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High throughput two-dimensional blue-native electrophoresis: A tool for functional proteomics of mitochondria and signaling complexes

The recent upsurge in proteomics research has been facilitated largely by streamlining of two-dimensional (2-D) gel technology and the parallel development of facile mass spectrometry for analysis of peptides and proteins. However, application of these technologies to the mitochondrial proteome has been limited due to the considerable complement of hydrophobic membrane proteins in mitochondria, which precipitate during first dimension isoelectric focusing of standard 2-D gels. In addition, functional information regarding protein:protein interactions is lost during 2-D gel separation due to denaturing conditions in both gel dimensions. To resolve these issues, 2-D blue-native gel electrophoresis was applied to the mitochondrial proteome. In this technique, membrane protein complexes such as those of the respiratory chain are solubilized and resolved in native form in the first dimension. A second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel then denatures the complexes and resolves them into their component subunits. Refinements to this technique have yielded the levels of throughput and reproducibility required for proteomics. By coupling to tryptic peptide fingerprinting using matrix-assisted laser desorption/ionization-time of flight mass spectrometry, a partial mitochondrial proteome map has been assembled. Applications of this functional mitochondrial proteomics method are discussed.

Keywords: Blue-native electrophoresis / Mitochondria / Protein complexes / Signaling
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1 Introduction

Contemporary advances in mitochondrial biology have revealed that, far from being merely a supplier of ATP for the cell, mitochondria play significant roles in redox cell signaling [1–3], apoptotic cell signaling [4], and cellular homeostasis [3]. The emerging paradigm is one of a complex cross-talk between mitochondrial and cellular functions, with a key role for reactive oxygen and nitrogen species as signaling molecules [1, 3]. In addition, mitochondrial dysfunction is reported as a primary mechanism underlying several pathological conditions [5], and mitochondria appear to be viable pharmacological targets for the treatment of such conditions [6, 7]. Clearly the organelle is a prime candidate for proteomic analysis, and this is now possible due to the timely development of accessible proteomic technology. This includes the ready availability of gene and protein sequence databases, the development of inexpensive, user-friendly MALDI-TOF instrumentation, and the streamlining of two-dimensional gel electrophoresis 2-DE to maximize throughput and reproducibility.

Currently, 2-DE is the method of choice for protein separation in proteomics, yet it remains perhaps the most limiting step in a proteomic analysis [8, 9]. Typically in the first dimension, proteins are separated based on their p*I* using IEF on an immobilized pH gradient in the presence of strong denaturants. In the second dimension, proteins in the IEF strip are then denatured and resolved on the basis of their M_r by SDS-PAGE [10].

From a mitochondrial perspective, two important characteristics of 2-DE have limited proteomic analysis of the organelle. First, many important mitochondrial proteins such as the complexes of the respiratory chain are hydrophobic membrane proteins, and will precipitate at the basic pole during IEF [11–13]. Second, all functional information regarding protein:protein interactions is lost due to the high stringency, denaturing conditions of both gel dimensions. Indeed, in a recent analysis of the mitochondrial proteome, Lopez *et al.* [11] report that only 7 of 92 proteins identified are constituents of the mitochondrial respiratory chain. This is despite the fact that the respiratory chain comprises at least 83 separate proteins which represent $>40\%$ of the total protein content of the mitochondrial inner membrane [14].

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Abbreviation: BN-PAGE, Blue-native polyacrylamide gel electrophoresis

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Clearly, novel protein separation techniques are required in order to examine the mitochondrial proteome. In this respect, Hanson *et al.* [13] recently proposed a 3-D method in which high-resolution sucrose density gradients are used to separate functional protein complexes prior to conventional IEF SDS-PAGE 2-D gels. However, the requirement for large amounts of mitochondrial protein (1–5 mg) and an overnight centrifugation step may limit the application of this technique for high throughput analysis of samples where material is limited, as frequently occurs with experiments using cell culture.

To address the issues of protein separation, we have adapted the method of 2-D blue-native (BN) electrophoresis [15] for analysis of the mitochondrial proteome. In this technique, membrane and other functional protein complexes are separated as intact enzymatically active complexes in a first dimension gel. The protein complexes are then transferred to a second dimension denaturing SDS-PAGE gel, to separate the complexes into their component subunits [15, 16]. Refinements to the original 2-D blue-native methodology have resulted in a high-throughput technique readily applicable to functional proteomic analysis of mitochondria, and to other functional protein complexes such as those involved in cell signaling.

