Applied Proteomics
Mitochondrial Proteins and Effect on Function

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Abstract—The identification of a majority of the polypeptides in mitochondria would be invaluable because they play crucial and diverse roles in many cellular processes and diseases. The endogenous production of reactive oxygen species (ROS) is a major limiter of life as illustrated by studies in which the transgenic overexpression in invertebrates of catalytic antioxidant enzymes results in increased lifespans. Mitochondria have received considerable attention as a principal source—and target—of ROS. Mitochondrial oxidative stress has been implicated in heart disease including myocardial preconditioning, ischemia/reperfusion, and other pathologies. In addition, oxidative stress in the mitochondria is associated with the pathogenesis of Alzheimer’s disease, Parkinson’s disease, prion diseases, and amyotrophic lateral sclerosis (ALS) as well as aging itself. The rapidly emerging field of proteomics can provide powerful strategies for the characterization of mitochondrial proteins. Current approaches to mitochondrial proteomics include the creation of detailed catalogues of the protein components in a single sample or the identification of differentially expressed proteins in diseased or physiologically altered samples versus a reference control. It is clear that for any proteomics approach prefractionation of complex protein mixtures is essential to facilitate the identification of low-abundance proteins because the dynamic range of protein abundance within cells has been estimated to be as high as $10^7$. The opportunities for identification of proteins directly involved in diseases associated with or caused by mitochondrial dysfunction are compelling. Future efforts will focus on linking genomic array information to actual protein levels in mitochondria. (Circ Res. 2002;90:380-389.)

Key Words: proteomics ■ two-dimensional gel electrophoresis ■ mitochondria ■ reactive oxygen species ■ mass spectrometry
Mitochondrial mutations and dysfunction have been implicated in numerous diseases including heart disease, myocardial preconditioning, and ischemia/reperfusion, neurological diseases, such as Parkinson’s, Alzheimer’s, and Huntington’s, aging, cancer, and various neuromuscular syndromes. The rapidly emerging field of proteomics can provide a powerful suite of techniques for the identification and characterization of mitochondrial proteins involved in these processes.

**Sample Complexity Reduction: The Key to Proteomic Analysis**

The analysis and characterization of complex mixtures of proteins are the central aims of proteomics. Whether the goal is creation of a detailed inventory of protein components in a single sample or identification of differently expressed proteins in disease samples versus controls, accurate resolution of thousands of proteins and their posttranslationally modified isoforms is an absolute requirement.

To date, two-dimensional (2-D) gel electrophoresis is the preferred method to resolve and array authentic proteins from cellular mixtures. Combined with high-throughput mass spectrometry (MS) techniques, 2-D gels allow the simultaneous analysis of thousands of protein species. For detailed descriptions of mass spectrometry methods applicable to proteomics, see previously published review articles. Peptide mass mapping using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry has become the preferred high-throughput technique for initial identification of protein spots from 2-D gels. Protein identifications made from peptide mass fingerprints can be confirmed using post source decay (PSD) MALDI-MS or tandem mass spectrometry (LC-MS/MS) fragmentation of individual peptides. The fragment ion spectra generated by these techniques can be used to search sequence and expressed sequence tag (EST) databases. An example of a typical MALDI-MS peptide profile and PSD-MALDI-MS spectrum generated in the laboratory of one of the authors (M.F.L.) from an in-gel digest of mitochondrial ATP synthase is shown in Figure 1.
shown in Figure 1. If the peptide mass fingerprints do not match any protein fingerprints present in the public databases, de novo amino acid sequence data can also be generated by fragmentation of individual peptides using LC-MS/MS or PSD-MALDI-MS.

As an alternative to the 2-D gel/mass spectrometry approach described earlier, methods for the characterization of protein mixtures independent of 2-D gels have been developed in recent years. The impetus for alternatives to gels has been the perceived need for higher throughput and methods that may be easily automated. Even in addition, questions about the limitations of 2-D gels with respect to the resolution of low-abundance or hydrophobic proteins have prompted the search for methods that are unbiased in these respects, although recent reports have demonstrated that these perceived limitations can be overcome.

The most recent estimates place the number of genes in the human genome at approximately 40,000, far less than had previously been predicted. Even with this revised estimate of a “low” number of genes, the number of proteins encoded by the genome will still be far too many to see on a single gel. Current high-resolution techniques can resolve on the order of 1000 to 3000 different polypeptide spots on a 2-D gel. Consequently, some reduction in the complexity of samples is needed to display the majority of cellular proteins on gels.

