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Blue Native electrophoresis to study mitochondrial and other protein complexes

Leo G.J. Nijtmans,[a,*] Nadine S. Henderson,[b] and Ian J. Holt[c]

[a] *Nijmegen Centre for Mitochondrial Disorders, University Medical Centre Nijmegen, Geert Grooteplein 10, PO BOX 9101, 6500 HB Nijmegen, The Netherlands*

[b] *Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794-8691, USA*

[c] *Dunn Human Nutrition Unit, Wellcome Trust, Cambridge CB2 2XY, UK*

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Abstract

The biogenesis and maintenance of mitochondria relies on a sizable number of proteins. Many of these proteins are organized into complexes, which are located in the mitochondrial inner membrane. Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a method for the isolation of intact protein complexes. Although it was initially used to study mitochondrial respiratory chain enzymes, it can also be applied to other protein complexes. The use of BN-PAGE has increased exponentially over the past few years and new applications have been developed. Here we review how to set up the basic system and outline modifications that can be applied to address specific research questions. Increasing the upper mass limit of complexes that can be separated by BN-PAGE can be achieved by using agarose instead of acrylamide. BN-PAGE can also be used to study assembly of mitochondrial protein complexes. Other applications include in-gel measurements of enzyme activity by histochemical staining and preparative native electrophoresis to isolate a protein complex. Finally, new ways of identifying protein spots in Blue Native gels using mass spectrometry are briefly discussed.

Keywords: Blue Native gel electrophoresis; Blue Native agarose electrophoresis; Mitochondria; Respiratory chain; Oxidative phosphorylation; Enzyme complexes; Histochemical staining; Pulse–chase

1. Introduction

Recently we entered the postgenome era [1] and the challenge now is to assign a function to each protein and describe its relationship to other proteins in the cell. Intracellular location and potential partners are useful starting points in our search for the function of a protein. Many proteins are spatially organized in a cell and form complexes with other proteins. This enables different steps in a biological process to be combined. Often the product of one catalytic reaction is the substrate for a subsequent reaction. By holding the enzymes in a complex, a series of enzymatic reactions is more efficient, so-called substrate channeling. Also any toxic intermediates can be kept within the protein complex, limiting damage to other parts of the cell.

To investigate membrane-bound protein complexes, Schägger and von Jagow developed a technique called Blue Native electrophoresis [2]. This technique, designed for the mitochondrial OXPHOS system, separates complexes without dissociating them into their constituent polypeptides. This was a difficult task as the respiratory chain complexes are embedded in the mitochondrial inner membrane: too little detergent and the complexes would not be released from the membrane, too much and the complexes would dissociate. Electrophoresis was also problematical; the high salt concentrations used in standard purification protocols of respiratory chain complexes perturb electrophoresis, and proteins (or complexes) need a charge to have mobility in an electric field. The charge usually comes from a chemical additive, such as sodium dodecyl sulfate (SDS), that binds to a protein and confers a uniform

* Corresponding author. Fax: +31-24-3616428.
E-mail address: l.nijtmans@ckslkn.azn.nl (L.G.J. Nijtmans).

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charge and thus electrophoretic mobility. However, SDS is such a strong detergent that it causes not simply dissociation of protein complexes, but also complete denaturation. Schägger and von Jagow elegantly solved these problems by including Serva Blue G, also known as Coomassie Blue G250 (Serva Biochemicals, Heidelberg, Germany) in the extraction and electrophoresis buffers. Serva Blue G gives a charge to the protein complexes without dissociating them. The detergent dodecyl-β-D-maltoside, which does not dissociate protein complexes, was used to solubilize the mitochondrial membranes. Extraction of the protein complexes was aided by inclusion of the zwitterionic salt aminocaprionic acid, which has a net charge of zero at pH 7 and, therefore, does not affect electrophoresis. The technique was named Blue Native polyacrylamide gel electrophoresis (BN-PAGE) owing to the color of the crucial compound (Serva Blue G). In the years since its development BN-PAGE has become a very important tool in mitochondrial research.