2 Materials and methods

2.1 Chemicals and animals

Unless otherwise indicated, all biochemicals were from Sigma (St. Louis, MO, USA) or Bio-Rad (Hercules, CA, USA). Male Sprague-Dawley rats (~250 g body mass) were from Harlan (Indianapolis, IN, USA), and maintained in temperature-controlled environment (70–72°F) on a 12 h light/dark cycle, with food and water available *ad libitum.*

2.2 Isolation of mitochondria

Liver mitochondria were isolated according to standard differential centrifugation procedures [17] in buffer comprising sucrose (250 mM), Tris (10 mM) and EGTA (1 mM), pH 7.4 at 4°C. Heart mitochondria were isolated from left ventricular tissue as previously described [18], in isolation medium comprising sucrose (300 mM), Tris (20 mM) and EGTA (2 mM), pH 7.3 at 4°C. Protein concentration was determined using the Folin-phenol reagent against a standard curve constructed using BSA [19]. For proteomic analysis, aliquots of 1 mg mitochondrial protein were centrifuged at 14 000 × *g* for 10 min in 1.5 mL plastic (Eppendorf) tubes at 4°C, shortly after mitochondrial isolation. Supernatants were discarded, and the pellets were then frozen in liquid N_2 and stored at –80°C for up to 1 week.

2.3 First dimension blue-native electrophoresis

Blue-native electrophoresis was performed using a modification of the method described by Schagger and von Jagow [15]. All buffers and solutions were pH 7.0 at 4°C. Mitochondrial pellets (1 mg protein) were resuspended in 100 μL of extraction buffer comprising aminocaproic acid (0.75 M) and BisTris (50 mM). To the suspension was added 12.5 μL *n*-dodecyl-β-D-maltoside (lauryl-maltoside, 10% w/v). Following incubation on ice for 20 min with vortex mixing every 5 min, samples were centrifuged at 14 000 × *g* for 10 min. To 100 μL of supernatant was added 6.3 μL of a 5% w/v suspension of Coomassie brilliant blue G-250 in aminocaproic acid (0.5 M). Samples were then stored on ice for no more than 30 min prior to gel loading.

A 5–12% gradient gel with 4% stacker was poured in Bio-Rad "mini-gel" plates according to Table 1. The anode buffer comprised BisTris (50 mM). The cathode buffer comprised Tricine (50 mM), BisTris (15 mM), and Coomassie brilliant blue G-250 (0.02% w/v). The gel, buffers and electrophoretic apparatus were chilled to 4°C before samples (35 μL/well, ~300 μg protein) were loaded. Molecular weight standards (high molecular weight calibration kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA) were resuspended in extraction buffer (200 μL *per* 250 μg vial) plus 25 μL of 10% lauryl-maltoside and 12 μL of 5% Coomassie blue, as above. A 30 μL aliquot (~32 μg protein) was loaded alongside protein samples. Electrophoresis was begun at 40 V, 4°C. After 1 h the cathode buffer was exchanged for one containing one-tenth the amount of Coomassie blue (0.002%), and electrophoresis was resumed at 110 V, 4°C for ~2 h. Electrophoresis was stopped when the blue dye front had reached a distance

Table 1. Preparation of first dimension gradient gel

Component	Gradient		Stacker
	5%	12%	4%
30% acrylamide/ 0.8% bisacrylamide	0.6 mL	1.33 mL	0.6 mL
H_2O	1.71 mL	0.28 mL	2.4 mL
Gel buffer	1.16 mL	1.16 mL	1.5 mL
Glycerol	–	0.6 mL	–
APS	23 μL	23 μL	70 μL
TEMED	3.5 μL	3.5 μL	9 μL

Volumes given are for a single Bio-Rad minigel (80 mm × 70 mm × 1.5 mm). 30% acrylamide/0.8% bisacrylamide was a proprietary mix (Protogel) from National Diagnostics (Atlanta, GA, USA). Gel buffer comprised aminocaproic acid (1.5 M) and BisTris (150 mM), pH 7.0 at 4°C. APS, ammonium persulfate, 10% w/v stock solution, freshly made.

of ~38 mm from the stacker/gradient gel interface. The gel was then cut into lanes for the second dimension using a fresh razor blade, with care being taken to include the heavily stained blue smudge at the base of the lane in the excised portion.

