Talanta ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Mitochondria proteome profiling: A comparative analysis between gel- and gel-free approaches

Rita Ferreira [a], Hugo Rocha [b], Vanessa Almeida [a], Ana I. Padrão [a], Cátia Santa [a], Laura Vilarinho [b], Francisco Amado [a,c], Rui Vitorino [a,*]

[a] QOPNA, Department of Chemistry, University of Aveiro, Aveiro, Portugal
[b] I&D Unit, Genetics Department, National Institute of Health Ricardo Jorge, Porto, Portugal
[c] School of Health Sciences, University of Aveiro, Portugal

ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form
29 March 2013
Accepted 8 April 2013
Available online 22 April 2013

Keywords:
Gel-based
Gel-free
Electrophoresis
LC-MS/MS
PTMs
Proteomics

ABSTRACT

Mitochondrial proteomics emerged aiming to disclose the dynamics of mitochondria under various pathophysiological conditions. In the present study we investigated the relative merits of gel-based (2DE and SDS-LC) and gel-free (2D-LC) protein separation approaches and protein identification algorithms (Mascot and Paragon) in the proteome profiling of mitochondria isolated from cultured fibroblasts, a sample traditionally used for diagnosis purposes.

Combining data retrieved from 2DE, 2D-LC and SDS-LC and search methods, a total of 696 non-redundant proteins were identified. An overlap of only 19% between the proteins identified by the three different methods was observed when Mascot and Paragon were used. Regarding protein ID, a consistency in the number of identified proteins *per* sample was noticed for 2DE approach. Independent of the methodological approach chosen, it was noticed that the predominance in mitochondria of hydrophilic proteins with 20–50 kDa and p*I* 5–6 and 8–9; however, 2D-LC and SDS-LC allowed the enrichment of proteins with a mass below 30 kDa and of basic proteins with p*I* values above 8. In conclusion, data from the present study highlight the power of integrating different separation technologies and protein identification algorithms.

1. Introduction

Mitochondrial dysfunctions are associated with many pathophysiological conditions, such as metabolic disorders, diabetes mellitus, cancer, neurodegenerative diseases and aging [1,2]. Among these, metabolic disorders involving defects in proteins/enzymes from pathways harbored in mitochondria are rare but related with high morbidity and mortality [3]. Many effects of these disease processes have been studied using classic biochemical methods focused on a particular protein [4]. Recent developments in proteomics have allowed a global perception into protein expression, localization, and interaction, and how they are modulated by pathophysiological conditions. Indeed, proteome profiling has been intensely used for the investigation of pathogenic mechanisms and functional correlations on protein levels in a non-biased manner [2,4,5]. Among the protein separation procedures used for mitochondria proteome profiling, two-dimensional gel electrophoresis (2DE) as well as multidimensional liquid chromatography (2D-LC) have been adopted [1,6–8]. While 2DE, a classical proteomic technology, separates proteins based on their isoelectric point (p*I*) and molecular weight (MW) with a limited dynamic range, 2D-LC partially overcomes the shortcomings of 2DE such as limitations in detecting proteins with extreme alkalinity, hydrophobicity, and molecular mass, allowing large-scale, discovery-driven proteomics [1,4,9–11].

Cultured skin fibroblasts are an attractive sample for diagnostic testing and research of metabolic diseases involving defects in mitochondrial proteins, considering the minimally invasive character of sampling and the large amount of cell material that can be obtained by culturing [12]. For the diagnosis of certain diseases, enzymatic measurements or molecular genetic analysis of tissue and/or cell samples is required [3] and for that, tissue biopsy is often needed. Although tissues like heart, skeletal muscle and liver are among the main targets of metabolic disorders, such as fatty acid oxidation defects [13], skin biopsy is usually preferred. Besides being ethically straightforward, the isolated fibroblasts can be used to measure ATP synthesis, oxygen consumption, electric membrane potential, and substrate oxidation rates [3,12,13]. Moreover, fibroblasts can be stored and retested when desired and might be used as a model system to study diseases' pathogenesis [3]. Despite all these advantages, few studies have

* Correspondence to: Department of Chemistry, University of Aveiro, 3810-193, Aveiro, Portugal. Fax: +35 1234370084.
E-mail address: rvitorino@ua.pt (R. Vitorino).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.04.026

been performed focused on mitochondrial protein profiling in cultured fibroblasts [1,6,7] and their use might be advantageous in the investigation of complex defects where several factors may contribute to disease [1].

In order to clarify the strengths and limitations of proteomics to study the mitochondrial proteome in fibroblasts and the potential implications for the comprehension of metabolic diseases' pathogenesis, we performed a comparative analysis of gel-based and gel-free protein separation approaches, and protein identification algorithms (Mascot and Paragon). We report the advantages of combining multidimensional approaches to get a deep protein profile screening of mitochondria isolated from cultured fibroblasts aiming a more comprehensive and effective manner to investigate the mitochondrial proteome. Nevertheless, from the separation approaches tested, a higher number of identified mitochondrial proteins were achieved with SDS-LC approach, apart from the protein identification algorithm chosen.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all reagents such as triethylammonium bicarbonate (TEAB), trifluoroacetic acid (TFA), protease inhibitor cocktail, formic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), urea, CHAPS were purchased from Sigma-Aldrich. Immobiline drystrip and ampholytes were from GE Healthcare. Sequencing grade modified trypsin (bovine) was from ABSciex (ABSciex, USA).