Since its inception, Blue Native electrophoresis has been adapted to a large number of applications that build on the basic principle. Here more extensive practical advice, including potential pitfalls, of BN-PAGE is provided. This article explains the basic techniques of first-dimension Blue Native gels, second-dimension SDS–PAGE, sample preparation, and Western blotting. We describe an adaptation to extend the range of molecular weight separation from 1 MDa to at least 8 MDa, using agarose in place of polyacrylamide. Then we discuss specific analytical applications of the technique. First, since complexes separated by BN-PAGE are still biochemically active, a specific staining method was developed based on histochemical assays. Second, how complexes or individual subunits can be isolated by electroelution is discussed. Third, special attention is paid to explain how Blue Native PAGE of metabolically labeled human cells can be applied to investigate the dynamics of complex assembly. Fourth, we illustrate how BN-PAGE is used to assess the molecular weight status of a protein. Finally, the application of mass spectrometry to identify protein spots is discussed briefly.

2. Description of the method

Blue Native two-dimensional electrophoresis allows analysis of both the concentration and the composition of (mitochondrial) protein complexes. In the first dimension (Fig. 1), separation of the complexes under native conditions occurs according to their molecular mass, and in the second dimension, where electrophoresis is performed under denaturing conditions, the *individual subunits* of the complexes are resolved, again on the basis of their molecular mass.

2.1. Sample preparation

Sample preparation depends on the tissue or cell type. Schägger has provided an excellent protocol for preparation from several human tissues [3]. The tissue is homogenized using a tight-fitting glass–Teflon homogenizer in a MOPS sucrose buffer containing pro-

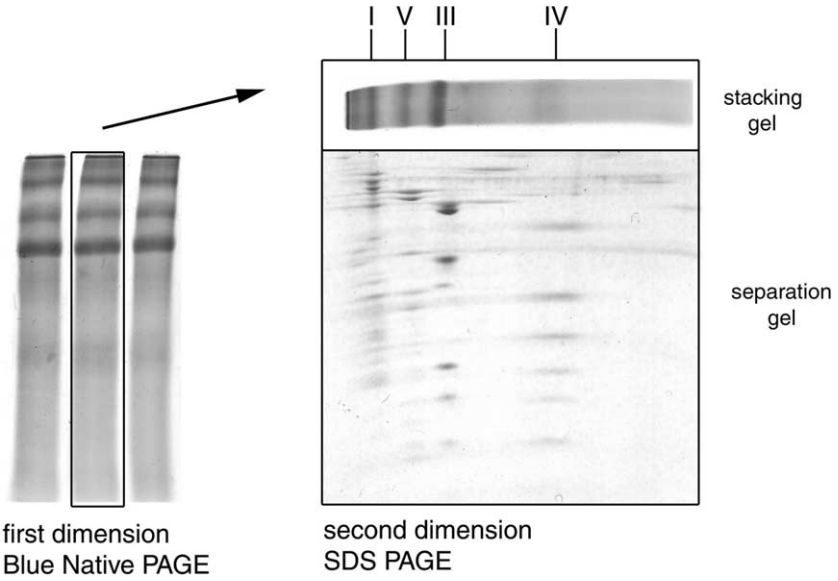

Fig. 1. Two-dimensional Blue Native/SDS–PAGE. Human heart mitochondrial particles were extracted and protein complexes separated on a 5–13% Blue Native polyacrylamide gel (left) (for, details see text). A lane was excised from the gel and rotated 90° counterclockwise. The gel strip was incubated with dissociation buffer and a 10% tricine SDS–gel was run in the second dimension. Complexes I, V, III, and IV of the respiratory chain are indicated by I, V, III, and IV, respectively (right).