2.4 Second dimension denaturing SDS-PAGE

The second dimension electrophoresis method was based on that of Schagger and von Jagow [15, 16], with significant refinements to the methodology. In particular, the transition from first to second dimension electrophoresis was modified to ensure more rapid, reproducible results.

During first dimension electrophoresis, a second dimension SDS-PAGE gel was prepoured in Bio-Rad minigel plates (1.5 mm spacers) according to Table 2 leaving a gap of 1 cm at the top of the gel. Into this gap was poured a heated (95°C) solution comprising agarose (0.7% w/v), SDS (0.5% w/v) and β-mercaptoethanol (15 mM). The glass plates were then raised slightly in their clamps, and the upper agarose-containing portion of the plates was positioned on a 30° angled metal heating block at 95°C, to maintain agarose fluidity. Lanes cut from the first dimension gel were then slid into place horizontally on top of the second dimension gel, using the hot agarose as a lubricant. The offset nature of the Bio-Rad plates provides a convenient staging area for the lanes to rest on before being slid down into the agarose. Two lanes (each ~38 mm long) were placed adjacently on a single second dimension gel, leaving sufficient room (~5 mm) for a M_r marker lane to be created using a single tooth from a gel comb. The assembly was then removed from the heating block and the agarose allowed to set. Following removal of excess agarose with a scalpel blade, a solution of SDS (1% w/v) and β-mercaptoethanol (15 mM) was added drop-wise to the top of the vertical gel, and the whole allowed to sit for 15 min to facilitate diffusion of the denaturants into the first dimension strips. Coordinated timing of the end of the first dimension electrophoresis with the preparations for second dimension gel assembly was essential, to avoid swelling or drying of the first dimension lanes prior to their insertion into the second dimension plates.

Table 2. Preparation of second dimension gel

Component	Lower (10%)	Upper (8%)
30% acrylamide/ 0.8% bisacrylamide	2.4 mL	0.93 mL
H_2O	2.0 mL	1.13 mL
Gel buffer	2.5 mL	1.16 mL
Glycerol	0.6 mL	0.28 mL
APS	25 μL	25 μL
TEMED	2.5 μL	2.5μL

Volumes given are for a single Bio-Rad minigel (70 mm × 90 mm × 1.5 mm). 30% acrylamide/0.8% bisacrylamide was a proprietary mix (Protogel) from National Diagnostics (Atlanta, GA, USA). Gel buffer comprised Tris (3 M), SDS (0.3% w/v), pH 8.45 at 25°C. APS, ammonium persulfate, 10% w/v stock solution, freshly made. Lower and Upper refer to portions of the gel, since both portions use the same buffer and therefore the upper gel is not a true stacker gel.

Gel plates were then placed in the electrophoretic tank and electrophoresis performed at 25°C, at 35 V for 1 h, and then 110 V for ~1.5 h. The anode buffer comprised Tris (200 mM), pH 8.9. The cathode buffer comprised Tris (100 mM), Tricine (100 mM), and SDS (0.1% w/v), pH 8.25 [16].

2.5 Gel staining and spot identification

Gels were stained using a mixture of Coomassie blue R-250 and G-250 (0.05% w/v each in 25% isopropanol, 10% CH_3COOH). Following destaining in 20% isopropanol + 5% CH_3COOH, protein spots were manually excised for identification by MALDI-TOF mass spectrometry [20]. Gel pieces were macerated and washed with 50% aqueous acetonitrile to remove SDS, salts and stain, dried to remove solvent and then rehydrated with trypsin solution (12 ng/μL; Roche, Basel, Switzerland) and incubated overnight at 37°C. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration (centricon device; Millipore, Bedford, MA, USA) the tryptic peptide mixture was desalted using a Zip-Tip (Millipore), and peptides eluted in 1–5 μL of acetonitrile. An aliquot (1 μL) of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Aldrich, St Louis, MO, USA) in 50% aqueous acetonitrile, and 1 μL of this mixture spotted onto a gold target plate for MALDI-TOF MS analysis using a PE-Biosystems Voyager Elite instrument (Framingham, MA, USA). Peptides were evaporated with a N_2 laser at 337 nm, and using a delayed extraction approach they were accelerated with a 20 kV injection pulse for time of flight analysis. Each spectrum was the cumulative average of 50–100 laser shots. MALDI-MS spectra were internally mass calibrated using the *m/z* 2163 trypsin autolysis product. Peptide peaks were de-isotoped and those exceeding 5% of full scale were submitted for analysis with publicly available "Mascot" software at http://www.matrixscience.com.

