Quantitative proteomics of mitochondrial membrane proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis, $^{16}$O/$^{18}$O stable isotope labeling and linear ion trap mass spectrometry

Horacio Serrano$^6$, Inmaculada Jorge$^6$, Pablo Martínez-Acedo$^6$, Pedro J. Navarro$^6$, Daniel Pérez-Hernández$^{6,8}$, Elisabet Miró Casas$^8$, David García-Dorado$^8$, Jesús Vázquez$^{6,1}$

$^6$Protein Chemistry and Proteomics Laboratory, Centro de Biología Molecular Severo Ochoa, Madrid, Spain, $^8$Universidad de Puerto Rico, Arecibo, Puerto Rico, $^1$Hospital Universitario La Princesa, Madrid, Spain and $^8$Servicio de Cardiología, Hospital Vall d’Hebron, Barcelona, Spain

Summary:
In spite of recent developments, the global analysis of membrane subproteomes is still a common obstacle. In this work we explore whether quantification of mitochondrial membrane proteins can be achieved by SDS-PAGE separation followed by in-gel digestion, $^{16}$O/$^{18}$O stable isotope labeling and peptide-centric identification and quantification by linear ion trap mass spectrometry. Using this approach we were able to identify more than fifty membrane proteins among a total of more than one hundred, from a preparation of 100 µg crude mitochondrial membranes. A part of this proteome was split into two and subjected to comparative analysis by $^{16}$O/$^{18}$O stable isotope labeling. A very narrow distribution of ratios was obtained, showing no evident deviations from the 1:1 ratio in more than two hundred quantifications. Our data demonstrated that significant changes in protein expression levels higher than 1.5-fold or lower than 0.6-fold could be detected at the p < 0.05 confidence level. Our results suggest that this method could be suitable for the large-scale identification and relative quantification of mitochondrial membrane proteomes, as well as other protein membrane preparations.

Keywords:
Membrane proteins, Differential Expression Proteomics; Mitochondria; $^{18}$O labeling; Linear Ion Trap

Introduction

About a third of all proteins encoded by genomes are estimated to be membrane proteins. Despite the importance of these proteins in living systems, the global analysis of membrane subproteomes is still a common obstacle (Hynek et al., 2006). Separation and quantification of plasma membrane proteins was initially attempted by using two-dimensional gel electrophoresis, but this approach has a number of technical drawbacks that are well known in the Proteomics community. Manipulation of membrane proteins is difficult due to their tendency to aggregation, and many of them fail to enter the isoelectric focusing gel in the first dimension and are hence lost; besides, other membrane proteins are known to remain undetected by conventional staining methods. Alternative methods have been proposed; in a recent report a combination of solvent-extraction and hydrophilic interaction chromatography followed by one-dimensional SDS-PAGE analysis was used for the analysis of the mitochondrial proteome (Carroll et al., 2006). However, the applicability of these methods for protein quantification by stable isotope dilution is still unknown.

The analysis of differential protein expression is fundamental for the understanding of biological processes and plays an increasingly important role in biological and medical research (Li et al., 2003).
Differential ¹⁶O/¹⁸O stable isotope labeling of proteins in comparative samples is an increasingly more commonly used method for subsequent quantitative analysis by mass spectrometry (Heller et al., 2003; Staes et al., 2004; Nelson et al., 2006). In a recent report we have demonstrated that linear ion trap mass spectrometry is a suitable method for peptide quantification by ¹⁸O labeling (López-Ferrer et al., 2006). Later, we presented a method to calculate labeling efficiency at the individual peptide level, improving the robustness and degree of automation of this technique (Ramos-Fernández et al., 2007). This method is being applied to the quantitative proteomics analysis of HUVEC cells in response to pro-angiogenic factors (Jorge et al., 2007; Navarro et al., 2007). This labeling technique has been used in combination with SDS-PAGE separation of proteins and in gel digestion for the analysis of phosphoproteins (Korbel et al., 2005). However, the latter work was done by using high-resolution mass spectrometry and their application to the analysis of membrane proteins has still not been tested.

In this work we analyze the feasibility of performing differential expression experiments on the mitochondrial membrane proteome by a combination of one-dimensional SDS-PAGE followed by “in-gel” digestion of the separated proteins, 18O/16O peptide labeling and peptide identification and quantification by linear ion trap mass spectrometry.