2.2. Cell culture

Skin fibroblasts from healthy individuals ($n=3$) were grown in Ham F10 nutrient mixture supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 1% penicillin, streptomycin and fungizone, in 75 cm^2 culture flasks. Ten culture flasks from each sample were grown to subconfluence before mitochondria isolation.

2.3. Mitochondria isolation and protein extraction

Isolation of mitochondria from the cell pellet was performed at 4 °C according to Schwab et al. [14] with minor modifications. Briefly, the cell pellet was suspended in isolation buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES and 5 g/L BSA, pH 7.5) and was then centrifuged at 500 × g for 2 min. The supernatant was discarded and the remaining pellet was suspended in 500 μL of isolation buffer. The cell suspension was homogenized in a tight-fitting Potter homogenizer (Teflon pestle). After centrifugation at 1500 × g for 10 min, the supernatant was kept on ice. The pellet was homogenized and centrifuged as described above. The two supernatants were pooled and centrifuged at 10,000 × g for 10 min. The resulting mitochondrial pellet was washed with 100 μL of BSA-free isolation buffer. Mitochondrial proteins were extracted according to the different separation procedures tested and protein content was determined with RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Following fibroblast mitochondria isolation, three different procedures were used for protein characterization (Fig. 1).

2.4. Two-dimensional electrophoresis (2DE)

Two-dimensional electrophoresis (2DE) was performed as previously reported with minor modifications [6]. In brief, 250 μg of mitochondrial protein extract were diluted to 250 μL with a rehydration solution containing 8 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer (pH 3–10 NL), 0.2% DTT and loaded on 13 cm IPG strips (pH 3–10 NL; GE Healthcare). Isoelectric separation was performed using the following focusing program: 12 h at 50 mV (rehydration), 2 h at 150 V (gradient), 1 h at 500 V (gradient), 1 h at 1000 V (gradient) and 3 h at 8000 V ("step-n-hold"). After IEF separation, the gel strips were applied on top of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (12.5%) and proteins were separated at 200 V constant current until the bromophenol blue front reached the bottom of the gel. At least three gels were generated for further gel image analysis and mass spectrometry characterization. The gels were stained with Coomassie Colloidal-G250, and scanned with Gel Doc XR (Bio-Rad). The "Spot Detection Wizard" function in PdQuest software (v8.0.1, Bio-Rad) was used to automate the process of finding protein spots and based on the automated spot detection results, misdetected spots were added or removed using visual inspection. All detected spots were manually excised for in-gel digestion.

2.5. One-dimensional gel electrophoresis (SDS-PAGE)

Seventy micrograms of protein were incubated with SDS sample buffer (10% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 6.8), 4% glycerol, bromophenol blue (w/v)) for 5 min at 100 °C. Samples were loaded onto Laemli gel (12.5%) and electrophoresis was carried out at a constant voltage of 200 V current until the bromophenol blue front reached the bottom of the gel. Gels were stained with Coomassie Colloidal-G250. Following image acquisition, the entire gel lanes were manually cut out lengthwise and divided into 16 gel slices for in-gel digestion.

2.6. In-gel/band protein digestion

Annotated spots or bands were in-gel digested with trypsin. Briefly, gel pieces were washed three times with 25 mM ammonium bicarbonate/50% acetonitrile and further dried in a Speed-Vac. Then, 10 μL of 25 μg/mL modified bovine trypsin (ABSciex, USA) in 25 mM ammonium bicarbon ate was added to the dried gel pieces and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid/50% acetonitrile, which was repeated three times, followed by dryness in a Speed-Vac. 2DE spot's tryptic peptides were resuspended in acetonitrile/formic acid solution and mixed (1:1) with a matrix consisting of α-cyano-4-hydroxycinnamic acid (5 mg in 1 ml of 50% acetonitrile/0.1% trifluoracetic acid). Aliquots of samples were spotted onto MALDI sample target plates. SDS-PAGE bands tryptic peptides were resuspended in acetonitrile/trifluoroacetic acid (5% and 0.01%, respectively) for reverse-phase LC separation.

2.7. In-solution protein digestion

Seventy micrograms of protein was used for digestion which was performed as described previously [15]. Protein was precipitated by incubation with cold acetone (6 volumes) overnight (−20 °C) and centrifuged at 20,000 × g for 30 min. Pellet samples were then ressuspended with triethyl ammonium bicarbonate buffer (TEAB) (1 M, pH 8.5) and RapiGest (Waters) to a final concentration of 0.5 M and 0.1%, respectively. Samples were reduced with 5 mM tris(2-carboxyethyl) phosphine (TCEP) for 1 h at 37 °C and alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) for 10 min at room temperature. Two micrograms of trypsin were added to each sample and the digestion was performed for 18 h at 37 °C. Samples were dried in a Speed-Vac and then ressupended for peptide separation in high pH reverse phase separation.

Please cite this article as: R. Ferreira, et al., Talanta (2013), http://dx.doi.org/10.1016/j.talanta.2013.04.026