2.6 Western blotting

2-D gels were electroblotted to nitrocellulose membranes overnight at 4°C, 30 V constant voltage, according to standard procedures [21], with addition of SDS (0.03% w/v) to the transfer buffer. The gel was soaked in cold transfer buffer for 5 min prior to assembly of the transfer cassette, to wash out excess Coomassie blue. Membranes were blocked with nonfat dry milk (10% w/v) in TBS plus 0.05% v/v Tween-20 (TBST). Cytochrome *c* was probed using a mouse monoclonal antibody (BD Pharmingen, San Diego, CA, USA) at 1/1000 dilution in TBST with 5% milk. This was followed by detection with a horse radish peroxidase linked secondary antibody and ECL (Amersham Pharmacia Biotech).

2.7 Cell culture and chloramphenicol treatment

Bovine aortic endothelial cells (BAEC) harvested from descending thoracic aortas were maintained at 37°C, 5% CO_2 in endothelial growth medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 ng/mL). Cells were used between passages 5–11. Upon reaching confluence, cells (75 cm^2 flask, $\sim 10^7$ cells) were treated with 20 μg/mL chloramphenicol for 48 h to inhibit mitochondrial protein synthesis [22, 23]. To isolate a mitochondrial fraction, cells (1 flask) were washed and then homogenized with 30 strokes of the tight pestle in a glass:glass Dounce homogenizer, in mitochondrial isolation buffer (as for liver mitochondria, see Section 2.2). The homogenate was centrifuged for 10 min at $3000 \times g$ to obtain a postnuclear supernatant, which was then centrifuged for 10 min at $24\,000 \times g$ to obtain the crude mitochondrial fraction. Mitochondrial pellets were then assayed for protein content and processed for blue-native electrophoresis as described in Section 2.3. Assays of the mitochondrial matrix marker enzyme citrate synthase [24] indicated that mitochondrial number did not change with chloramphenicol treatment (result not shown).

3 Results

The technique of 2-D blue-native gel electrophoresis is especially suited for analysis of the mitochondrial proteome, since it separates many mitochondrial proteins that cannot be resolved using conventional IEF SDS-PAGE 2-D gels. Figures 1 and 2 show partial proteomic maps of rat heart and liver mitochondria, and exemplify several important characteristics of blue-native gels. Firstly, a large proportion of spots, namely those of the respiratory chain complexes, can readily be identified by their position on the gel alone and do not require identifi-

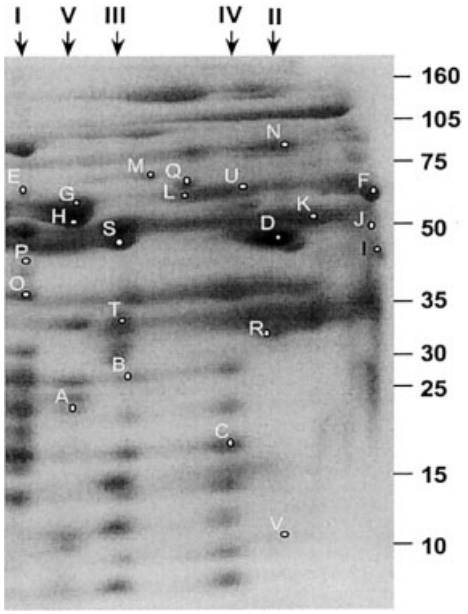

Figure 1. 2-D BN-PAGE functional proteomic map of rat heart mitochondria. Upper panel shows a typical 2-D BN-PAGE gel of rat heart mitochondria (~300 μg protein) prepared as detailed in Section 2.3. Roman numerals along top of gel indicate the position of the five respiratory complexes in the first dimension. Spots were cut where indicated and identified by MALDI-TOF as detailed in Section 2.5. Spot identification is shown in Table 3.