Material and methods

A protein extract from a preparation of rat heart mitochondrial membranes (100 µg), prepared from cardiac mitochondria as described (Holmuhamedov et al., 1998; Ruiz-Maena et al., 1999) were redissolved in 40 µl buffer consisting of 8 % (w/v) SDS, 10 % (v/v) glycerol, 25 mM Tris-Cl (pH6.8), 5 % (v/v) β-mercaptoethanol, 0.01 % (w/v) bromophenol blue, and were separated using SDS-PAGE (12 % polyacrylamide gel, 8 x 8 cm). The gel was stained with Coomasie Brilliant Blue R-250 (Bio-Rad). The gel lanes were horizontally cut into 5 pieces (containing approx. 20 µg of protein each), and each piece were subjected to digestion with porcine trypsin (Promega) at a substrate/protease ratio of 20:1 (w/w), as previously described (Shevchenko et al., 2006). The resulting peptides from each piece were dried down and analyzed separately by RP-HPLC-MS/MS. For differential labeling, dried peptide fractions were incubated with immobilized trypsin (Pierce) in a reaction mixture containing 20 % (v/v) acetonitrile, 100 mM ammonium acetate (pH 6.0), immobilized trypsin at a substrate/protease ratio of 200:1 (v/w), and ¹⁸O or ¹⁶O water at a substrate/water ratio of 1:1 (w/w). The samples were incubated overnight at 37 ºC and mixed together just before MS/MS analysis.

Peptides were analyzed by using a Surveyor LC system coupled to a LTQ linear ion trap mass spectrometer (Thermo-Fisher) as described previously (Martínez-Ruiz et al. 2005; Ortega-Pérez et al. 2005), with minor modifications. Peptides were concentrated and desalted on a RP precolumn (0.32 x 30 mm, BioBasic-18, Thermo Electron) and online eluted on an analytical RP column (0.18 x 150 mm BioBasic-18, Thermo Electron), operating at 2 µl/min and using a 170-min gradient from 5 % to 40 % B (solvent A: 0.1 % formic acid (v/v); solvent B: 0.1 % formic acid (v/v), 80 % acetonitrile (v/v)). The linear ion trap was operated in a data-dependent ZoomScan- and MS/MS-switching mode using the six most intense precursors detected in a survey scan from m/z 400 to 1600. Zoom target parameters, number of microscans, normalized collision energy, and dynamic exclusion parameters were as described in López-Ferrer et al. (2005).

Protein identification in Rattus norvegicus-unipro.fasta database was carried out as described using SEQUEST (López-Ferrer et al. 2004) and statistical analysis and determination of error rates were performed after doing a normal and inverted database search by using the Probability Ratio method (Martinez-Bartolomé et al., 2007 submitted; Navarro et al., 2007). Peptide quantification from ZoomScan data were performed as described (López-Ferrer et al., 2006; Ramos-Fernández et al., 2007), using a program written in our laboratory (QuiXoT).

Results and Discussion

We were interested in the analysis of the mitochondrial membrane proteome by multidimensional chromatography techniques; this approach required in solution digestion of the proteome followed by ion exchange fractionation of the resulting peptide pool and subsequent analysis by RP-HPLC-MS/MS, as described in other works (López-Ferrer et al., 2004). After unsuccessful attempts to digest the mitochondrial membrane proteome by conventional in solution digestion techniques (data not shown),
we analyzed whether multidimensional analysis of these membrane proteins could be performed by using SDS-PAGE as a first separation step, followed by in-gel digestion of different gel regions and RP-HPLC-MS/MS analysis of peptides obtained from these fractions. For this end, a protein extract from a mitochondrial membrane preparation was subjected to SDS-PAGE; the gel was cut into five pieces, each piece was digested, and the resulting peptides analyzed separately by RP-HPLC-MS/MS (five runs), as described in the Material and Methods section. The MS/MS spectra were searched against Rattus norvegicus-uniprot database, as well as against an identical, inverted database, constructed by reversing the amino acid sequence of each one of the proteins. 1154 MS/MS spectra, corresponding to 524 unique peptides were selected for positive identification at a 5 % false discovery rate. They corresponded to 128 unique proteins. Analysis of the results according to Gene Ontology categories revealed a high proportion of mitochondrial membrane proteins. As shown in figure 1, 69 % of identified proteins were of mitochondrial origin, from which about a half of the proteins were from the inner or outer membrane (figure 1). More than fifty membrane proteins could be readily identified by this approach.

