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Proteomic Approaches to Identify and Characterize Alterations to the Mitochondrial Proteome in Alcoholic Liver Disease

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Abstract

Mitochondrial dysfunction is recognized as a contributing factor to a number of diseases including chronic alcohol induced hepatotoxicity. While there is a detailed understanding of the metabolic pathways and proteins of the liver mitochondrion, little is known of how changes in the mitochondrial proteome contribute to the development of hepatic pathologies. In this short overview the insights gained from study of changes in the mitochondrial proteome in alcoholic liver disease will be described. Profiling the liver mitochondrial proteome has the potential to shed light on the alcohol-mediated molecular defects responsible for mitochondrial and cellular dysfunction. The methods presented herein demonstrate the power of using complementary proteomics approaches, i.e. 2-D IEF/SDS-PAGE and BN-PAGE, to identify changes in the abundance of mitochondrial proteins following chronic alcohol consumption. This proteomic data can then be integrated into a logical and mechanistic framework to further our understanding of the role of mitochondrial dysfunction in the pathogenesis of alcohol induced liver disease.

Keywords

Alcohol; Liver; Mitochondria; Proteomics; Isoelectric-Focusing; Blue Native Gel Electrophoresis (BN-PAGE)

1. Introduction

It is widely recognized that a key component in the development of alcohol induced liver disease is the diminished capacity of liver to generate and maintain sufficient levels of ATP. This resulting decrease in bioenergetic capacity is thought to contribute to decreased hepatocyte viability and ultimately the pathological changes that occur in alcohol-dependent hepatotoxicity. The importance of mitochondrial dysfunction in this disease process has long been appreciated due to chronic alcohol mediated defects on the oxidative phosphorylation system, which leads to decreased ATP synthesis (1,2). Moreover, evidence indicates that these chronic alcohol induced alterations in mitochondria structure and function also contribute to increased production of reactive oxygen and nitrogen species in mitochondria (3). This increase in mitochondrial oxidant production could result in post-translational oxidative modifications

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and inactivation of mitochondrial macromolecules, particularly proteins, thereby further compromising mitochondria function in the chronic alcohol consumer.

While mechanisms responsible for alcohol dependent mitochondrial dysfunction have been studied, the impact of chronic alcohol consumption on the overall content of mitochondrial proteins, i.e. the global mitochondrial proteome, has only recently been investigated (4,5). Early studies by Cunningham and colleagues demonstrated that chronic alcohol consumption decreases the synthesis of the 13 mitochondrial encoded proteins that are components of complexes I, III, IV, and V (6,7) due to defects in mtDNA (4,8,9) and a decrease in functional mitochondrial ribosomes (10,11). It is important to note however that there are well over 600 proteins that comprise the mitochondrial proteome (12,13), and that close to 100 of these are components of the oxidative phosphorylation system, most of which are encoded by the nuclear genome. As very little information is available on the effects of chronic alcohol consumption on the total mitochondrial proteome, especially effects on nuclear encoded proteins, we have used two complementary proteomics approaches, 2-D isoelectric focusing (IEF)/SDS-PAGE and blue native-PAGE (BN-PAGE) to improve our understanding of the impact these changes have on mitochondrial functionality and their contribution to the development of alcohol induced liver pathology.

2. Materials

2.1. Isoelectric focusing (IEF)

1. Buffer for rehydration of IEF gel strips: 7 M Urea, 2 M Thiourea, 2% CHAPS, 0.5% N-dodecyl- β -D Maltoside (i.e. lauryl maltoside), and 0.002% Bromophenol Blue. Store at -20°C in 1.0 mL aliquots.
2. Equilibration buffer for IEF gel strips: 6 M Urea, 2.0% SDS, 0.375 M TrizmaBase, 20% Glycerol, and 0.002% Bromophenol Blue, pH 8.8 (with HCl). Store at -20°C in 2.0 mL aliquots. Before using, warm to 37°C to re-dissolve urea into solution.
3. DTT stock solution: 1 M DTT in water. Store at -20°C in 50 μL aliquots. Do not re-freeze and re-use DTT aliquots for future experiments.
4. Ultrapure, low melting temperature agarose (AquaP ϕ rTM LM, National Diagnostics, catalog # EC-204) for sealing IEF gel strips onto SDS-PAGE gels: 1.0% agarose (w/v) in 1 X SDS-PAGE running buffer. Make fresh on day of experiment.
5. The Invitrogen ZOOM IPG runner (catalog # ZM0001) is used to perform IEF in combination with Invitrogen ZOOM strips (catalog # ZM0011, pH 3–10), ZOOM IPG Runner Cassettes (catalog # ZM0003), and the ZOOM Dual Power Supply (catalog # ZP10002). Please refer to manufacturer's manual for additional details on set-up and use.
6. The ampholyte carriers used for IEF are Ampholines Electrophoresis Reagent (Sigma, catalog # A5174, pH 3–10). Other carrier ampholines or ampholytes can be substituted.