tease inhibitors (440 mM sucrose, 20 mM Mops, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) and an enriched mitochondrial fraction is collected by centrifuging at 20,000*g* for 20 min. The standard way to dissolve mitochondrial pellets is by swirling them with a small spatula in a solution of 1 M aminocaproic acid, 50 mM Tris (pH 7.0), and 1.5–5% dodecylmaltoside, depending on the tissue (see Table 1). After centrifugation at 100,000*g* for 15 min the supernatant is collected and combined with Serva Blue G (5% in 1 M aminocaproic acid (see Table 1) prior to loading. Mitochondrial membranes can also be isolated from cultured cells [4]. Briefly, two 75-cm^2 flasks of fibroblasts are cultured until cells are approximately 90% confluent (2 million cells). The cells are harvested using trypsin, washed twice with cold phosphate-buffered saline (PBS), and resuspended to a concentration of 1×10^6 cells in 100 μl PBS in a 1.5-ml Eppendorf tube, and 100 μl of a cold digitonin solution (8 mg/ml in PBS) is added to the cell suspension. The cells and digitonin solution are kept on ice for 10 min to dissolve the membranes. One milliliter of cold PBS is added to the cells (to dilute the digitonin) which are spun for 5 min, at 10,000*g* (14,000 rpm in a microcentrifuge), at 4 °C. The supernatant is removed and the pellet washed once more in 1 ml cold PBS. At this point the pellets can be stored at −80 °C or immediately solubilized. To solubilize, the pellets are vigorously pipetted in 100 μl buffer containing 1.5 M aminocaproic acid, 50 mM Bis–Tris/HCl, pH 7.0. Next, 20 μl of the detergent dodecylmaltoside (10% w/v) is added and the cells are incubated on ice for 5 min. The cells are centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant is transferred to a new Eppendorf tube and 10 μl sample buffer (750 mM aminocaproic acid, 50 mM Bis–Tris/HCl, pH 7.0, 0.5 mM EDTA, and 5% Serva Blue G) is added. This sample is ready to load on a gel, but can alternatively be stored for at least a month at −80 °C. Typically 10–50 μl of this solution is loaded onto a gel. We usually use minigel systems (e.g., 8 × 10 cm) with a thickness of 1.5 mm in the first dimension [4].

The high salt concentration (1.5 M aminocaproic acid) or the type of detergent used to solubilize the mitochondrial pellet can affect the stability of a complex, e.g., pyruvate dehydrogenase complex (PDC) [5]. The problem of instability can be addressed by modifying the salt concentration or the type or amount of detergent employed; precise conditions depend on the particular complex of interest. It has also been shown that Triton X-100 and the mild detergent digitonin give different aggregation states of respiratory chain complexes [6].

Table 1
Buffers for sample preparation

Buffer	Heart (5 mg)	Muscle (20 mg)	Brain (10 mg)
MOPS sucrose buffer (μl)	250	250	250
Aminocaproic acid/ Bis–Tris (μl)	20	40	40
Dodecylmaltoside (μl)	3	15	20
Serva Blue G (μl)	1.5	7.5	10

2.2. First dimension

Ordinarily, the first dimension is a native polyacrylamide gel. To separate the respiratory chain complexes from cells or tissues a gradient gel of 5–13% acrylamide is used. This gradient gives good separation for proteins between 100 and 1000 kDa. Other gradient gels may be preferred to focus on a specific complex or subcomplex. For example, a 10–20% gradient gel was employed to resolve protein complexes between 20 and 200 kDa [7]. Agarose gels are more appropriate for the analysis of complexes larger than 1 MDa (see below). Whatever the gradient or gel matrix, we routinely use a minigel system (e.g., Bio-Rad, Hercules, CA, Mini-PROTEAN II) for PAGE and any horizontal gel rig (e.g., Flowgen) for agarose to shorten electrophoresis run times. Additional benefits are that a minigel system saves on chemicals and sample. The latter is particularly important in the case of precious human tissue from diseased individuals.

2.2.1. Buffers

Acrylamide solution: 48 g acrylamide and 1.5 g bisacrylamide in 100 ml Milli-Q water.

Gel buffer (3×): 150 mM Bis–Tris, 1.5 mM aminocaproic acid, adjusted to pH 7.0 with HCl at 4 °C.

Anode buffer: 50 mM Bis–Tris/HCl, pH 7.0, at 4 °C.

Cathode buffer A: 50 mM Tricine, 15 mM Bis–Tris/HCl, pH 7.0, at 4 °C.

Cathode buffer B: cathode buffer A + 0.02% Serva Blue G.