2-D Gels Are High-Resolution Protein Arrays

Two-dimensional gels are a classic technology that has enjoyed a renaissance in the past 10 years. Because of the highly parallel nature of the technique, hundreds to thousands of proteins can be visualized simultaneously. Therefore, 2-D electrophoresis is an ideal tool for the resolution of complex protein mixtures. When coupled with mass spectrometry, individual polypeptide components can be accurately identified. A 2-D gel is also an array of authentic proteins, ie, charge, molecular weight, and posttranslational modifications are preserved during electrophoresis. This allows direct analysis of protein isoforms that may be involved in particular metabolic or disease processes. In addition, the quantitative differences between proteins in mixtures can be determined from 2-D gel images. This allows the direct detection of differentially expressed gene products. The importance of measuring protein levels has become increasingly clear as several reports have shown that transcriptional profiles do not necessarily parallel the actual protein levels in cells and cannot be used to predict posttranslational modification of proteins. Therefore, differentially expressed protein identification may turn out to be the most straightforward route to identification of drug and diagnostic targets.

There has been some debate regarding the perceived limitations of 2-D gels in the detection of low-abundance or very hydrophobic proteins. However, evidence presented in a recent study demonstrates that sample fractionation in combination with the correct solubilization reagents can overcome the bias toward high-abundance, cytosolic proteins and reveals the presence of hydrophobic and basic membrane proteins. In addition, high-percentage acrylamide gels can facilitate identification of low molecular weight proteins. Therefore, a 2-D gel–based approach is a feasible one for studying the relatively limited subset of proteins found in and associated with mitochondria.

Membrane and Hydrophobic Protein Solubilization for 2-D Gels

Membrane proteins have always proven difficult to resolve on 2-D gels. Several reports have described improved solubilization protocols using sequential extraction, novel detergents, and fractionation protocols for the enrichment of hydrophobic proteins. Using these techniques, the authors resolved integral membrane proteins from yeast and Escherichia coli samples. These novel methods are clearly applicable to the solubilization and resolution of difficult mitochondrial membrane proteins.

High-Sensitivity Stains for 2-D Gels

Currently, there are no stains that can span the approximately 7 or 8 orders of magnitude dynamic range of cellular proteins. However, the development of high-sensitivity, mass spectrometry “friendly” stains has improved the probability of detecting and identifying low-abundance proteins in 2-D gels. A recent development, SYPRO Ruby protein stain is a fluorescent ruthenium-based stain that binds noncovalently to proteins in gels (Molecular Probes). This stain can detect low nanogram amounts of proteins in gels, is very compatible with in-gel digests for mass spectrometry, and has a linear dynamic range of 2 to 2000 nanograms, spanning the ranges of both Coomassie and silver stains. An advantage of the extended dynamic range is a potentially increased accuracy of quantitative comparisons in differential display experiments.

Subcellular Fractionation

Subcellular fractionation is a useful technique for isolating proteins associated with organelles such as mitochondria. Researchers have used centrifugation or other fractionation techniques to isolate nuclei, and as a result nuclear matrix proteins, as well as proteins associated with a variety of other cellular organelles or compartments including mammalian lysosomes, endosomes, rough endoplasmic reticulum (ER), plasma and mitochondrial membrane, mitochondrial ribosomes, centrosomes, and cyanobacterial thylakoid membranes. A pertinent question to any investigation of the proteomics of subcellular fractions is a discussion of the purity of the fraction. For example, mitochondrial preparations can be contaminated with cytosolic, microsomal, and ER proteins. It is not even clear how closely these proteins are associated in vivo and whether the connection is
biologically relevant. Because the contaminating proteins will be identified using mass spectrometric approaches, the key is consistent preparation of fractions. This will prevent erroneous assumptions when different fractions are compared.

