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Review

# Detection and analysis of protein–protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (Membrane) protein complexes and supercomplexes

It is an essential and challenging task to unravel protein–protein interactions in their actual *in vivo* context. Native gel systems provide a separation platform allowing the analysis of protein complexes on a rather proteome-wide scale in a single experiment. This review focus on blue-native (BN)-PAGE as the most versatile and successful gel-based approach to separate soluble and membrane protein complexes of intricate protein mixtures derived from all biological sources. BN-PAGE is a charge-shift method with a running pH of 7.5 relying on the gentle binding of anionic CBB dye to all membrane and many soluble protein complexes, leading to separation of protein species essentially according to their size and superior resolution than other fractionation techniques can offer. The closely related colorless-native (CN)-PAGE, whose applicability is restricted to protein species with intrinsic negative net charge, proved to provide an especially mild separation capable of preserving weak protein–protein interactions better than BN-PAGE. The essential conditions determining the success of detecting protein–protein interactions are the sample preparations, *e.g.* the efficiency/mildness of the detergent solubilization of membrane protein complexes. A broad overview about the achievements of BN- and CN-PAGE studies to elucidate protein–protein interactions in organelles and prokaryotes is presented, *e.g.* the mitochondrial protein import machinery and oxidative phosphorylation supercomplexes. In many cases, solubilization with digitonin was demonstrated to facilitate an efficient and particularly gentle extraction of membrane protein complexes prone to dissociation by treatment with other detergents. In general, analyses of protein interactomes should be carried out by both BN- and CN-PAGE.

**Keywords:** Blue-native-PAGE / Colorless-native-PAGE / Chloroplasts / Digitonin / Mitochondria
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**Abbreviations: BN**, blue-native; **$C_{12}E_8$**, octaethyleneglycol mono-*n*-dodecyl ether; **CN**, colorless-native; **DDM**, β-D-dodecylmaltoside; **DM**, β-D-decylmaltoside; **LHCII**, light-harvesting complex II; **OG**, β-D-octylglucoside; **OST**, oligosaccharyltransferase; **OXPHOS**, oxidative phosphorylation; **PSI**, photosystem I; **PSII**, photosystem II; **RAMP**, ribosome-associated membrane protein; **SRP**, signal recognition particle; **TIM**, translocase of the inner membrane of mitochondria; **TOM**, translocase of the outer membrane of mitochondria; **TRAP**, translocating chain-associated membrane protein, **Triton X-100**, *p*-isooctylphenoxy-polyethoxyethanol

## 1 Introduction

After the elucidation of a large number of genomes from prokaryotic and eukaryotic organisms, a prime task in biochemistry is the characterization of the functions of the respective gene products (proteins) in the context of the actual proteome of a cell or of physiologically meaningful subproteomes like those of organelles. Crucially, many or even most proteins exert their functions by stable or transient interactions with other proteins either as homo- or heterooligomeric protein complexes. This can be ultimately attributed to the so-called 'macromolecular crowding' in the cell interior, since 20–30% of the intracellular volume is occupied by macromolecules not

available to other molecules, leading to physicochemical properties very distinct from those of diluted solutions. Due to this general phenomenon, interactions between macromolecules as well as their segregation into different compartments are favored [1, 2]. These characteristics are thought to be of particular relevance in biological membranes being the cell compartments with the highest concentration of biomolecules [3, 4] like the extremely protein-rich mitochondrial cristae membranes and the thylakoids of chloroplasts [5, 6]. One of the most significant emerging issues is the supramolecular organisation of the enzymes of whole or partial metabolic pathways as the so-called 'metabolons' which appear to enable enzymatical advantages like 'substrate channelling' and whose conceptualisation and experimental investigation have actually a long history [7, 8].

Gel-based electrophoretic approaches are especially valuable tools in functional proteomics [9]. First, entire proteins are separated and made accessible to further fractionation and characterisation, *e.g.* by MS. Second, depending on the gel system hydrophilic proteins in solution or detergent-solubilized proteins can be directly applied and separated in a single step, thus representing rather comprehensively the proteome of the biological starting material. This means that in principle protein–protein interactions can be analyzed on a proteome-wide scale without restriction to a few proteins of a complex protein mixture by affinity chromatography and coimmunoprecipitation employed to detect protein binding partners [10, 11]. Alternatively, tandem affinity purification (TAP) [10–16] and other sophisticated fishing procedures [17] are able to comprehensively detect protein complexes. However, the necessary manipulation of the starting material by tagging of native proteins might influence some physiological protein–protein interactions. Furthermore, the elaborate yeast two-hybrid analysis is able to identify binary protein complexes on a proteome-wide scale [18–20]. As major drawbacks, these methods produce significant numbers of false-positive and false-negative interactions as well as are not or limitedly suitable for all kinds of biological samples and a detailed analysis of protein complexes like the investigation of the subunit stoichiometry [10–20].