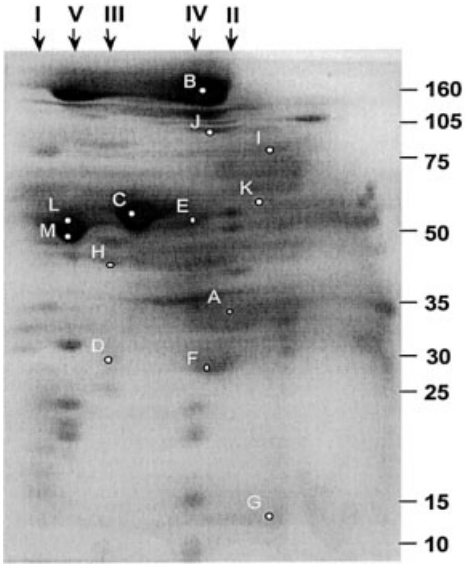

Figure 2. 2-D BN-PAGE functional proteomic map of rat liver mitochondria. Upper panel shows a typical 2-D BN-PAGE gel of rat liver mitochondria (350 μg protein) prepared as detailed in Section 2.3. Roman numerals along top of gel indicate the position of the five respiratory complexes in the first dimension. Spots were cut where indicated and identified by MALDI-TOF as detailed in Section 2.5. Spot identification is shown in Table 4.

Table 3. Identity, molecular mass, and criteria for identification of spots in Figure 1

Spot	Protein	Mass (kDa)	No. peptides matched/unmatched	Score
A	ATP Synthase subunit d	18.8	5/16	53 [53]
B	Chain s, Complex III	21.6	6/13	98 [70]
C	Subunit 4, Complex IV	19.5	7/15	112 [70]
D	Acyl-CoA Dehydrogenase	47.8	5/7	76 [71]
E	Propionyl-CoA Carboxylase, β-chain	58.4	7/11	86 [60]
F	ATPase β-chain	50.7	12/17	110 [60]
G	ATPase β-chain	55.3	11/30	115 [60]
H	ATPase β-chain	50.7	11/19	147 [71]
I	Isocitrate dehydrogenase	46.8	5/6	82 [71]
J	α-actin	41.8	4/7	170 [71]
K	Citrate Synthase	44.7	4/7	66 [60]
L	H5P60	57.9	8/12	114 [71]
M	Very long chain Acyl-CoA dehydrogenase	70.7	9/12	115 [60]
N	Flavoprotein subunit of Complex II	71.6	7/14	70 [60]
O	39 kDa subunit, Complex I	39.5	5/22	–[b]
P	Creatine Kinase	47.3	8/15	136 [71]
Q	Monoamine Oxidase A	58.8	6/17	58 [53]
R	Dodecanoyl CoA isomerase	32.3	–[a]	–[a]
S	Complex I I I core protein 2	48.3	–[a]	–[a]
T	Cytochrome C1 heme protein	27.3	–[a]	–[a]
U	Aldehyde dehydrogenase	54.3	15/24	197 [71]
V	Cytochrome *c*	12.4	–[c]	–[c]

The number of peptide masses from the spectra that were identified/unidentified is shown in the 4th column. The 5th column shows the MOWSE score from the MS-Fit software at www.matrixscience.com. The number in square brackets is the score at which statistical significance ($p < 0.05$) occurred for that particular search.

a) Identified by position on the gel, from the corresponding gel for liver mitochondria (Fig. 2)
b) Identified from an expressed sequence tag database search using software at prospector.ucsf.edu (no MOWSE score significance available)
c) Identified from Western blot (see Fig. 3)

Table 4. Identity, molecular mass, and criteria for identification of spots in Figure 2

Spot	Protein	Mass (kDa)	No. peptides matched/unmatched	Score
A	Enoyl-CoA Hydratase	28.3	6/11	94 [70]
B	Carbamoyl phosphate symthetase	164.5	36/47	300 [70]
C	Glutamate dehydrogenase	61.3	15/19	219 [71]
D	Cytochrome C1 heme protein	27.3	4/9	72 [71]
E	Aldehyde dehydrogenase	54.3	9/17	111 [71]
F	Dodecanoyl-CoA isomerase	32.3	7/18	76 [71]
G	Cytochrome *c* *	12.4	–[b]	–[b]
H	Complex III core protein 2	48.3	8/19	74 [71]
I	Glucose regulated protein	71.9	17/28	164 [71]
J	Dimethylglycine dehydrogenase	96.0	11/18	122 [71]
K	Sulfite oxidase	54.3	6/8	88 [71]
L	ATP synthase α-chain	55.3	–[a]	–[a]
M	ATP synthase β-chain	50.7	–[a]	–[a]

The number of peptide masses from the spectra that were identified/unidentified is shown in the 4th column. The 5th column shows the MOWSE score from the MS-Fit software at www.matrixscience.com. The number in square brackets is the score at which statistical significance ($p < 0.05$) occurred for that particular search.

a) Identified by position on the gel, from the corresponding gel for heart mitochondria (Fig. 2)
b) Identified from Western blot (see Fig. 3).

cation by sequencing or peptide fingerprinting. This is because the 1-D pattern of respiratory complexes is well established, and the vertical alignment of the spots in the second dimension yields familiar spot patterns that agree with published subunit compositions of purified respiratory complexes [15, 25).