2.2.2. Casting of the gel

Gel dimensions are 8.0 × 10.0 cm. The spacers used in the first dimension are 1.5 mm. The gel solutions are prepared from stock solutions (see Table 2). Gel and gradient preparation is carried out at 4 °C to slow polymerization [2,3].

2.2.3. Electrophoresis

Samples are electrophoresed slowly at first until they have entered the stacking gel, typically 30 V for 30 min, after which the voltage is increased to 80 V. To remove excess Serva Blue G, cathode buffer B is replaced by cathode buffer A after the front migrates to the middle of the gel. Electrophoresis continues at 80 V until the blue dye front reaches the end of the gel [2,8].

Table 2
Casting of gradient Blue Native gel

Component[a]	Stacking gel (4%)	Gradient gel (5%)	Gradient gel (13%)
Acrylamide solution	0.4 ml	1.01 ml	2.60 ml
Gelbuffer (3×)	1.67 ml	3.33 ml	3.33 ml
Glycerol	—	—	2.0 ml
H_2O	2.87 ml	5.39 ml	1.98 ml
APS[a] (10%)	55 μl	60 μl	30 μl
TEMED	5.5 μl	6 μl	3 μl
Total volume	5 ml	10 ml	10 ml

[a] APS (10%), amonium persulfate solution; TEMED, tetramethylenediamine.

2.3. Second dimension

A lane is cut out of the first-dimension gel with a razor blade (Fig. 1). The strip is rotated through 90°, placed on a glass plate, and incubated with a dissociating solution (1% SDS and 1% 2-mercaptoethanol), for 1 h at room temperature. Excess dissociating solution is drained away using a filter paper and the glass plates are assembled according to the manufacturer's instructions. Careful removal of dissociating solution is essential as 2-mercaptoethanol inhibits gel polymerization. Often the first-dimension spacers are thicker (e.g., 1.5 mm) than those of the second-dimension gel (e.g., 0.75 mm). Thus, the first-dimension strip is compressed between the glass plates, making it relatively easy to cast the second-dimension separation gel without contact occurring between the two. After polymerization, the stacking gel is poured around the first-dimension strip, creating a bridge to the separation gel. One needs to be careful not to trap air bubbles beneath the first-dimension strip because this will distort the movement of proteins through the gel. The second dimension is a SDS–polyacrylamide gel. Depending on the molecular weight range of interest a glycine [9] or tricine SDS–polyacrylamide gel [10] can be used. A 10% tricine SDS–gel without a spacer gel is a good starting point as it can resolve polypeptides across the range 20–200 kDa. The addition of a spacer gel and an increase in acrylamide concentration extend the useful separation range to 1–100 kDa [10].

2.4. Western blotting

Western blotting is performed according to Towbin et al. [11] using a glycerin-based buffer. Immunodetection of protein complexes from a blot of one-dimensional BN-PAGE does not always give good results. One reason for this is that epitopes recognized by the antibody may be hidden in the complex. Another potential problem is nonspecific signal resulting from the blue dye, and the high local concentration of dye bound to the target protein may interfere with antibody binding. Incubating the gel with dissociating reagent before blotting can improve immunodetection, as epitopes are liberated that were previously hidden. Stripping the blot by incubating it 30 min at 50 °C in stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris–HCl, pH 6.7) to remove Serva Blue G decreases nonspecific binding of antibody. However, in our hands a blot of the second-dimension SDS–gel almost always enhances the signal obtained.

2.5. Blue Native agarose

Standard 5–13% BN-PAGE gives good resolution of complexes between 100 kDa and 1 MDa. However, complexes greater than 1 MDa cannot enter even a 4% acrylamide gel. Below 4% the gels become fluffy and difficult to handle. Pyruvate dehydrogenase complex is approximately 7 MDa; therefore it is not possible to resolve the complex by BN-PAGE. Therefore we substituted agarose for acrylamide to resolve PDC. The other crucial modification was a lowered salt concentration (0.05 M aminocaproic acid) to prevent dissociation of the complex. A high salt concentration aids extraction of membrane-embedded complexes but is unnecessary in the case of PDC, which is located in the mitochondrial matrix. The exact protocol of Blue Native agarose gel electrophoresis (BN-AGE) is described by Henderson and colleagues [5]. Briefly, a very thin (3–4 mm) gel of 2.5–4% high-gel-strength agarose (SeaKem Gold, Flowgen) was poured into a standard horizontal minigel apparatus (Flowgen). Running buffer was blue cathode buffer B. Electrophoresis was performed at relatively high current (5–10 mA) to minimize smearing of complexes. Second-dimension SDS–PAGE was performed as described above, except using thicker spacers (1.5 mm) due to the thickness of the agarose. Since the agarose gel must be thin, this does not allow for loading of a large volume of sample (typically 8–10 μl).