Importantly, genome analyses predict that about 20–30% of the cellular proteins are integral membrane proteins [21] and in addition many soluble proteins are peripherically associated, *e.g. via* direct interactions to integral membrane proteins. The biochemical analysis of integral membrane proteins requires the extraction of biomembranes with detergents which efficiently solubilize hydrophobic proteins but may disrupt protein–protein interactions and affect enzymatical activities. Hence, only mild detergents which are predominantly nonionic ones like β-D-dodecylmaltoside (DDM), *p*-isooctylphenoxy-polyethoxyethanol (Triton X-100) and digitonin are suitable for the analysis of protein complexes (Table 1). Likewise, the electrophoretic analysis of intact, enzymatically active protein complexes is only possible under nondenaturing conditions in contrast to the SDS-PAGE [22, 23] or standard IEF [24], both enabling the separation of proteins according to their mass and p*I*, respectively. This is a challenging task since hydrophobic proteins are likely to aggregate artificially during the electrophoretic run [25]. Various gel electrophoretic approaches had been employed which proved to be appropriate for the analysis of particular membrane and soluble protein complexes like native IEF (*e.g.* [26, 27]) and others (*e.g.* [28, 29]). The nondenaturing disodium *N*-dodecyl-8-iminodipropionate-160 (Deriphat) gels [30–40] as well as the green-native gel system [41–46] have been successfully utilised to analyse the photophosphorylation complexes of detergent extracts from thylakoids of higher plants and cyanobacteria.

By far the most versatile and powerful nondenaturing gel system, apparently capable of separating all kinds of membrane and soluble protein complexes even from complex mixtures, turned out to be the so-called blue-native (BN)-PAGE (BN-PAGE) and to lesser extent the sister gel system of it, the colorless-native (CN-) PAGE also synonymously termed as clear-native-PAGE. The two related methods were originally developed as microscale tools to separate the oxidative phosphorylation (OXPHOS) complexes from bovine heart and yeast by directly applying DDM extracts of isolated mitochondria [47, 48].

This review summarises the most important techniques to detect and analyse protein–protein interactions by BN- and CN-PAGE and aims to give a broad overview of the important achievements of the two electrophoretic systems to elucidate protein–protein interactions in organelles and prokaryotes. The purpose is not to provide detailed protocols of BN(CN)-PAGE and subsequent procedures because there are already excellent and refined reports available [47–53]. I apologize to the authors whose relevant papers could not be cited or not described in much detail because of limited space.

## 2 BN-PAGE

The versatility of BN-PAGE originates from two peculiar features which are critical to separate protein complexes with different physicochemical properties occurring in various biological samples like mitochondria, chloroplasts, other organelles, cytosolic fractions and bacterial

 

**Table 1.** Detergents used for solubilization of integral membrane protein complexes for analysis by BN- and CN-PAGE

| Detergent | Sample | BN-PAGE examples [references] | CN-PAGE examples [references] | Section (appearance of references) |
|---|---|---|---|---|
| Nonionic detergents | | | | |
| DDM | Mammalian mitochondria | [47–53, 57, 92–104, 144, 145, 165–167, 169–171, 175–190, 216, 230, 315, 390–395] | [48] | 2, 3, 4.1–4.3, 5.2, 6 |
| | Yeast mitochondria | [47, 65, 105, 172, 173, 230] | [105] | 2, 3, 4.1–4.4 |
| | Filamentous fungal mitochondria | [193, 232, 233, 280–283] | | 2, 4.1, 4.2, 4.4, 5.1 |
| | Plant mitochondria | [66–76, 147, 203–205, 316, 317, 385] | | 2, 4.2, 5.2, 6 |
| | Algal mitochondria | [77–82] | | 2, 4.3, 5.3 |
| | Protist mitochondria | [76, 83–91] | | 2, 4.3 |
| | Plant chloroplasts (thylakoids) | [200–205, 251–253, 327–335, 340] | [125] | 2, 4.1, 4.5, 5.3 |
| | Algal chloroplasts (thylakoids) | [215, 341] | | 4.3, 4.5, 5.3 |
| | Mammalian microsomes | [155] | | 4.1, 5.4 |
| | Yeast microsomes | [371] | | 5.4 |
| | Plant microsomes | [372] | | 5.4 |
| | Cyanobacterial membranes | [40, 206, 243–250, 342–346] | | 4.2, 4.4, 4.5, 5.3 |
| | Cyanobacterial outer membrane | [217] | | 4.4, 5.5, 6 |
| | Bacterial membranes | [54, 59, 137, 376–379] | | 2, 4.1, 4.3, 4.4, 5.5 |
| DM | Mammalian mitochondria | | this review (Fig.3C) | 5.2 |
| | Plant chloroplasts (thylakoids) | [62, 109] | | 2, 3, 4, 4.1–4.3, 5.3, 6 |
| | Algal chloroplasts (thylakoids) | [62] | | 2, 5.3 |
| | Plant inner chloroplast envelope membranes | [161, 290] | | 4.2, 5.1 |
| | Cyanobacterial membranes | [62] | | 2, 5.3 |
| OG | Yeast mitochondria | [105] | [105] | 3 |
| | Plant microsomes | [157, 158] | | 4.1, 5.4 |
| | Cyanobacterial membranes | [206] | | 4.2, 5.3 |
| | Cyanobacterial outer membrane | [217] | | 4.4, 5.5, 6 |
| 6-*O*-(*N*-Heptylcarbamoyl)methyl-α-D-glucopyranoside (HECAMEG) | Yeast mitochondria | [105] | [105] | 3 |
| Dodecanoyl-sucrose | Plant chloroplasts | [109] | | 3, 4, 5.2, 5.3, 6 |
| Triton X-100 | Mammalian mitochondria | [47, 50, 51, 144, 145, 168, 226, 389] | | 2, 3, 4.1–4.4, 5.2, 6 |
| | Yeast mitochondria | [144, 278] | [105, 106] | 3, 4.1, 5.1, 5.2 |
| | Plant mitochondria | [147] | | 4.1, 5.2 |
| | Human plasma membranes | [207] | | 4.2 |
| | Mammalian microsomes | [362] | | 5.4 |
| | Plant chloroplasts (thylakoids) | [251, 327] | | 4.5, 5.3 |
| | Plant outer chloroplast envelope membranes | [291] | | 5.1 |
| | Cyanobacterial membranes | [206] | | 4.2, 5.3 |
| | Bacterial membranes | [54, 137] | | 2, 4.1, 4.3, 5.5 |
| Nonidet P-40 | Yeast mitochondria | [105] | [105] | 3 |
| $C_{12}E_8$ | Yeast microsomes | [366, 367] | | 5.4 |
| Lubrol | Yeast mitochondria | [105] | [105] | 3 |
| Digitonin | Mammalian mitochondria | [50, 51, 57, 60, 104, 110, 112, 142, 145, 156, 159, 235, 286–288, 309, 310, 322, 323] | [110, 112] this review (Fig. 3D) | 2, 3, 4, 4.1–4.4, 5.1, 5.2, 6 |
| | Yeast mitochondria | [58, 107, 138–142, 144, 145, 149, 174, 218–226, 255–262, 279, 318–321, 388] | [107, 111, 113–121] | 2, 3, 4, 4.1–4.5, 5.1, 5.2, 6 |