**Charge Fractionation**

Protein mixtures can be conveniently fractionated by charge by using narrow-range pH gradients during isoelectric focusing \(^{77–80}\) or by using a combination of instrumentation and narrow-range pH gradients. \(^{81–83}\) These strategies result in a series of overlapping protein patterns created with very narrow-range pH separations. This approach is well suited to the study of mitochondrial proteins because narrow-range, basic pH gradients can resolve many membrane proteins. Traditional liquid chromatography techniques can also be used to fractionate protein mixtures by charge before separation on 2-D gels. A disadvantage is that these methods require greater amounts of sample. An example of this approach is the separation of *E. coli* proteins using anion exchange chromatography with the subsequent resolution of each fraction on 2-D gels. \(^{84}\)

**Affinity Fractionation**

Affinity ligands can be useful for fractionating protein mixtures before 2-D gel separation. Affinity separations can be used to remove abundant proteins such as in the removal of albumin or IgG from serum or plasma with Cibachron Blue resins, \(^{85,86}\) or other techniques. \(^{87}\) Conversely, affinity ligands can be used to enrich desirable or low-abundance proteins in a mixture using heparin, \(^{88,89}\) hydroxyapatite, \(^{90}\) various lectins, among them concanavalin A, \(^{91,92}\) immobilized metal-ion affinity chromatography (IMAC) resins, \(^{93}\) and other ligands. \(^{94–98}\) The IMAC approach has successfully been used to enrich for calcium-binding proteins from mitochondria. \(^{93}\) An extension of this is to use an individual member of a protein complex as an affinity ligand or “bait” to “fish” for the rest of the proteins in the complex. Characterization of actin-binding proteins from muscle \(^{99}\) and the purification and identification of proteasomes \(^{100}\) are examples of this type of approach.

**Other Fractionation Techniques**

Other techniques for protein enrichment or fractionation before 2-D gels include membrane protein enrichment by differential detergent fractionation or sodium chloride treatment, \(^{101–104}\) immunoprecipitation, \(^{105}\) or size fractionation, \(^{106,107}\) which can isolate specific protein groups.

**Liquid Chromatography and Protein Chip-Based Methods**

Numerous efforts have been undertaken to develop proteome analysis protocols that do not require the running of 2-D gels. Several of these approaches have the advantage that they are potentially very high-throughput, although to date none have demonstrated resolution of complex mixtures equivalent to that which can be achieved in 2-D gels.

**Multidimensional Liquid Chromatography**

Initial efforts to characterize proteins in mixtures without using 2-D gels as a first step have focused on multidimensional liquid chromatography to decrease sample complexity coupled with mass spectrometry for protein identification. \(^{108,40–42,45–46}\) These approaches have all suffered from the disadvantage that they are not quantitative, hence not useful for differential display analyses. In addition, most of these approaches fall short of the resolution achieved with 2-D gels and are still biased with respect to pI and molecular weight. This is because very acidic proteins will be underrepresented in the mixture due to fewer tryptic cleavage sites, and low molecular weight proteins will be underrepresented due to the production of fewer peptides. \(^{42}\) A clear advantage is that most of these methods are very automation-friendly.

**Affinity-Tagged Peptides**

A refinement of the multidimensional liquid chromatography approach involves the biotinylation and affinity capture of cysteiny1 peptides in an effort to minimize the bias toward larger proteins with more numerous peptides. \(^{109,110}\) Drawbacks to this approach are that it is not quantitative and that the purified peptides do not all ionize and fragment equally well in the mass spectrometer, leading to underrepresentation of these proteins in the analysis. \(^{110}\)

Recently, a method was developed that takes these approaches one step further and allows quantitative analysis of protein mixtures. \(^{111}\) With this technique, sample protein mixtures to be compared are affinity-tagged with isotopically coded linkers or ICAT. The protein mixtures are then subjected to trypsin digestion and the tagged peptides are isolated by affinity chromatography and then analyzed by mass spectrometry. Isotopically tagged light and heavy forms of identical peptides are quantified by measuring the relative signal intensities of each pair. The relative advantages and disadvantages of this approach can be summarized in several points.

The ICAT technique has become very popular because it theoretically allows high-throughput quantitative protein identification without using 2-D gels. This can be a tremendous time-saver and eliminates labor-intensive steps.

A serious caveat is that only a small proportion of peptides contain cysteine so the technique is biased toward those proteins with several or more (relatively rare) cysteine residues. A significant number of proteins contain no cysteine residues at all, and such proteins will not be detectable by this technology. In addition, the dynamic range of the technique can be limited, depending on the mass spectrometer used. \(^{111}\)

Unfortunately, the technique is also not truly quantitative in that the chromatographic fractionation of deuterated and nondeuterated peptides is subject to measurement errors of up to 500%. This is due to the fact that the differentially tagged peptides do not co-elute exactly. \(^{112}\) Finally, posttranslational modifications cannot be observed with this approach.