3. Applications

3.1. In-gel activity assays

In the original article describing BN-PAGE, Schägger and von Jagow demonstrated that isolated OXPHOS complexes retain enzyme activity [2]. The enzyme activity of complex III after extraction from a Blue Native gel was comparable to that of a chromatographic preparation after activation by phospholipids. Complex IV had cyanide-sensitive ferrocytochrome *c* activity, although the K_m shifted, probably due to binding of the negatively charged dye to cytochrome *c*. Likewise, ATP hydrolysis activity could be measured in ATP synthase extracts, but F_1–F_0 coupling was seemingly perturbed as only 20% inhibition was achieved with addition of oligomycin. Subsequently it was reported that F_1 can be detected as a

single entity [12] and that it again retains ATP hydrolysis activity [13]. To visualize OXPHOS complexes in a quantitative way Zerbetto and colleagues exploited the fact that protein complexes retain enzyme activity on BN-PAGE [14]. The authors treated gels with solutions for histochemical staining of specific complexes. The use of these histochemical staining methods made it possible to combine enzyme activity with the mobility of the complex in the gel. A major advantage of the method is that nonspecific activities do not interfere, because they are unlikely to have the same size and mobility as the complex of interest. Where a complex has an altered mobility the effect on catalytic activity can be readily ascertained (Fig. 2). For instance, when F_1 dissociates from the F_1, F_0-ATPase it maintains its dephosphorylating activity (Fig. 2).

In-gel activity measurements are, at best, imprecise; phospholipid content and binding of inhibitor proteins may adversely affect results. The threshold for detection is quite high and a range of protein concentrations should be tested. For example, if an assay of a patient-derived sample suggests a 50% decrease in activity then we would recommend that on a second gel double the protein is loaded to test whether this gives the same activity as the control samples. In general, samples from skeletal muscle or heart give the highest activity, whereas in-gel histochemical signals from cultured cell samples are much weaker. Therefore, it is only appropriate to compare like samples.

3.1.1. Procedure

Gels are incubated overnight at room temperature with the following solutions:

Complex I: 2 mM Tris–HCl, pH 7.4, 0.1 mg/ml NADH, and 2.5 mg/ml NTB (nitrotetrazolium blue).

Complex II: 4.5 mM EDTA, 10 mM KCN, 0.2 mM phenazine methasulfate, 84 mM succinic acid and 50 mM NTB.

Complex IV: 5 mg 3.3′-Diamidobenzidine tetrahydrochloride (DAB) dissolved in 9 ml phosphate buffer (0.05 M, pH 7.4), 1 nM catalase (20 μg/ml), 10 mg cytochrome *c*, and 750 mg sucrose.

Complex V: 35 mM Tris, 270 mM glycine, 14 mM $MgSO_4$, 0.2% $Pb(NO_3)_2$, and 8 mM ATP, pH 7.8.

Gels are washed in distilled water and scanned or photographed immediately. Fixing the gels should be avoided as this causes the disappearance of complex V bands.