 

**Table 1.** Continued

| Detergent | Sample | BN-PAGE examples [references] | CN-PAGE examples [references] | Section (appearance of references) |
|---|---|---|---|---|
| | Filamentous fungal mitochondria | [108, 280–283] | [108] | 3, 4, 4.1–4.3, 5.1, 5.2 |
| | Plant mitochondria (or outer membranes) | [109, 147, 148, 160, 196, 213, 214, 284, 285, 311, 324] | [109] | 3, 4, 4.1–4.3, 5.1–5.3, 6 |
| | Plant chloroplasts (thylakoids) | [109, 263–265, 327, 336–339] | [109] | 3, 4, 4.1–4.3, 4.5, 5.1–5.3, 6 |
| | Algal thylakoids | [124, 129] | [124] | 3, 4, 5.3 |
| | Plant outer chloroplast envelope membranes | [291] | | 5.1 |
| | Mammalian microsomes | [362, 363] | | 5.4 |
| | Yeast microsomes | [365, 369, 370] | | 5.4 |
| | Mammalian peroxisomes | [229, 292] | | 4.4, 5.1 |
| | Bacterial membranes | [59, 137, 373–375, 380] | | 2, 4.1, 4.3, 4.4, 5.5 |
| Zwitterionic detergents | | | | |
| CHAPS | Human rod outer segment membranes | [133] | | 4 |
| CHAPSO | Human postnuclear membranes | [134] | | 4 |
| FOS-choline | Cyanobacterial outer membrane | [217] | | 4.4, 5.5 |

membranes. First, the running pH of BN-PAGE is fixed at 7.5 which is in the physiological range of most intracellular compartments. This allows the migration of protein assemblies which are susceptible to acidic and/or basic conditions that lead to dissociation of subunits and/or loss of enzymatical activities. Second, the anionic triphenylmethane dye CBB G-250 (Fig. 1) is introduced by its presence in the cathode buffer and often by addition to the sample shortly before the run. Significantly, the CBB dye binds to essentially all membrane proteins and many soluble ones in a manner that retains many protein–protein interactions. This confers a negative net charge to the protein complex and largely diminishes any artificial aggregation of detergent-solubilized hydrophobic membrane proteins during the electrophoretic run by electrostatical repulsion without in need of detergents present in the gel. The binding of anionic CBB dye occurs variably but rather uniformly in case of membrane proteins resulting in Coomassie/protein ratios of ~1 g/g as a rule of thumb [54]. Consequently, protein complexes and individual proteins migrate to the anode due to excessive negative charge and are separated by the sieving effect of the polyacrylamide gel according essentially to the particle size (molecular mass) [47–51]. Of course, the electrophoretic behaviour of the protein species is more or less influenced by their native (nondenatured) shape and their intrinsic charge, the more so if CBB dye cannot or only marginally bind [47, 48, 51, 54]. Notwithstanding, the accuracy to determine molecular masses of membrane and soluble protein complexes was proven in many cases