A second observation is that many spots which are not part of the oxidative phosphorylation machinery also appear on the maps. Since the majority of publications on the technique of 2-D BN-PAGE have focused on the proteins of the respiratory chain [15, 26–29], it is likely that the utility of this technique in studying other mitochondrial proteins has been overlooked. This includes both membrane and nonmembrane proteins.

The differences between Figs. 1 and 2 highlight important differences between the mitochondria of heart and liver. Liver mitochondria appear to have a lower complement of respiratory complexes, and more of other proteins. For example carbamoyl phosphate synthetase, an important urea cycle enzyme, is more highly expressed in the liver, consistent with the major role for this organ in urea metabolism. Another important characteristic evident from these data is that novel functional interactions between mitochondrial proteins can be identified. For example, it is shown that creatine kinase (CK, ~50 kDa) appears to associate with complex I (~880 kDa) in the first dimension. While this may be due to a direct interaction between CK and complex I, it is also possible that either a multimeric form of CK migrates at a similar M_r to complex I in the first dimension, or that CK is bound to another, as yet, unidentified protein, causing it to migrate at the higher molecular weight.

The ability of 2-D BN-PAGE to characterize functional interactions is emphasized in Fig. 3, which shows a Western blot for cytochrome *c.* Consistent with its well established role in electron transport, cytochrome *c* appears to be distributed as three pools: (i) bound to complex III, (ii) bound to complex IV, and (iii) free. The complex IV bound pool appears by far the most abundant, and may represent the fact that mitochondria are prepared for 2-D BN-PAGE in the absence of respiratory substrate. Thus cytochrome *c* would be primarily in the oxidized form that binds readily to complex IV. The utility of this technique in examining cytochrome *c* location during apoptosis will be discussed later.

A further demonstration of functional associations revealed by 2-D BN-PAGE is shown in Fig. 4. In this experiment, cells were treated with the mitochondrial protein synthesis inhibitor chloramphenicol [22, 23]. The respiratory chain complexes are assembled from subunits encoded by both mitochondrial and nuclear DNA, raising the question of whether inhibiting mitochondrial protein synthesis alone would lead to partial assembly of the nuclear encoded subunits into "subcomplexes". Figure 4 suggests that this is indeed the case, since the α and β subunits of complex V (ATP synthase) which are nuclear encoded appear to assemble into an F_1 ATPase particle in the absence of the mtDNA encoded subunits 6 and 8, which are components of the membrane-bound F_0 domain [22].

4 Discussion

The most important finding of this study is that 2-D BN-PAGE is a powerful tool for analysis of the mitochondrial proteome that, based upon the pioneering work of Schagger and von Jagow [15], was previously not amenable to high throughput analysis. Furthermore, a mitochondrial proteome has not been mapped using this technique beyond the major components of the respiratory chain. Our main refinement to the original methodology is in the transition from the first to the second dimen-