2.2. Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE)

2.2.1. 1-D BN-PAGE

1. Cathode Buffers: a) High-Blue Cathode Buffer; 50 mM Tricine, 15 mM BisTris, and 0.02% Coomassie Brilliant Blue G-250, pH 7.0. b) Low-Blue Cathode Buffer; 50 mM Tricine, 15 mM BisTris, and 0.002% Coomassie Brilliant Blue G-250, pH 7.0. Store at 4°C (*see* Note 1).
2. Anode Buffer: 50 mM BisTris, pH 7.0. Store at 4°C (*see* Note 1).

3X Gel Buffer: 1.5 M Aminocaproic acid, 150 mM BisTris, pH 7.0. Store at room temperature (*see* Note 2).

3. Extraction Buffer: 0.75 M Aminocaproic acid, 50 mM BisTris, pH 7.0. Store at 4°C (*see* Note 1).
4. Coomassie Brilliant Blue G-250 suspension: 0.5 M Aminocaproic acid and 5% Coomassie Brilliant Blue G-250, pH 7.0. Store at 4°C (*see* Note 1).
5. Lauryl Maltoside solution: 10% (w/v) N-dodecyl- β -D-maltoside in water. Store at -20°C.
6. Molecular weight standards for 1-D BN-PAGE: High-molecular weight native marker kit (Amersham Biosciences, catalog # 17-0445-01). Dissolve contents of 1 vial into 200 μ L of extraction buffer (#4), 25 μ L Lauryl Maltoside (#6), and 12 μ L Coomassie Brilliant Blue (#5). Store at -20°C.

2.2.2. 2-D BN-PAGE

1. Cathode Buffer: 100 mM Tris, 100 mM Tricine, and 0.1% SDS, pH 8.25. Store at room temperature.
2. Anode Buffer: 200 mM Tris, pH 8.9 with HCl. Store at room temperature.
3. 2-D BN-PAGE Gel Buffer: 3 M Tris and 0.3% SDS, pH 8.45 with HCl. Store at room temperature.
4. SDS/ β -Mercaptoethanol solution: 20 μ L β -mercaptoethanol, 200 μ L 10% SDS into 1780 μ L ultrapure H₂O. Make fresh on day of experiment.
5. Agarose solution for sealing 1-D BN-PAGE gel strips onto SDS-PAGE gels: 100 mg low melting temperature agarose (AquaP σ r™ LM, National Diagnostics, catalog # EC-204), 1 mL 10% SDS, 100 μ L β -mercaptoethanol into 9.0 mL ultrapure H₂O. Make fresh on day of experiment.

2.3. Total Protein Staining for Gels

1. Coomassie Blue stain: 0.3 g Coomassie Blue R-250, 100 mL Glacial Acetic Acid, and 250 mL Isopropanol, and 650 mL ultrapure H₂O. Use 100 mL per one mini-gel. Stain gel overnight and do not re-use stain. Gels are destained the following day using 10% glacial acetic acid solution. A small piece of a paper towel is added to the gel container to absorb the Coomassie Blue as it “leaches” from gel. Change blue-stained paper towels every 2–4 hr until clear background on gels is achieved.
2. SYPRO® Ruby Protein Gel Stain (Invitrogen, catalog # S-12000): After electrophoresis, fix gel in 100 mL of a 40% methanol and 10% glacial acetic acid solution for 1 hr. After fixation step, discard fix solution and incubate gel with 60 mL SYPRO® Ruby solution overnight. After staining gel, discard SYPRO® Ruby solution and de-stain gel in a 10% methanol/7% glacial acetic acid solution for 1–2 hr before visualizing gels. It is recommended that gels are de-stained for at least 16–24 hr using multiple changes in de-stain solution to minimize background (i.e. decrease speckling) of gels. A number of imaging platforms have been validated for visualizing SYPRO® Ruby stained gels. Please refer to the SYPRO® Ruby product

¹Adjust the pH of these solutions at 4°C because gels are run in the cold room (4°C).