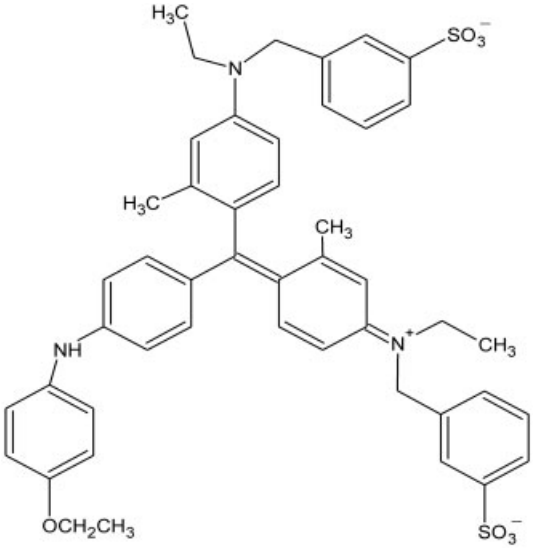


**Figure 1.** CBB G-250.

by separation of membrane and soluble protein complexes with known masses [48, 50, 51] and corroborating approaches like sucrose-density centrifugation, gel permeation and analytical ultracentrifugation, which are more elaborate and less precise (*e.g.* [54–58]). In general, the apparent molecular masses of all CBB-binding protein species with a p*I* $< 8.6$ and those which do not bind CBB dye with a p*I* $\leq 5.4$ fit very well to calibration curves revealing a linear dependence of the logarithmic apparent mass on the migration distance [48, 51].

 

The upper size limit to separate protein complexes by BN-PAGE is ~10 000 kDa and not around 1 500 kDa as erroneously stated sometimes [52, 59], since the largest multiprotein complex of mitochondria, the pyruvate dehydrogenase complex, can enter separation gradient gels with 3% polyacrylamide concentration at the top [50, 51, 60]. In principle, other gel matrices like agarose with larger pore sizes than achievable by polyacrylamide can be adopted which may potentially allow the separation of large protein assemblies with masses significantly above 10 000 kDa. A BN agarose gel was shown to separate the pyruvate dehydrogenase complex but its ability to resolve protein mixtures with large molecular mass ranges was inferior [61] to that of a polyacrylamide gradient gel [50, 51, 60, 62]. It seems possible that improved agarose-polyacrylamide composite gels (*e.g.* [63]) can provide a suitable matrix for the BN electrophoresis technique to separate giant protein complexes. Likewise, by increasing the polyacrylamide concentration at the bottom up to 18–20% [50, 51], small proteins and protein complexes of ~10–50 kDa can be separated, allowing the separation performance to focus on desired and narrower mass ranges.

It is of special note that dissociation of rather weak protein–protein interactions induced by the anionic CBB dye during BN-PAGE has to be taken into account, particularly when prepurified delipidated membrane protein complexes are applied. Strikingly, detergent-solubilized spinach $CF_OF_1$-ATP synthase remains intact during BN-PAGE employing a cathode buffer with low dye concentration of 0.002%, but quantitatively bifurcates into the $CF_O$- and $CF_1$-subcomplexes in case of a ten-fold higher dye concentration of 0.02% [64] which is usually used for analysis of detergent extracts [47, 48]. Thus, the combination of neutral detergents with the anionic CBB dye can mimic properties of mild anionic detergents [64].

The original protocol of BN-PAGE was introduced to analyse the five individual OXPHOS complexes in a single step after solubilization of isolated bovine heart and yeast mitochondria with nonionic detergents in 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol (Bis/Tris)-buffered 6-aminocaproic acid solution [47]. The separated OXPHOS complexes retained their enzymatical activity fully or to a significant extent after electroelution from the gel, demonstrating the native conditions and justifying the term BN-PAGE [47–49]. In the following years up to now, this original protocol has been used without major modifications to examine the subunit composition of the individual mitochondrial OXPHOS complexes from a large variety of eukaryotes like yeast [47, 65], plants [66–76], algae [77–82], protists [76, 83–91], the nematode *Caenorhabditis elegans* [92], human cell culture [93–95] and human tissues [96–98]. The alternative solubilization of mitochondrial membranes with digitonin leading to the separation of intact OXPHOS supercomplexes is discussed in more detail in Sections 5.1 and 5.2 (see also Tables 1, 2). Likewise, BN-PAGE emerged as a superior analytical tool for the clinical diagnosis of mitochondrial OXPHOS defects (*e.g.* [95–97, 99–104]), the more so since milligram amounts of biopsies as well as mitoplasts or isolated mitochondria from cell cultures of patient cells are sufficient to get meaningful results, allowing the combined monitoring of the assembly status of the OXPHOS complexes and their enzymatic activities by subsequent in-gel activity staining (see Section 4.2).

Moreover, BN-PAGE proved to be a robust, manageable, as well as cost- and time-efficient method providing superb results for the analysis of membrane and soluble protein complexes from virtually all kinds of biological starting material, as described in more detail below. It is also often used as a final purification step and/or as tool for the determination of molecular mass, oligomeric state and subunit composition of prepurified protein complexes (*e.g.* [47–51, 54, 55]).