More recently, a method for differential stable isotope labeling of peptides using per-methyl esterification of carboxylic acids such as are present in aspartic and glutamic acids and in the carboxy termini has been developed. \(^{113}\) This technique should allow less biased labeling of peptides for identification by mass spectrometry. Whether the resolution and dynamic range of this newer isotope-coded affinity tagging technology for proteomics will be equivalent or better
than that achieved with 2-D gels/mass spectrometry remains to be seen. However, this technique will most likely still be subject to the potential errors in quantitation observed with ICAT.

**Protein Chips or Microarrays**

The advent and success of genomic microarrays for high-throughput analysis of transcriptional profiles have prompted analogous approaches to proteomic analysis. In the past few years, numerous articles have been published detailing different approaches to protein chip technology. Most of these studies involve the application of proteins or antibodies in arrays on glass slides or membranes. The technology is still in its infancy but has the potential to allow the analysis and characterization of thousands of proteins and their interactions in a single experiment. This would be a tremendous advantage for simultaneously screening large numbers of samples. The drawbacks of current methods are that post-translational modifications, which are known to be instrumental in the development of many diseases, will be difficult or impossible to track by these techniques. In an innovative departure from the traditional concept of protein chips, some researchers are implementing the microfluidic printing of arrayed chemistries on individual protein spots blotted onto membranes. This technique will effectively allow each spot on a 2-D gel blot to act as a protein chip.

**Building Mitochondrial Databases**

The literature on the proteomics of mitochondria is sparse. Few studies have been published to date, but this is bound to change quickly as intense interest is focused on the area of oxidative stress, disease, and mitochondrial proteins. The initial reports have been focused on building databases or maps of mitochondrial proteins from 2-D arrays. This approach is driven by the concept that detailed maps can serve as a reference for studies of mitochondrial dysfunction and disease. Rabilloud et al. created a database of 46 human placental proteins identified from 2-D gels by mass fingerprinting with MALDI-TOF complemented by protein sequencing and immunodetection. A limitation of this study was the contamination of the placental mitochondrial preparation by secreted proteins such as choriomammotropin that are present in granules approximately the same size and density as mitochondria and that co-purified during the subcellular fractionation procedure. More recently, the same group has focused on identifying the antioxidant defense system in rat mitochondria. Lopez et al. created a database of approximately 70 rat liver mitochondrial proteins using a combination of affinity fractionation, 2-D gels, and mass spectrometry. Most recently, Scheffler et al. identified approximately 60 proteins in mitochondria isolated from cultured neuroblastoma cells using 2-D gels and mass spectrometry. The three studies cited above shared the limitation that the proteins that were resolved on broad-range pI 2-D gels were analyzed. This meant that in most cases, the protein pI was limited to a range of 3 to 10, and the molecular weight was limited to approximately a range of 200 to 10 kDa. This necessarily excludes various mitochondrial proteins including those that are more basic. Mitochondria also contain many low molecular weight proteins that are underrepresented in these studies. On the other hand, because it has been estimated that the number of different polypeptides found in mitochondria is only on the order of one thousand, there should theoretically be no problem resolving the majority of mitochondrial proteins on a single 2-D gel. This supports the general approach of combining 2-D gels with other techniques such as charge and affinity fractionation to enhance resolution and therefore allow the visualization of most of the mitochondrial proteome.

Using an alternative approach, Patterson et al. identified approximately 100 proteins from a combination of protein databases and expressed sequence tags. In this study, purified mouse liver mitochondria were treated with atractyloside to cause opening of the permeability transition pore complex; the mitochondria were subsequently digested with trypsin and the cysteinylation peptides were purified by affinity capture. The purified peptides were analyzed by data-dependent LC-MS/MS.
Figure 3. Enlarged areas from 2-D gel images of mouse heart mitochondrial proteins. KO indicates SOD2 knockout; WT, wild type. Mitochondria were isolated as described in Melov et al. Proteins were prepared, run on 2-D gels, and stained with silver stain as described in Pluskal et al. Acidic proteins are to the left, basic proteins to the right, higher molecular weight at the top, and lower molecular weight at the bottom. Each panel shows duplicate gels from each treatment. Each gel was the result of a single mitochondrial preparation isolated from a single mouse neonatal heart. Arrows indicate a polypeptide present in WT but absent in KO.

ever, a number of factors limit this study including the fact that all peptides do not ionize with identical efficiency and thus the study will be biased toward those that do. Also, the LC-MS/MS approach is not quantitative, so the relative abundances of the represented proteins cannot be determined.