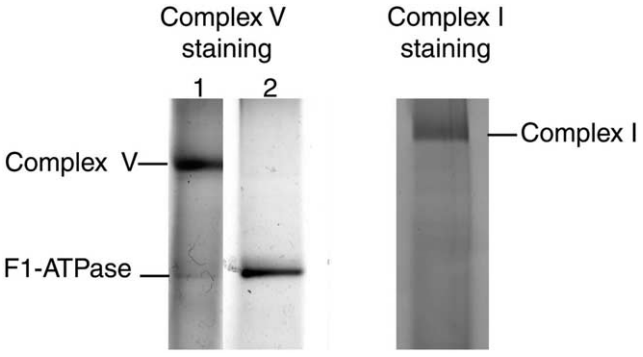

Fig. 2. In-gel activity assay of F_1-ATPase and complex I. Blue Native gels were histochemically stained for ATP hydrolysis activity (left) or complex I activity (right) (for details see text). Crude mitochondrial fractions of A549 lung carcinoma cells (lane 1) and of B2.ρ^0 cells, which are devoid of mitochondrial DNA, were analyzed (lane 2). Because of the lack of mitochondrial gene products in B2.ρ^0 cells the F_1-ATPase can form but not the F_0, F_1-ATPase, whereas in the wild type F_0, F_1-ATPase (holoenzyme) is assembled.

3.2. Preparative native electrophoresis

To demonstrate the activity of native protein complexes Schägger and von Jagow electroeluted protein from gels [2]. As the complexes retained enzyme activity after electroelution it was inferred that they remained intact. This was confirmed by running electroeluted samples on a second native gel, where the majority of the complexes were in the form of holoenzyme. An electrophoresis-based purification protocol was devised subsequently [8]. The procedure yields highly pure protein complex that can be used in antibody production or for more detailed analysis at the subunit level. Protein purified in this way should also be suitable for mass spectrometry, crosslinking, or electron microscopy.

3.2.1. Procedure

Larger, thicker gels (e.g., 14 × 14 × 0.16 cm) are generally employed for preparative native electrophoresis to maximize yield. Electrophoresis is started at 100 V until the sample has entered the gel and continued for several hours at 500 V [8]. To minimize dissociation of the complexes, electrophoresis should be carried out at in a cold room and should be stopped immediately when the desired separation has been achieved. The band of interest is cut out of the gel, and protein recovered using an electroeluter (e.g., Bio-Rad Model 422 electroeluter). Electroelution buffer is 25 mM tricine, 7.5 mM Bis–Tris, pH 7.0, and electroelution is performed at 100 V for 5–18 h at 4 °C (elution efficiency can be followed by the migration of the blue-stained protein) (for a more detailed description readers are referred to [8]).

3.3. Dynamics of mitochondrial biogenesis

BN/SDS–polyacrylamide 2D electrophoresis gives a picture of the multisubunit protein complexes of mitochondria. In addition, the dynamics of protein synthesis and complex assembly can be addressed using the technique in combination with pulse–chase labeling of proteins in cultured cells. Crude mitochondrial mem-

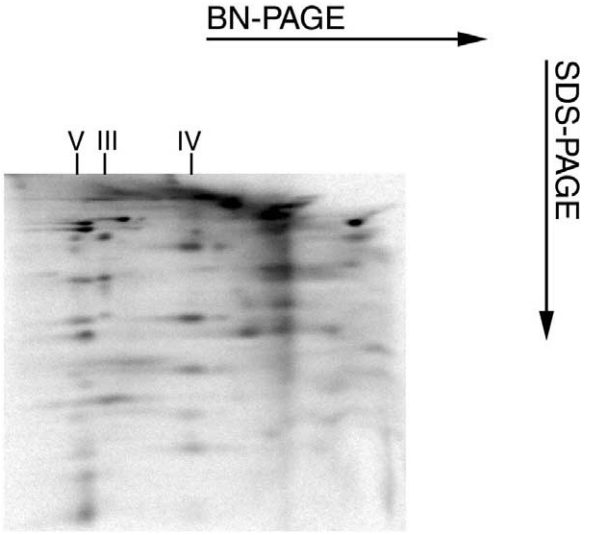

Fig. 3. Two-dimensional Blue Native/SDS–PAGE of radioactively labeled mitochondrial pellets. A549 cells were grown in the presence of [^{35}S]methionine. After 1 h the label was removed and the cells were grown for 24 h in the absence of label. Mitochondrial pellets were isolated and a two-dimensional gel was run (for details see text). Complexes V, III, and IV are indicated.

brane fractions are prepared by the standard protocol (see Sample preparation) from cells labeled with [^{35}S]methionine. Separation of proteins in the first and second dimensions also follows the basic protocol [7]. The radioactively labeled proteins are detected by exposing the gel to an X-ray film or phosphor imager screen (Fig. 3).