## 3 CN-PAGE

CN-PAGE was introduced in 1994 soon after the development of BN-PAGE and is nothing but the BN gel system without employment of CBB dye [48]. Thus, protein complexes migrate solely driven by their intrinsic charge towards the anode and only acidic protein species (p*I* $< 7$) enter the gel [48], which restricts the versatility and depending on the protein mixture leads to reduced resolving power compared with BN-PAGE. The electrophoretic mobility of the protein species is determined not only by their size but also by their p*I* in contrast to BN-PAGE conditions [48]. In general, soluble acidic proteins or protein-lipid-detergent particles solubilized from membranes with a p*I* $\leq 5.4$ have enough negative charge resulting in the same mobility as during BN-PAGE [48]. CN-PAGE was first used as a preseparation step of DDM-solubilized bovine heart OXPHOS complexes followed by 2-D BN-PAGE leading to very pure protein complexes. Because of the above-mentioned features, CN-PAGE has been employed much more seldom than BN-PAGE and essentially all respective reports can be cited here [27, 48, 105–128]. Recently, CN-PAGE was termed 'clear-native-PAGE' as a shorter synonym [112–121]. The electrophoretic mobility of the OXPHOS complexes of mammals and filamentous fungi was found to be significantly reduced compared with BN-PAGE conditions leading to inferior but satisfying resolution [48, 108, 110, 112], whereas those of the yeast [105, 107, 111, 113–121] and plant [109] counterparts are very similar during both electro-

 

**Table 2.** Summary of the most successful proteomic studies in terms of identified proteins employing BN- or CN-PAGE combined with mass spectrometric protein identification

| Species | Detergents | Electrophoretic analysis | Number of identified nonredundant proteins | Notes | [Reference] Section (appearance in the text) |
|---|---|---|---|---|---|
| Mitochondria | | | | | |
| Rat (kidney, liver, heart, skeletal muscle and brain) | Digitonin | 2-D BN/SDS-PAGE | Total: 125/30 subunits of the five OXPHOS complexes, 92 non-OXPHOS proteins (72 as constituents of complexes), three contaminating myelin proteins | Preservation of OXPHOS supercomplexes | [156] 4.1 5.2 |
| Bovine heart | Digitonin | 2-D BN/SDS-PAGE | Total: 71/32 subunits of the five OXPHOS complexes, 39 non-OXPHOS proteins | Preservation of a particular high yield of OXPHOS supercomplexes compared with Refs. [145, 146] | [60] 2, 4.3, 5.2 |
| Human heart | DDM | 2-D BN/SDS-PAGE | Total: 65/53 subunits of the five OXPHOS complexes, 12 non-OXPHOS proteins | Solubilization of individual OXPHOS complexes | [98] 2, 4.3 5.2 |
| Rice shoots | DDM | 2-D BN/SDS-PAGE | Total: 49/28 subunits of four OXPHOS complexes, 21 non-OXPHOS proteins | Solubilization of individual OXPHOS complexes | [73] 2, 4.3, 5.2 |
| *Arabidopsis thaliana* cell culture, membrane-enriched fraction | DDM | BN-PAGE | Total of various gel-based approaches: 180 (BN-PAGE data not specified) | Solubilization of individual OXPHOS complexes | [317] 5.2 |
| Yeast | Triton X-100 | 2-D CN/SDS-PAGE | Total: 38 subunits of a putative dehydrogenase supercomplex and a few contaminants | | [106] 3, 4.2 |
| Chloroplasts (thylakoids) | | | | | |
| *Arabidopsis* stroma fraction | None | 2-D CN/SDS-PAGE | Total: 241/mostly as constituents of protein complexes | CN-PAGE preserved stroma protein complexes better than BN-PAGE | [123] 3, 4.1, 4.3 |
| *C. reinhardtii* thylakoids | Digitonin | 2-D BN/SDS-PAGE | Total: 37 subunits of protein complexes (mostly photophosphorylation) | More gentle cell disruption than in Ref. [129], preservation of dimeric $CF_0F_1$-ATP synthase | [124] 3, 4, 4.3, 5.3 |
| *C. reinhardtii* thylakoids | Digitonin | 2-D BN/SDS-PAGE | Total: 30/27 subunits of photophosphorylation complexes and RubisCO, 3 subunits of OXPHOS complexes | Differential display of two culture conditions | [129] 4, 4.3, 5.3 |
| ER (microsomes) | | | | | |
| Human platelet microsomes | DDM | 2-D BN/SDS-PAGE | Total: 62/mostly as constituents of protein complexes | | [155] 4.1, 5.4 |
| Dog pancreas microsomes (purified RAM) | Digitonin | 2-D BN/SDS-PAGE | Total: 32/mostly as constituents of 6 protein complexes | Preservation of two OST supercomplexes | [363] 5.4 |
| Bacteria | | | | | |
| *M. thermautotrophicus* membrane and cytosolic fractions | Digitonin DDM | 2-D BN/SDS-PAGE | Total: 361/many as constituents of protein complexes | Digitonin solubilization preserved some supercomplexes | [59] 2, 4, 4.4, 5.5 |
| *E. coli*, inner and outer envelope membranes | DDM | 2-D BN/SDS-PAGE | Total: 54/Inner membrane: 42/ Outer membrane: 12/ mostly as constituents of protein complexes | | [137] 4, 5.5 |
| Soluble cell extract of *Pseudomonas sp. strain phDV1* | None, density-gradient fractionation | 2-D BN/SDS-PAGE | Total: 49/many as constituents of protein complexes | Differential display of two culture conditions | [382] 5.5 |

 

phoresis systems. Of special interest is the observation that the photophosphorylation complexes of the green alga *Chlamydomonas reinhardtii* [124], other thylakoid protein complexes [109, 125] and apparently also soluble stroma complexes [27, 109, 122, 123, 126] have a comparable electrophoretic behavior in BN- and CN-PAGE suggesting rather acidic p*I*s of the respective proteins. Indeed, stromal Clp protease complexes of higher plants, which have the same apparent molecular mass of ~325 kDa obtained by BN- and CN-PAGE, were analyzed by native IEF determining a p*I* of ~5 [27].

