics of these processes. The most common analytical methods available to protein chemists for the discovery of these areas of enquiry ultimately involve the indirect identification of proteins by analyzing their peptide fragments. Most methods can be classed into one of two broad categories: (1) biochemical separation of intact polypeptides prior to their reduction to peptides such as in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), or (2) digestion of more or less complex mixtures of proteins followed by separation and identification of individual peptides [1]. Because the peptide sequence coverage of proteins is typically incomplete, the second of these strategic directions often fails to discriminate between peptides that originated in differentially abundant isoforms of a monogenic collection of proteins, or even the products of different but highly homologous genes. However, the differences between monogenic isoforms provide a rich source of biological information. We [2] and others [3] have shown that accurate mass spectrometric quantification of small isotopic enrichments due to isotopic labeling is possible, and we have demonstrated the application to living mice. Here, we report that a combined approach of whole animal ^{13}C stable isotope labeling [2], 2D-PAGE protein separation, radioactive isoform spot quantification, and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) reveal that different protein isoforms of murine liver mitochondrial aldehyde dehydrogenase (ALDH2) exhibit markedly dissimilar turnover rates. The separation of intact polypeptide isoforms by 2D-PAGE was necessary to achieve this result, which would not have been possible using a pre-separation-digest shotgun proteomics approach.

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2. Experimental

The methods and materials correspond exactly to those previously described [2].

2.1. Reagents

All reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany) when not otherwise specified. All reagents were of reagent grade purity and organic solvents were HPLC grade. Iodogen tubes, the BCA Protein Assay Reagent Kit, and β-(4-hydroxyphenol)ethyliodoacetamide were obtained from Pierce (Rockford, IL, USA). Benzonase was purchased from Merck KGaA (Darmstadt, Germany). ^{125}I was purchased from Amersham Buchler (Braunschweig, Germany). Eighteen centimeters immobilized pH gradient gels of pH ranges 5.5–6.7 and 4–7 were purchased from Amersham BioSciences (Freiburg, Germany).

2.2. Mice experiments

Briefly, living male C57BL/6 mice (age range: 80–84 days) were labeled with [^{13}C]glucose (from Campro-Scientific GmbH (ISOTEC), Berlin, Germany) as sole carbon source by intravenous infusion. Control mice were infused with [^{12}C]glucose under otherwise identical conditions. One hour after the infusion was inserted the glucose tracer infusion was started with 2 mg g^{-1} h^{-1} over 30 min followed by 1 mg g^{-1} h^{-1} and continued over 10 h, which produces an effective labeling phase of 8 h, accounting for an initial transient phase of approximately 2 h (during which labeled amino acids are produced from the glucose label). Then the mouse was sacrificed and the liver immediately excised and stored at −80 °C.

2.3. 2D IEF-SDS-PAGE

The mouse liver (approximately 300 mg) was ground under liquid nitrogen and subsequently extracted in 1.2 mL of isoelectric focusing buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% Triton X-100, 10% (v/v) glycerol, 65 mM dithiothreitol). Protein concentration was determined by Bradford assay, and 200–400 μg of protein was loaded onto immobilized pH gradient strips, pH 4–7, by passive rehydration in duplicate per mouse.

2.4. Mass spectrometry

Protein spots were excised from 2D-PAGE gels in-gel digested (incubation at 37 °C overnight) with an excess of Promega sequencing grade modified trypsin. The samples were loaded onto Bruker AnchorChip^{TM}-MALDI-Targets and were prepared using α-cyano-4-hydroxycinnamic acid (HCCA) as matrix. Sample drying was carried out at room temperature. Mass spectrometric measurements were carried out on a Bruker Utraflex time-of-flight mass spectrometer (Bruker Daltonics, Bremen), which was equipped with a SCOUT-MALDI Source for multisample handling, a pulsed UV-laser, a two-stage gridless reflector, a 2 GHz Digitizer, and Multi-Channel-Plate detectors for linear and reflector mode measurements. All measurements were carried out in positive ionization mode using a reflector voltage of 25 kV.

Mass spectra were acquired as sums of ion signals generated by the sample irradiation with 300 laser pulses. Spectra were internally mass calibrated using trypsin auto digestion peptide signals (*m/z* 842.50, 1045.56, 2211.10, 2283.17) as reference values. Mass measurement accuracies were typically ≤50 ppm. For the identification of the proteins the peptide masses extracted from the mass spectra were searched against the NCBI non-redundant protein database (www.ncbi.nlm.nih.gov, version Oct. 2006) using MASCOT software version 2.1 (Matrix Science, London). The search was performed using carbamidomethyl as fixed modification of cysteines, oxidation as an optional modification of methionines, one allowed missed cleavage, and a mass accuracy of 75 ppm.

2.5. FSR determination

Estimation of precursor isotopic enrichments, relative isotopologue abundances, and fractional synthesis rates (FSR) were carried out as previously described [2]. Briefly, the average mass of the population of isotopologues measured for any peptide ion by MALDI-TOF-MS can be used to provide an accurate measure of the amount of heavy stable-isotope atoms incorporated into that peptide. The enrichment level of different amino acids in the precursor pool of free amino acids is modeled, and this information is used in combination with the predicted amino acid sequence of the peptide ion to estimate which fraction of molecules were synthesized during the labeling period. Performing this analysis over multiple peptides provides an averaged estimate of the FSR of the entire protein.

2.6. Radioactive measurements

Radioactive protein spot quantification was performed on the ProteoTope platform, which is a combination of methods developed by ProteoSys AG, Mainz, Germany, including radioiodination, 2D-PAGE, and high sensitivity radio-imaging [4]. Proteins were labeled with radioactive ^{125}I followed by radioactive quantification of protein spots in a 2D-PAGE gel.

3. Results and discussion

Silver-stained 2D-PAGE gels containing liver proteins were made from a mouse labeled with [^{13}C]glucose and a control mouse which had received an identical concentration of [^{12}C]glucose. Five separate spots were identified as corresponding to mouse ALDH2 (Tables 1 and 2). Fig. 1 shows the position of these spots in a 2D gel. Fractional synthesis rates were calculated for each spot separately in order to estimate the fraction of polypeptides that were synthesized during the experimental labeling period (Table 1, Mouse 1). Example ion patterns are shown in Fig. 2, which demonstrates the distortion towards higher mass of the isotopic pattern caused by [^{13}C]glucose labeling of different spots from Mouse 1 compared to a non-labeled