Relative quantification of peptide pairs is performed as follows. For each peptide species identified in the survey scan, the linear ion trap is programmed to execute a ZoomScan spectrum followed by a MS/MS spectrum; the first spectrum is used for quantification and the second one is used for peptide identification (López-Ferrer et al., 2006). Only ZoomScan spectra for peptides identified by their MS/MS spectrum are taken into account (López-Ferrer et al., 2006). This is done automatically by QuiXoT, a program written in C# that opens automatically the raw files and peptide identification results and shorts out the ZoomScan spectra corresponding to these species (Navarro et al., 2007). Peptide quantification from the ZoomScan spectra is performed by QuiXoT using an algorithm described previously (Ramos et al., 2007). The program fits the entire isotopic envelope to the sum of theoretical isotopic envelopes from four independent species, including the non-labeled species from the unlabeled sample, as well as the non-labeled and mono- and di-labeled species from the labeled sample, according to a kinetic model that takes into account the rate of $^{18}$O incorporation (Ramos et al., 2007). This allowed a precise calculation of labeling efficiency of each one of the quantified peptides, as well as the determination of the proportion of peptide coming from the unlabeled and labeled samples. Calculation of labe-
ling efficiency for each one of the quantified peptide pairs, particularly in large-scale experiments, is very important to avoid potential artifacts in this kind of techniques, since false protein expression changes may arise from incomplete incorporation of the isotope label to one of the peptide pairs. The performance of this method to control for incomplete labeling artifacts has been demonstrated in a previous work (Ramos et al., 2007).

To analyze labeling efficiency, this parameter was plotted against the ratio of peptide intensities in the two samples in a log₂ scale (figure 2A); we have found this plot particularly appropriate to check the extent of isotopic labeling and its influence on relative peptide quantification. As shown in figure 2A, almost all peptides had a labeling efficiency higher than 0.6, and could therefore be used for quantification, since, as published previously, at these high efficiency levels the small variations that incomplete labeling produce on the peptide ratio are accurately taken into account by introducing a labeling efficiency correction (Ramos et al., 2007). QuiXoT calculates a statistical weight that measures the accuracy of individual quantifications; higher weights correlate with a lower deviation in the ratio (figure 2B). Low-quality quantifications were automatically filtered out by using a statistical weight threshold of 50 (figure 2B); application of this filter eliminated only 5% of the total number of quantifications. As shown in figure 2C, the calculated log₂ ratios were closely clustered around zero (ratio 1:1) and the distribution of log₂ peptide ratios could be perfectly fitted to a normal distribution, which is the null-hypothesis for an experiment with no differential expression events, as described by others (Staes et al., 2004; López-Ferrer et al., 2006; Jorge et al., 2007; Ramos et al., 2007). The Gaussian distribution was fitted with a mean of -0.16 and a SD of 0.31. The log₂ ratio distribution was very narrow, and our data demonstrated that significant changes in protein expression levels higher than 1.5-fold or lower than 0.6-fold could be detected at the p < 0.05 confidence level.

Our results demonstrate that differential expression proteomics on the mitochondrial membrane proteome is possible by using SDS-PAGE separation, in gel-digestion; stable ¹⁶O/¹⁸O isotope labeling and linear ion trap mass spectrometry. Direct comparison of protein expression levels of two samples analyzed by monodimensional SDS-PAGE does not, in general, allow detecting protein expression changes in complex mixtures due to the low protein resolution of this technique and the interference of abundant proteins. However, our results suggest that by using this method as a first dimension together with stable isotope labeling, results similar to those obtained by multidimensional chromatography could be attained. Although we have focused this work in a preparation of mitochondrial membranes, we think that this method is of general applicability for mem-
brane proteins. Besides being particularly suitable for membrane proteins, since the presence of SDS in the gel electrophoresis step allows a good separation of these proteins and their posterior in gel digestion, this approach had several advantages over conventional in solution protocols. Among others, it allowed the analysis of detergent-containing samples, being this technical detail particularly relevant, since detergents are included in most protein extraction protocols, particularly those of membrane proteins. Also, SDS-PAGE was used as a first separating step, making it unnecessary to perform cation exchange chromatography separation of the peptide pool. Besides, all the peptides from each one of the proteins were analyzed together in the same HPLC fraction, thus allowing a more straightforward protein identification and interpretation of quantification results. Finally, confirmation of tentative differential expression events can be easily performed in separate lanes by concentrating the analysis on gel zones where the protein is expected to migrate, making it unnecessary to repeat the whole experiment. We are applying this method to the analysis of changes in the mitochondrial membrane proteome from rat heart in response to pathophysiological events such as hypertension and other myocardium-related events.

References


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