Most importantly, it is clear that some protein–protein interactions are affected to some extent by the anionic CBB dye, which can lead, for example, to the dissociation of protein complexes into smaller units [64]. Indeed, the application of CN-PAGE to analyse digitonin extracts of fungal [107, 108, 120] and mammalian mitochondria [110, 112] was reported to separate significantly higher yields of preserved OXPHOS supercomplexes than obtained by BN-PAGE, which is described in Section 5.2. Similarly, soluble protein complexes of the highly purified stroma of *Arabidopsis* chloroplasts were found to be less stable during BN-PAGE [27, 123] especially when high concentrations of CBB dye were added before electrophoresis [123]. In the latter study, CN-PAGE enabled the separation of many proteins of which 241 could be identified and most appeared to be part of protein complexes, ranking this work as one of the most successful gel-based approaches in organellar proteomics even only because of this high number of identified proteins [123] (Table 2). To avoid potential aggregation of membrane proteins from detergent extracts during the electrophoretic run, mild detergents were added to the CN gels, *e.g.* 0.01% DDM [48], 0.01% Triton X-100 [108–110] and 0.003–0.025% digitonin [108–112, 124]. When membrane proteins are analysed the need and effect of varying concentrations of proper detergents present in the gel should be always tested to assess on one hand potentially artificial aggregation during the gel run, and on the other hand the stability of protein complexes preserved in the detergent extract [108–111]. Another advantage of CN-PAGE is the improvement of in-gel activity staining since there is no interference by background color or impaired substrate accessibility by protein-bound CBB dye [105, 107, 110, 112] (see Section 4.2).

## 4 Detection and analysis of protein–protein interactions by native gel electrophoresis

The most important parameters determining the success of detecting protein–protein interactions are the efficiency/mildness of the sample preparation. In detail, the solubilization with gentle detergents in the case of membrane-containing starting materials like organelles and bacteria as well as the quality/integrity of the biological sample itself, which can be influenced for example by the isolation procedure [124, 129] (see Section 5.3) of the organelles/membranes as well as the storage time of frozen tissues before processing [60, 110] (see Section 5.2), are critical. The search for appropriate solubilization conditions, which can substantially vary between diverse biological membranes due to distinct lipid/protein compositions and ratios, includes the employed buffer (ionic strength, pH, temperature), the applied detergent as well as the detergent/protein ratio, being described in much detail elsewhere [51, 130–132]. An overview of detergents successfully used for BN(CN)-PAGE analysis is given in Table 1. Importantly, most (if not all) detergents capable of retaining protein–protein interactions seem to be compatibe, since for example membrane protein complexes solubilized with zwitterionic detergents like CHAPS [133] and 3[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHAPSO) [134] were separated by BN-PAGE. If possible, it is recommendable to avoid particular high ionic strengthes of cations like potassium which may induce aggregation of CBB dye and of highly mobile ions like chloride in the samples applied to BN-PAGE and/or CN-PAGE, respectively [47–51]. Nonetheless, the potential to tolerate high concentrations of such ions in the samples is astonishing. For example, detergent-extracts of hydrogenosomes containing up to 2 M NaCl were successfully analyzed by BN-PAGE [135, 136]. Thus, the buffer condition of the applied samples, whose electrophoretic performance has to be tested carefully in each particular case, allows sufficient range of trial.

### 4.1 Determination of molecular masses of protein complexes using BN- and CN-PAGE

The mobility of most protein species during BN-PAGE is essentially governed by their size resulting in linear dependence of the logarithmic apparent masses on the migration distance (*e.g.* [48, 51, 54, 137]). As a rule, the apparent masses of homo- and heterooligomeric protein assemblies can be reliably expected to be the sum of the respective multiple masses derived from the stoichiometric composition of the protein subunits or protein subcomplexes since relevant protein-depending characteristics are very similar for monomeric and oligomeric structures. For example, the oligomeric state of bacterial and mitochondrial membrane transport proteins [54, 138–143], monomers, dimers and higher oligomers of mitochondrial ATP synthases (homo-supercomplexes) [50, 51, 79–82, 108–110, 112, 124, 144–147], the stoichiometry of

 

respiratory supercomplexes composed of complexes I, III and IV [108–110, 112, 144–149], as well as chloroplast $CF_OF_1$-ATP synthase monomers and dimers [124] were demonstrated to be identifiable by means of their apparent masses. Importantly, the electrophoretic mobility of membrane protein complexes can slightly vary dependent on the amounts of bound lipids as exemplified by bovine heart ATP synthase solubilized with DDM or digitonin [50, 51, 145, 146]. The same may occur using very low detergent/protein ratios leading to increased apparent masses by less delipidation [51].