The labeling protocol is straightforward and uses few cells and little radiolabel. The purification of a crude mitochondrial pellet is simple and does not involve many steps. These features become important when several time points are to be analyzed from multiple cell lines.

In the low-molecular-weight range a lot of proteins are labeled, making interpretation difficult. One way around this problem is to label only mitochondrially encoded proteins by first inhibiting cytosolic protein synthesis with cycloheximide [15]. The turnover of mitochondrial membrane-bound complexes is, in general, slower than that of soluble matrix proteins and therefore longer chase times are required for the analysis of this class of protein complexes (Fig. 3).

3.3.1. Metabolic labeling protocol

Two million fibroblasts or five million cybrid cells [13] are plated on 90- to 100-mm dishes (one plate per time point). After 24 h, the cells are rinsed with PBS prewarmed to 37 °C and 4 ml/plate prewarmed DMEM, without methionine and 10% dialyzed serum (37 °C) are added. The cells are incubated 1 h at 37 °C. When proteins are radiolabeled in the presence of an inhibitor of either cytosolic protein synthesis (100 μg/ml cycloheximide, 100 μg/ml emetine) or mitochondrial protein synthesis (15 μg/ml doxycycline, 40 μg/ml chloramphenicol), the drug is added 10 min before the radiolabel [15].

3.3.2. Pulse–chase

Radioactive methionine is added at 80 μCi per plate (20 μCi/ml), and the cells are incubated for the desired pulse time, e.g., 1 h. The cells are then washed twice with PBS (37 °C) and 8 ml of medium (37 °C) containing unlabeled methionine added. The cells are chased according to the time schedule for the experiment. Informative time points are 1 h pulse and 0, 1, 2, 6, and 20 hours chase [7]. Labeled cells are harvested by removing the medium (RADIOACTIVE!) and rinsing with cold PBS, and detached by trypsin treatment. Digitonin pellets are prepared according to the standard BN-PAGE protocol described earlier. Pellets are processed for gel loading immediately or stored at −80 °C.

3.4. Molecular weight status

Techniques to study protein–protein interactions include the yeast two-hybrid system [16], affinity chromatography [17], and coimmunoprecipitation [18]. The disadvantage of these techniques is that they are not well suited to hydrophobic membrane proteins. Because Blue Native PAGE is specially designed for membrane proteins, this technique is a good alternative to the above techniques. It may also be used to substantiate an association suggested by another technique.

When the molecular weight of a native protein in the first dimension is different from its molecular weight in the denaturing second dimension, it is very likely that the protein is part of a complex and associated with other proteins, or multiple copies of itself (Fig. 4). Other proteins associated with this protein of interest can be identified because they have the same mobility in the first dimension. For instance in Fig. 4, HSP60 has a mobility of 60 kDa in the second dimension. This corresponds to the expected molecular weight of a monomeric molecule. In the first dimension, however, it has mobilities of 380 and 450 kDa. These correspond to a HSP60 heptamer and a HSP60 heptamer plus a HSP10 heptamer, respectively. Cox4p association with the HSP60 complex has also been demonstrated using BN-PAGE. Of course, proteins that have the same mobility in the first dimension may be part of different complexes that are the same size. Moreover, results have to be evaluated carefully because hydrophobic membrane proteins have a tendency to form aggregates, which are not complexes. Therefore, proving two proteins are in the same complex requires additional evidence.

3.5. Identification of spots by mass spectrometry

A recent development in biology is high-throughput mass spectrometry [19]. This technique allows rapid