²The 3X gel buffer can be stored at room temperature. However, the pH must be adjusted at 4°C because gels are run in the cold room (4°C).

information sheet provided by Invitrogen to obtain the excitation sources and emission filters for optimal visualization of protein in gels.

3. Methods

It is hypothesized that chronic alcohol-induced alterations to the mitochondrial proteome negatively affects mitochondrial bioenergetics and signaling, which contribute, in part, to the development of liver pathology. To address this question our laboratories have begun to profile the mitochondrial proteome using both conventional 2-D IEF/SDS-PAGE and BN-PAGE. Recent advancements in the field have now made it possible to identify post-translational modifications to mitochondrial proteins by ROS/RNS, identify defects in the assembly of multi-protein complexes in mitochondria, and separate the more hydrophobic respiratory complex proteins of the inner membrane using newly refined gel electrophoresis methods (14).

A scheme depicting our strategy for detecting alterations to the mitochondrial proteome in models of chronic alcohol-induced liver injury is shown in Figure 1. Briefly, mitochondria isolated from the liver of control and alcohol-fed animals can be subjected to conventional 2-D IEF/SDS-PAGE and BN-PAGE. The high-resolution protein “maps” generated via these electrophoresis methods can then be analyzed for changes in protein abundance or a variety of well-defined ROS/RNS-mediated post-translational modifications using immunoblotting techniques. Finally, proteins of interest can then be identified using several mass spectrometry techniques. Proteomic studies from our laboratories are considered significant in that novel changes in several key energy metabolism pathways including fatty acid oxidation, TCA cycle, and oxidative phosphorylation system were found to be altered in mitochondria isolated from animals exposed to ethanol chronically (4,5,15). These alterations were in response to alcoholic mediated metabolic stress and correlated to those pathways with a direct impact on the etiology of disease. A detailed discussion of these techniques is provided in the sections below.

3.1. Preparation of Mitochondrial Samples

1. Mitochondria are prepared from fresh liver tissue by standard differential centrifugation techniques (16) and should exhibit tightly coupled respiration with respiratory control ratios in the range of 4–8. Moreover, mitochondrial protein yield should average 25–30 mg mitochondrial protein/g wet weight liver and cytochrome *c* oxidase and citrate synthase activities should be determined as additional markers of mitochondria purity and yield (4,15). It is imperative that mitochondria used for proteomics studies are isolated by methodologies that result in functional and pure mitochondrial preparations. After isolation and determination of the mitochondrial protein concentration, the appropriate volume to achieve 1.0 mg protein is pipetted into a 1.5 mL microcentrifuge tube and mitochondria are spun at 12,500 rpm for 10 min to form a 1.0 mg protein pellet. The supernatant is removed and pellets are stored, dry at –80°C before used in experiments. Mitochondria samples can then be re-suspended in the appropriate buffer for BN-PAGE proteomic experiments. For IEF focusing studies mitochondria are typically stored in suspension at a concentration of at least 10 mg protein/mL.

3.2. Running 1-D IEF Gels

1. Mitochondria samples are thawed and kept on ice immediately before use. The optimal protein concentration for a 2-D IEF/SDS-PAGE gel is 50–200 µg protein per gel to achieve maximal resolution of proteins with minimal streaking. Repeat protein determination using standard methods (i.e. Lowry or Bradford protein assay) as protein concentration can change over time. A protein concentration of no less than

5 mg/mL is preferred as this will allow minimal dilution of reagents in IEF rehydration buffer.

2. Remove rehydration buffer and DTT aliquots from freezer and thaw to room temperature. Do not keep these reagents on ice as urea and DTT will precipitate from solution.
3. Into 1.0 mL of rehydration buffer add 10 μ L of pH 3–10 ampholines, 10 μ L of 200 mM tributylphosphine (BioRad, catalog # 163-2101), and 40 μ L of 1 M DTT. Mix well and keep at room temperature.
4. IEF strips can be rehydrated overnight with a total volume of 150–160 μ L containing the mitochondrial sample. The following example conditions are given below for the rehydration of strips with buffer and a protein sample that has a concentration of 10 mg/mL. Samples are allowed to extract in rehydration buffer for at least 30 min at room temperature before loading samples onto IEF gel strips. Gently vortex samples every 5–10 min during extraction.