As outlined above, the determination of the real masses of proteins separated by CN-PAGE is limited to rather acidic protein species, which seems to work well for analysing membrane and soluble protein complexes from plant mitochondria and plastids [27, 109, 122, 123, 125, 126]. In any case, CN-PAGE data should be compared with parallel BN-PAGE analysis of the samples, which is alternatively possible by combining a 1-D CN-PAGE with a subsequent BN-PAGE in the 2-D [48, 109, 110, 112, 125, 128]. The elucidation of the oligomeric state of separated protein complexes, whose electrophoretic mobilities are reduced compared with those under BN-PAGE conditions, is nevertheless similarly reliable as shown in the case of digitonin-solubilized ATP synthase oligomers and respiratory supercomplexes of filamentous fungi and mammals [108, 110, 112]. An easy possibility to detect differences of the electrophoretic mobilities and/or stabilities of protein complexes in CN gels is the parallel run of respective samples with addition of CBB dye shortly before the electrophoresis thus mimicking BN-PAGE conditions. This was performed recently by Rexroth *et al.* [124] to assess the stability of chloroplast $CF_OF_1$-ATP synthase dimers for both electrophoresis systems. Likewise, DDM- or digitonin extracts of bovine heart mitochondria with added CBB dye applied to the CN gel provide a reliable membrane protein mass standard. Herewith, the OXPHOS complexes and supercomplexes, respectively, as well as the pyruvate dehydrogenase complex are separated under BN-PAGE conditions, which directly enables the calibration of apparent masses up to ~10 000 kDa in CN gels.

The oligomeric state of some glycoproteins could be determined by BN-PAGE (*e.g.* [150–156]), but the mobility of glycoproteins may be reduced like those of $V_O$- and $V_OV_1$-ATPases [152, 156] or plasma membrane receptors [153, 154]. In the case of heavily glycosylated proteins like the two β-D-octylglucoside (OG)-solubilized tomato proteins separated by BN-PAGE as species of ~400–420 kDa [157, 158], the apparent masses might be even higher than the real one of the polypeptide chains and the attached carbohydrate moieties combined, simply because binding of CBB dye is restricted to the polypeptide chain and the covalently bound carbohydrates may increase overproportionally the Stokes radius.

### 4.2 In-gel activity assays to probe the enzymatical activity of separated protein complexes

The preservation of enzymatical activities is a crucial indicator of native conditions maintained from isolation of the biological starting material and solubilization with mild detergents in case of membranes until the protein complexes or individual proteins are separated during the gel run. As mentioned above, the mitochondrial OXPHOS complexes were shown to be active after electroelution from BN gels [47–49, 159]. Indeed, the electroelution of separated protein complexes is an important option employed in several studies (*e.g.* [47–49, 159–163]), but more appropriate for preparative purposes. Noteworthy, CBB dye bound to electroeluted (membrane) protein complexes can be removed by gel filtration and replaced with a mild detergent, thus BN-PAGE appears to be a generally utilisable method for the isolation and subsequent characterization of protein complexes in their native state [162]. Very beneficial for analytical applications are zymogram techniques, available for more than 300 different enzymes, of which most provide in case of enzyme activity a coloured precipitation at the gel position of the enzyme band [164]. In principle, all these protocols are applicable to CN-PAGE, and likewise a large number to the more versatile BN-PAGE if the blue CBB dye does not interfere too much with enzyme reaction or signal detection. The most often employed in-gel staining assays are those revealing enzymatic activity of the OXPHOS complexes from mitochondrial detergent-extracts; NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome oxidase (complex IV), and ATP hydrolase (complex V), which have been used in BN gels [*e.g.* 52, 53, 65, 81–83, 104, 108–110, 148, 159, 165–198] and in CN gels [105–107, 110, 112–121]. The semiquantitative measurement and the assessment of specific activities of reactive bands is possible [112, 172], which provide valuable informations for the clinical diagnosis of OXPHOS diseases by monitoring catalytic and/or structural defects of OXPHOS complexes and allowing even the discrimination between those defects [186]. The enzymatic activity of the plastid NAD(P)H dehydrogenase complex [199–202] of higher plants, which is homologous to the mitochondrial respiratory complex I, was revealed by specific staining in BN gels of DDM-solubilized barley [203, 204] and tobacco thylakoids [205]. Similarly, the activity staining of the *cbb*$_3$ cytochrome oxidase of *Rhodobacter capsulatus* was achieved in BN gels

 

after solubilization with DDM, Triton X-100, digitonin, but not with OG [206], representing a fine example to screen suitable detergents for native solubilization of protein complexes. Another interesting study is the separation of a cell surface NADH oxidoreductase complex from Triton X-100 solubilized plasma membranes of human osteosarcoma cells, displaying in-gel NADH reductase activity which was sensitive to a specific inhibitor [207]. Furthermore, a number of in-gel activity assays for soluble enzymes, namely aconitase, isocitrate lyase, malic enzyme, superoxide dismutase, $NADP^+$-dependent isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, two malate-generating enzymes and four oxaloacetate-forming enzymes were successfully employed to examine cytosolic fractions of Gram-negative *Pseudomonas fluorescens* applied to BN-PAGE [208–212].

Taken together, the activities of many enzymes, which are often homo- or heterooligomeric protein complexes, are retained during BN- and CN-PAGE, providing significant support for the physiological relevance of the separated protein species in the respective cases.