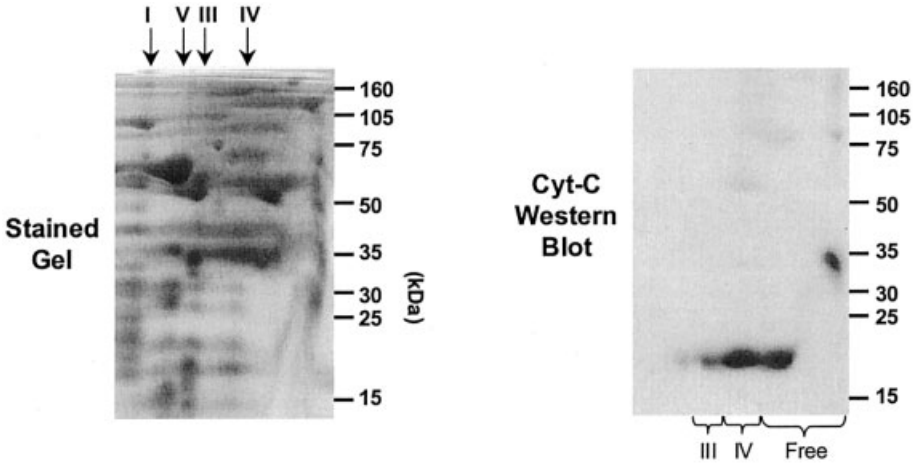

Figure 3. Anti cytochrome *c* Western blot from 2-D BN-PAGE gel of rat heart mitochondria. Upper panel shows stained gel of heart mitochondria, prepared as in Fig. 1. Lower panel shows cytochrome *c* Western blot of a duplicate gel, prepared as described in Section 2.6. Roman numerals above gel indicate position of respiratory complexes in first dimension. Annotations below the blot denote pools of cytochrome *c*: complex III-linked, complex IV-linked, and free.

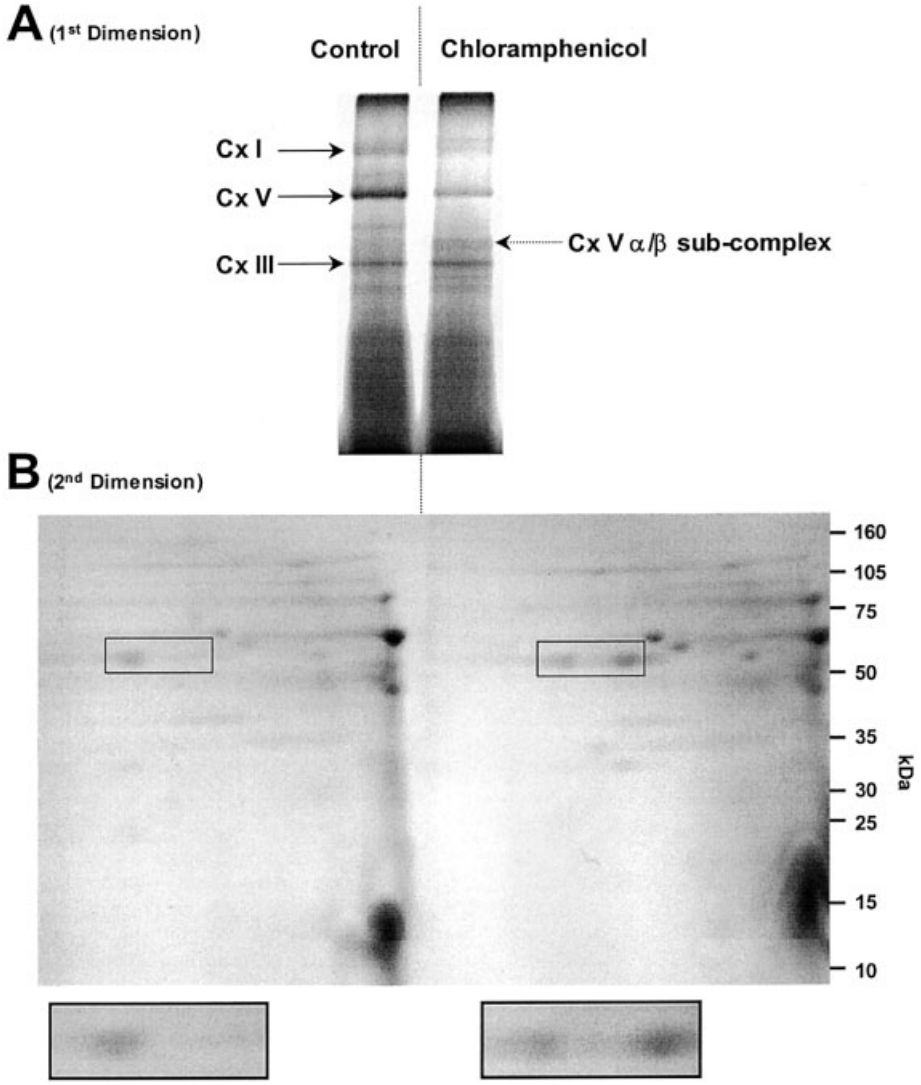

Figure 4. Functional proteomic analysis of mitochrondrial respiratory subcomplexes and the effects of chloramphenicol. BAEC were cultured, treated with chloramphenicol, and mitochondria isolated and run on 2-D BN-PAGE as detailed in Section 2.7. A, First dimension BN-PAGE gels. Intact respiratory complexes, and the complex V α/β subcomplex, are indicated by the arrows. B, Second dimension separation of the samples shown in panel A. First dimension strip were oriented with top to the left, bottom to the right. Two samples were run adjacent in the second dimension gel. Enlargements below main gel show areas outlined by boxes, and indicate assembly of α and β subunits of ATPase into a subcomplex. Identity of these proteins was confirmed by MALDI-TOF analysis of excised spots.