Protein Amount	Quantity of Sample	Quantity of Rehydration Buffer
50 μ g	5 μ L	155 μ L
100 μ g	10 μ L	150 μ L
200 μ g	20 μ L	140 μ L

5. Following the procedure described by the specific manufacturer for the instrumentation being used slowly load 160 μ L of sample onto the strip. Samples will load along the entire lane by capillary action. Pipette slowly to minimize the introduction of bubbles into the gel lane as this could result in uneven rehydration of the IEF strips and loss of sample. Load all samples into cassette lanes before removing IEF gel strips from freezer.
6. After loading samples, remove the IEF strip pack from the freezer and keep strips on ice to minimize condensation. For each sample, carefully remove an IEF strip from the plastic backing, holding it with clean forceps by the marked negative (–) end. Holding the IEF strip from the (–) end and with the gel side up, gently slide the IEF gel strip into the cassette well until the strip reaches the (+) end of the cassette.
7. After rehydration of the IEF gel strips is complete, assemble the electrophoresis apparatus according to the manufacturer's instructions.
8. Perform IEF gel electrophoresis using the following conditions: 175 V for 20 min, ramp to 2000 V for 45 min, 2000 V for 30 min, ramp down to 500 V for 30 min, and 500 V for 2 hr. As IEF proceeds, the bromophenol blue dye front will migrate from the top to the bottom of the IEF strips and a green/blue/yellow band will appear at the anode (+) after IEF.
9. After IEF, seal cassettes in plastic wrap and freeze at -80°C until ready to perform second dimension SDS-PAGE.

3.3. Running 2-D IEF/SDS-PAGE Gels

1. For second dimension SDS-PAGE gels a wide range of equipment is commercially available that is compatible with the first dimension IEF. Homogenous (10 or 12%) or gradient (8–15%) acrylamide gels can be used for 2-D SDS-PAGE. It is important

that spacer plates are selected that provide sufficient width for inserting the IEF gel strips.

2. Remove IEF gel strips from freezer and allow IEF gel strips to thaw for a few minutes before beginning the equilibration step. For equilibration, prepare 1.0 mL equilibration buffer per IEF gel strip. Remove equilibration buffer and DTT aliquots from freezer and thaw at room temperature. To 1.0 mL of equilibration buffer add 50 μ L of 1 M DTT.
3. To remove the IEF gel strips from cassette, remove plastic covering gently and using forceps place each strip into a 15 mL conical tube, gel side up, and cover with 1.0 mL of equilibration buffer. Gently rock strips with equilibration buffer for 15 min. During the equilibration step prepare the agarose solution and keep warm. The agarose solution is used to “seal” the IEF gel strips to the top of SDS-PAGE gel.
4. After equilibration, remove strips from conical tubes, cut small plastic end from the anode (–) side, rinse strips in 1 X SDS-PAGE running buffer, and slide the IEF gel strip between the plates of the SDS-PAGE gel. Gently pipette the warm agarose solution over strip to “seal” the IEF gel strip on top of the SDS-PAGE gel. Do not allow bubbles to form between the IEF gel strip and the top of the SDS-PAGE gel. Slide a “tooth” from a 1.5 mm gel comb between glass plates at the (–) end of the gel strip. This will serve as the “well” for molecular weight markers. Gels are placed upright for 15–30 min to allow the agarose to set before beginning electrophoresis.
5. Once the agarose has set, gently remove the gel comb “tooth” and load broad range molecular weight markers to the well. Assemble the gel apparatus per the manufacturer’s instructions, fill inner and outer chambers with standard 1 X SDS-PAGE gel electrophoresis running buffer, and run gels for 1–2 hr at 100 V or until the dye front reaches the bottom of gel.
6. After electrophoresis, gels can be stained for total protein using conditions described above (Coomassie Blue or SYPRO® Ruby) or subjected to western blotting. Typically we stain the 2-D IEF/SDS-PAGE gels with SYPRO® Ruby stain to increase sensitivity of low abundance proteins. An example of a 2-D IEF/SDS-PAGE gel stained with SYPRO® Ruby stain is presented in Figure 1.

3.4. Running 1-D BN-PAGE Gels

1. BN-PAGE is a specialized method of electrophoresis which facilitates the high-resolution analysis of membrane protein complexes in their native state from biological membranes. A detailed discussion of the development and theory of BN-PAGE for analysis of the oxidative phosphorylation system proteins is presented in the seminal papers of Schägger and von Jagow (17,18).
2. For BN-PAGE the procedure more recently described by Brookes et al. (19) is used with the 1-D BN-PAGE typically performed with 5–12% or 5–16.5% gradient gels with 1.5 mm spacer plates. Recipes for gels are as follows (*see Note 3*).