Differential Display Analysis of Mitochondrial 2-D Gel Protein Arrays for Target Identification

A direct approach to drug and diagnostic marker detection is to compare the protein patterns in disease and control samples. To this end, several differential display studies on the proteomics of mitochondrial dysfunction have been recently published. Landin et al. investigated the perturbation of mitochondria after overdose with acetaminophen. By use of a combination of 2-D gels, mass spectrometry, and immunodetection, a mitochondrial acetaminophen-binding protein was identified as aldehyde dehydrogenase. More recently, changes in mouse heart mitochondrial proteins from transgenic mice deficient in creatine kinase were reported. The authors reported several clear changes in the protein patterns from transgenic and wild-type mice including a decrease in levels of the mitochondrial proteins ScMit-CK and aconitase. This study demonstrates the advantage of using a proteomic approach for the identification of protein changes associated with transgenic mouse models because the use of genomic methods for this purpose remains time-consuming and indirect.

Mitochondrial Dysfunction, Diseases, and Opportunities for Proteomic Analysis

Mitochondrial mutations and dysfunction have been implicated in numerous diseases, pathologies, aging, and tumorigenicity. Mitochondrial DNA deletions or point mutations that cause a reduced capacity for oxidative phosphorylation result in a number of specific syndromes most often including neuromuscular dysfunction. Mitochondria play a critical role in the health and survival of cells by providing the energy that fuels the maintenance, repair, and turnover of cellular components. Deterioration of mitochondrial function is therefore increasingly thought to play a major role in aging and neurological diseases as a result of the buildup of damage caused by reactive oxygen species (ROS) produced by the mitochondrion itself during oxidative phosphorylation. ROS production is a function both of the inefficiency of transfer of electrons through the respiratory chain and the level of antioxidant defenses in the cell.

The availability of appropriate animal models has now set the stage and facilitated the in-depth study of the proteomics associated with these processes. Homologous recombination has been used to generate several different mouse models of mitochondrial disease. These models fall into three separate categories: ROS toxicity, ATP deficiency, and mtDNA depletion/transcription deficiency. We will now briefly discuss proteomic analysis of mitochondria isolated from a model of ROS toxicity.

Differential Protein Display of Mouse Heart Mitochondrial Proteins

Some preliminary comparisons of mouse heart mitochondrial proteins carried out by the authors illustrate the effectiveness of using the 2-D gel approach for protein differential display studies before identification of potential targets with mass spectrometry. Mitochondrial superoxide dismutase is a protein responsible for detoxifying superoxide produced within the mitochondria during normal respiration. Mice have been created that lack this protein (SOD2 nullizygous mice). The resulting phenotype includes a dilated cardiomyopathy and mural thrombi that develops within 3 days of birth. The cardiomyopathy can be rescued with antioxidant treatment, which also uncovers a severe spongiform encephalopathy, which can also be rescued on appropriate antioxidant treatment in a dose-dependent fashion. Figure 2 shows images of Coomassie Blue–stained 2-D gels of mitochondrial proteins isolated from wild-type (WT) and SOD2 nullizygous or knockout (KO) mice. The arrows within the boxed areas indicate a number of protein differences, both qualitative and quantitative. Each of these polypeptides is present in sufficient quantity for excision and processing by mass spectrometry and identification is currently underway. Figure 3 demonstrates the reproducibility of the 2-D technique when applied to isolated mitochondria from separate individuals. Each of the silver-stained gel images is the result of a single mitochondrial preparation from an individual mouse neonatal heart. The arrows in the four duplicate images of
each type clearly indicate a polypeptide absent in KO that is present in WT.

Future Directions

It is clear that the science of mitochondrial proteomics is currently a nascent but rapidly growing field. The opportunities for identification of proteins directly involved in diseases caused by mitochondrial dysfunction are clear and compelling. A future objective is, without a doubt, to link the copious amount of genomic array information that is being generated daily to actual protein levels in mitochondria.\textsuperscript{127,128} The synergies between these types of data are a clear result of the complementarities. Transcriptional profiles can identify groups of genes that are induced or repressed with respect to a treatment or disease, but only direct identification of the protein isoforms in a given physiological condition can decipher the potential roles played by posttranslational modifications.

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References


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