sion. This process originally took ~4 h, and comprised soaking the first dimension lane in SDS/β-mercaptoethanol for 2 h followed by pouring a three-layered gel underneath. This was logistically challenging since trapping of air bubbles beneath the frst dimension lane frequently occurred. Also β-mercaptoethanol is a strong inhibitor of gel polymerization, resulting in horizontal streaking of spots due to diffusion in an unpolymerized gel layer immediately beneath the first dimension strip. Also significant loss of sample occurs due to diffusion of proteins out of the gel strip and into the denaturing solution.

In the method outlined here, the entire operation is performed in minigel format, and the transition from the first to the second dimension takes ~30 min. Denaturing of proteins is achieved in three ways: (i) diffusion of denaturants from the agarose solution; (ii) heat; and (iii) a second addition of denaturants to the gel prior to running. The prepouring of the second dimension gel, and the lack of an unpolymerized layer beneath the first dimension lane, yields a more reproducible end result, in which horizontal diffusion of spots is minimized. Overall, these methodological advances make the technique more user-friendly and applicable to high throughput analysis of small biological samples. Coupled with the logistical issues described above, and since the primary application of 2-D BN-PAGE to date has been the proteins of the mitochondrial respiratory chain [15, 26, 28, 29], it is likely that the utility of this technique in studying other mitochondrial proteins has been overlooked. Figures 1 and 2 illustrate the wider scope of this method, with 2-D BN-PAGE being applicable to the mitochondrial proteome at large.

The data in Fig. 3, showing functional associations between cytochrome *c* and respiratory complexes III and IV, are particularly interesting in the context of the recent

discovery that mitochondrial cytochrome *c* release contributes to apoptotic signaling [4]. One possibility that has not been widely considered, is that cytochrome *c* release may be a two-step process involving release from binding sites on the respiratory complexes prior to translocation across the outer membrane. The use of 2-D BN-PAGE to study the distribution of cytochrome *c* in mitochondria of apoptotic cells may thus provide novel insights into its release mechanism, and determine which pools of cytochrome *c* (free *vs.* complex bound) are, in fact, released.

Furthermore, the application of this technique to examine functional associations between proteins is exemplified in Fig. 4. It has previously been shown that inhibition of mitochondrial protein synthesis does not influence synthesis and uptake of nuclear encoded subunits of mitochondrial complexes, and in fact increases the stability of nuclear encoded respiratory gene transcripts [22, 23, 30]. These observations, coupled with the data presented here, suggest that even in the absence of mtDNA encoded subunits, the respiratory complexes are capable of assembly into subcomplexes that may have limited functionality.

Clearly this has important implications for inherited mitochondrial DNA mutations, in which one or more mtDNA encoded subunits are absent. Any enzymatic activity of respiratory subcomplexes would partially explain the disparity between loss of gene and loss of function that is often seen in mitochondrial DNA diseases.

This method also lends itself readily to the study of post-translational modifications to mitochondrial respiratory complexes and other proteins. These may include phosphorylation, or more recently discovered oxidative and nitrosative post-translational modifications including reaction with lipid derived aldehydes or tyrosine nitration [31, 32]. The central role of the mitochondrion in both nitrosative and redox cell signaling [1, 33] highlights a possible role for these modifications in the cross-talk between signaling pathways.

The example applications of 2-D BN-PAGE presented here serve to demonstrate this technique as a true example of “functional proteomics”. That is to say, important information regarding protein:protein interactions is contained in the gels. This is immediately evident in the fact that almost all of the proteins identified migrate in the first dimension at a higher molecular weight than the second dimension, meaning they are functionally coupled to other proteins. Thus, as an alternative to commonly used immunoprecipitation techniques, 2-D BN-PAGE may prove an invaluable tool in the extrapolation of complex cell signaling pathways, which involve multiple protein: protein interactions and post-translational modifications. In the post-genomic era, and what is soon to be the post-proteomic era, this ability of 2-D BN-PAGE to elucidate protein:protein interactions represents the next logical step in the decoding of the functional proteome.

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