³These gel volumes can be proportionally increased to prepare a resolving gel that has an extended length. This is typically done if the 1-D BN-PAGE gels are to be run overnight to improve the resolution (i.e. separation) of the complexes. Similarly, when gels are run overnight the Cathode buffer can be switched to a “blue-free” buffer, i.e. 50 mM Tricine, 15 mM BisTris, pH 7.0, with no added Coomassie Blue G-250. This helps to minimize the high blue background when 1-D BN-PAGE gels are destined for imaging and densitometry purposes.

	Resolving gel solutions		Stacking gel solution	
	<i>Light-5%</i>	<i>Heavy-12%</i>	<i>or Heavy-16.5%</i>	<i>4%</i>
Protogel	0.66 μ L	1.60 μ L	1.91 μ L	0.60 μ L
Water	1.97 μ L	0.56 μ L	-	2.40 μ L
3X Gel Buffer	1.34 μ L	1.34 μ L	1.16 μ L	1.50 μ L
Glycerol	-	0.47 μ L	0.40 μ L	-
10% AMPS	26.0 μ L	26.0 μ L	23.0 μ L	70.0 μ L
TEMED	4.0 μ L	4.0 μ L	3.50 μ L	9.0 μ L

- On the day of the experiment, remove the 1.0 mg mitochondrial pellets from the -80° C freezer and keep on ice. Add 100 μ L of extraction buffer and 12.5 μ L 10% N-dodecyl- β -D-maltoside to each 1.0 mg mitochondrial pellet. Gently resuspend mitochondrial protein by pipetting to extract and dissociate the protein. Place samples on ice for 30–60 min with gentle vortexing every 5–10 min. After this extraction step, centrifuge samples at 12,500 rpm for 5 min at 4° C degrees to pellet any non-dissolved material, remove supernatant to a fresh microcentrifuge, and then determine the protein concentration of the extract. A typical protein concentration of sample prepared by the procedure described here is 5–8 mg/mL.
- After determining the protein concentration in each sample, add 6.3 μ L of the cold Coomassie Brilliant Blue G-250 suspension (Solution #5, section 2.2.1) to each tube of mitochondrial extract and gently vortex. Load samples immediately onto gel for 1-D BN-PAGE (*see* Note 4).
- After assembling the gel apparatus per manufacturer's directions, fill wells of the stacking gel with cold Hi-Blue cathode buffer (Solution #1a, section 2.2.1), and load 75–250 μ g of protein per lane. Fill the inner (i.e. upper) buffer chamber with cold Hi-Blue cathode buffer (Solution #1a, section 2.2.1) and the outer chamber with cold anode buffer (Solution #2, section 2.2.1). Electrophoresis is performed in the cold room at 40 V for 1 hr or until samples have migrated into the resolving gels. At this time the cathode buffer is changed to cold Low-Blue cathode buffer (Solution #1b, section 2.2.1) and electrophoresis is continued for an additional 3–4 hr or until dye front reaches the bottom of the gel.
- At this point, gels can be stained with Coomassie blue to visualize the content of the intact oxidative phosphorylation complexes in samples or gels can be processed for 2-D BN-PAGE by the procedures described below. A representative 1-D BN-PAGE gel is shown in the upper left panel of Figure 1.

3.5. Running 2-D BN-PAGE Gels

- To resolve the individual polypeptides that comprise each oxidative phosphorylation complex, the intact gel lane containing all five complexes is cut from the 1-D gel, rotated 90° , and laid on top of a Tris-Tricine/SDS-PAGE gel. The recipe for preparing

⁴For the separation of large membrane protein complexes it is recommended that the concentration of the Coomassie Blue G-250 in the sample be $\frac{1}{4}$ of the lauryl maltoside concentration for electrophoresis (17,18). Therefore, based on the given stock solutions and protocol for the extraction of inner membrane proteins this works out to be approximately 6.3 μ L of a 5% (w/v) Coomassie Blue G-250 solution. Addition of a molar excess of dye to protein also helps to remedy one problem of native electrophoresis, i.e. the tendency of membrane proteins to form aggregates in the presence of detergents. However, the problem of aggregation is minimized by the inclusion of the Coomassie Blue G-250. This dye binds to the hydrophobic regions on the proteins surface, which induces a negative surface charge thereby reducing protein aggregation. In addition, the anionic nature of Coomassie Blue G-250 induces the needed "charge shift" on proteins such that they will migrate to the anode at pH 7.5 (17,18).

one, 1.5 mm thick 2-D BN-PAGE gel is as follows leaving a 1.0 cm gap at the top of the gel.