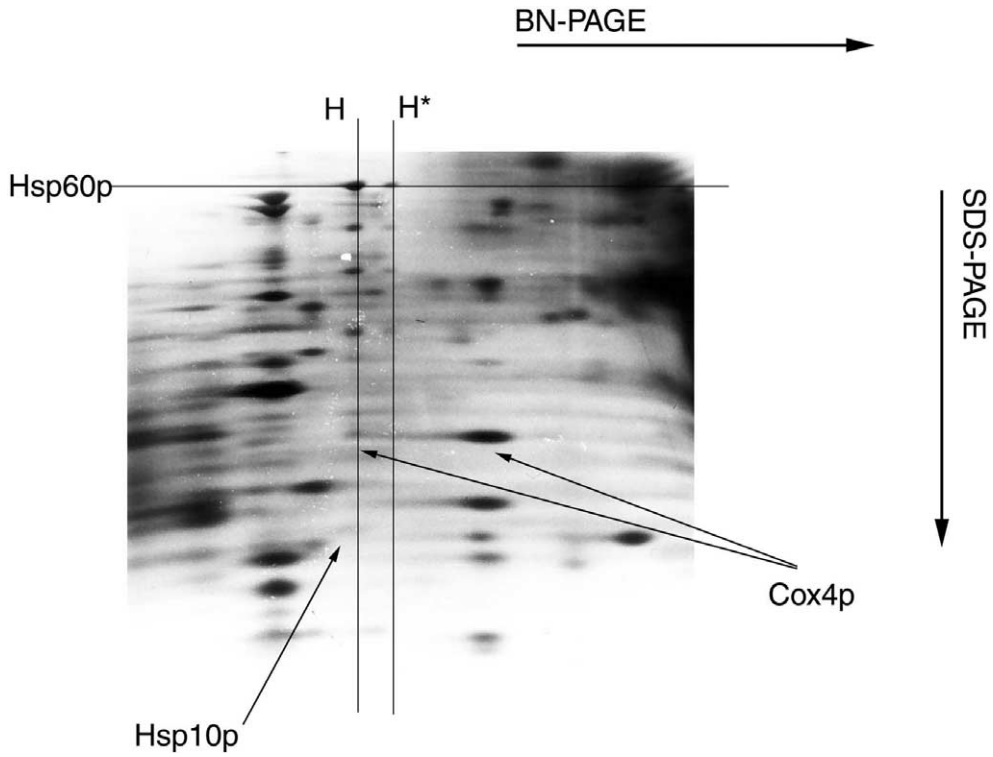

Fig. 4. Organization of proteins in high-molecular-weight complexes. Crude mitochondrial pellet was prepared from Molt-4 cells and separated by two-dimensional BN/SDS–PAGE. The discrepancy between the denatured and native molecular weights of Hsp60p indicates that this protein is organized in a complex. The denatured Hsp60p is indicated by a line and the HSP60 complexes are indicated as H and H*. Hsp10p and Cox4p are indicated with arrows.

identification of a spot on a gel. Great sensitivity and fast detection make this a very powerful tool. Generally the gels used are isoelectric focusing gels in combination with SDS–polyacrylamide. However, the technique can also be applied to BN-PAGE either with or without a second-dimension SDS–PAGE.

3.5.1. General description

Gels are stained with silver, Coomassie Blue R-250 [20], or Sypro Ruby (Molecular Probes, Eugene, OR). Proteins are alkylated using dithiothreitol or iodoacetamide. The next step is in-gel digestion with a protease, usually trypsin. Tryptic fragments are extracted from the gel and applied to the mass spectrometer. The pattern of tryptic fragments can be compared to a database and usually yields unambiguous identification of a protein; however, considerable care needs to be exercised to avoid keratin or protease contamination.

4. Concluding remarks

Although Blue Native electrophoresis was developed in 1991 [2] it was a few years before other groups discovered the many benefits of the method. Now many laboratories studying mitochondria employ the technique. It is expected that the use of BN-PAGE will increase further as it can be set up easily using apparatus found in most laboratories and it has a wide range of applications. An important case in point is mitochondrial dysfunction. Dysfunction may stem from proteins involved in respiratory complex assembly, such as Surpf1p [21] and Cox10p [22]; proteins involved in iron homeostasis, e.g., Frataxin [23]; proteins involved in copper homeostasis, e.g., Sco1p and Sco2p [24,25]; proteins involved in proteolysis, e.g., paraplegin [26]; and proteins involved in mitochondrial DNA replication, e.g., Twinkle [27]. The molecular mechanisms and possible interactions of these proteins still remain to be elucidated. It is expected that BN-PAGE will continue to contribute to solving these and related research questions.

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