2-D BN-PAGE Gel Buffer	2.98 mL
Protogel	2.98 mL
H ₂ O	2.31 mL
Glycerol	0.72 mL
10% AMPS	60.0 µL
TEMED	6.00 µL

2. To apply the 1-D BN-PAGE gel strip on to the top of the SDS-PAGE resolving gel, raise-up the gel plates in the clamps and place the top, i.e. 1–2 cm gap on an angled hot plate or heat block. Pour approximately 4–5 mL of hot agarose (Solution #5, section 2.2.2) into the gap at the top of the gel sandwich, and using the back plate as a “staging area” and the hot agarose as a “lubricant”, gently slide the gel lane down between the plates until the strip lays on top of the SDS-PAGE resolving gel. Once the gel is in place, insert a single “tooth” of a gel comb on the end to serve as a well for molecular weight markers.
3. Remove the gel plate assembly from the hot plate and allow the agarose to set for 20–30 min. Excess agarose is then removed from the top of the gel with a scalpel blade and then the gel is overlaid with a thin layer of the SDS/β-mercaptoethanol solution every 5–10 min for 30–45 min to denature proteins.
4. Fill the inner chamber with Cathode buffer (Solution #1, section 2.2.2) and the outer chamber with the anode buffer (Solution #2, section 2.2.2), load molecular weight markers into well, and run gels at 30 V for 45 min followed by 110 V for 1 ½ to 2 hr.
5. After electrophoresis, gels can be stained for total protein using the conditions described above (Coomassie Blue or SYPRO® Ruby) or subjected to western blotting.

3.6. Image Analysis of Gels

1. Instructions for image analysis of gels are detailed in the following publications from our laboratories (4,5,15). In brief, scanned TIFF images for 1-D and 2-D BN-PAGE gels can be analyzed using either Scion Image Beta 4.02 (Scion Corp) or Quantity One (BioRad Laboratories) using the directions provided for each software program. For 2-D IEF/SDS-PAGE image analysis, gels are scanned, saved as TIFF files, and analyzed for differences in protein abundance between control and ethanol samples using PDQuest Image Analysis software (BioRad Laboratories).

3.7. Mass Spectrometry for Identification of Proteins

1. Proteins found by image analysis to change in relative abundance in response to a treatment are cut from gels and subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry by standard methodologies as described in (4,19) to identify proteins. The peptide masses identified using MALDI-TOF mass spectrometry as entered into the MASCOT search engine (see www.matrixscience.com) and the NCBI data base is searched to match the tryptic peptide fingerprint with a parent protein.

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Figure 1. Analysis of the mitochondrial proteome using 2-D IEF/SDS-PAGE and BN-PAGE proteomics

2-D IEF/SDS-PAGE is used to generate high resolution maps of the soluble matrix proteins (right panel), whereas 1-D and 2-D BN-PAGE is used to define changes to respiratory chain proteins (left panels). The right side panel illustrates a 2-D IEF/SDS-PAGE separation of proteins from whole liver mitochondria using the Invitrogen IPGRunner® System in combination with pH 3–10 IEF gel strips, whereas the left side panels demonstrate the BN-PAGE separation of inner membrane proteins. Roman numerals are used to identify the 5 oxidative phosphorylation complexes in the BN-PAGE gels. During non-denaturing 1-D BN-PAGE the 5 complexes remain intact (first step) whereas under the denaturing conditions used for 2-D BN-PAGE the individual proteins of each complex are resolved by size (second step). Thus, those proteins that comprise each complex are aligned vertically within the gel. Note that both gel platforms can be subjected to image analysis to look for changes in protein abundances or subjected to western blotting (WB) to reveal post-translational modification (PTMs) by reactive oxygen and nitrogen species, as well as electrophilic lipids, as a number of antibodies have been developed against these modifications [P-SX, protein thiol modifications; P-4HNE, 4-hydroxynonenal protein adduct; P-CHO, protein carbonyls; P-NO₂, 3-nitrotyrosine protein modification].