### 4.3 2-D SDS- and BN-PAGE

The comigration of proteins in native gels is a prerequisite and a first evidence to assign them as components of a protein complex. A direct way to detect homo- and heterooligomeric protein complexes separated in the native gel is immunoblots probed with antibodies against candidate proteins (*e.g.* [58, 111]). For a more accurate analysis, 2-D gels reveal the subunits or subcomplexes of protein complexes preserved in the 1-D. Denaturing 2-D SDS-PAGE, which dissociates protein complexes completely into their constituting subunits, resolves the subunit pattern in a vertical line below the position of the protein complex in the 1-D gels (Fig. 2). This technique has been widely used in combination with BN- and CN-PAGE (*e.g.* [47–52, 59, 60, 64–110, 112, 123–125, 128, 129, 137–148]). Importantly, the subunits of true complexes display the same band shape with identical focal points along the vertical line. These hallmarks are easy to recognise in the subunit pattern of high-abundant protein complexes but may be more difficult to see in 2-D SDS gels of highly complex protein mixtures like in case of the two hitherto most ambitious proteomics studies employing BN-PAGE [59] and CN-PAGE [123] in the 1-D, which identified 361 proteins of the methanogenic archaeon *Methanothermobacter thermautotrophicus* corresponding to ~20% of the predicted proteome [59], and 241 stroma proteins of *Arabidopsis* encompassing at least an expression range of five orders of magnitude, respectively [123] (Table 2).

Another useful procedure, especially for the analysis of intricate supercomplexes with dozens of subunits, is the combination with a BN-PAGE in the 2-D running with low concentrations of mild detergents added to the cathode buffer. This achieves a less rigorous dissociation than induced by SDS, leading to the separation of intact protein complexes and/or specific subcomplexes in the 2-D BN gel. This type of 2-D gel was first employed by Schägger and Pfeiffer [145] to dissociate digitonin-solubilized bovine heart respiratory supercomplexes $I_1III_2IV_{0-4}$ into their individual complexes I, $III_2$ and IV, as well as ATP

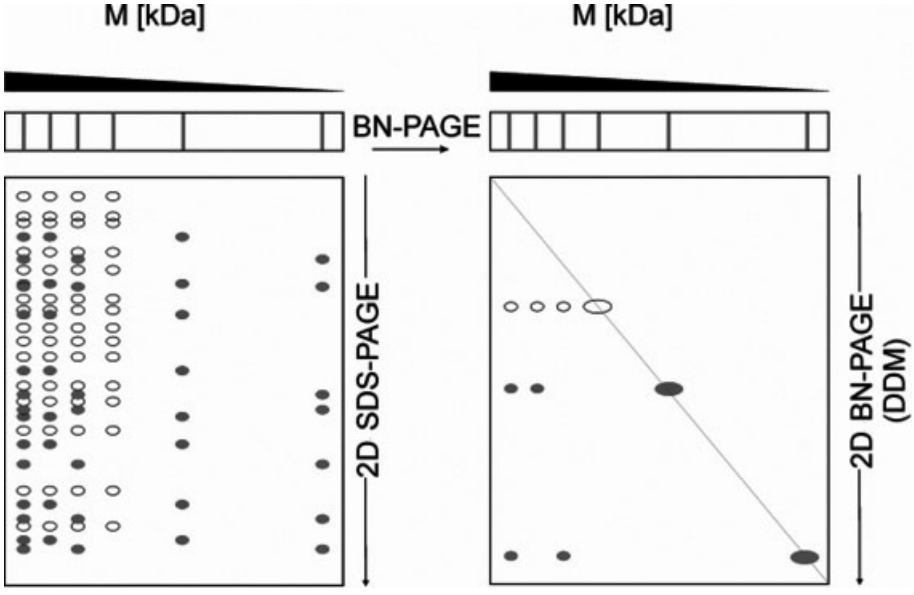


**Figure 2.** BN-PAGE in the 1-D which separates protein complexes according to their molar mass combined with (left) 2-D SDS-PAGE to analyze the subunit composition of protein complexes or (right) 2-D BN-PAGE with mild detergents added to the cathode buffer to analyse subcomplexes. Protein complexes with the same mobility in both dimensions migrate on a diagonal (right). In case of OXPHOS supercomplexes, the addition of 0.02% DDM to the cathode buffer leads to dissociation into the individual complexes (right).

 

synthase dimers into monomers by application of 0.02% DDM to the cathode buffer. Protein species with the same mobility as in the 1-D, *e.g.* individual respiratory complexes, migrate on a diagonal. Notably, 0.03% Triton X-100 in the cathode buffer instead of DDM resulted in a more gentle dissociation preserving some amounts of the small supercomplex $I_1IV_1$ which originated from the most abundant supercomplex $I_1III_2IV_1$ in the 1-D gel. This indicated a direct complex I-IV interaction in the supercomplex $I_1III_2IV_1$ which could be strongly supported by Schäfer *et al.* [159] in a single particle structure of the bovine heart supercomplex $I_1III_2IV_1$ electroeluted from 1-D BN-PAGE. Recently, using 2-D BN/BN-PAGE the membrane- and matrix-protruding subcomplexes of plant complex I were detected in low yields by partial dissociation of complex I, which facilitated the identification of the novel carbonic anhydrase subunits [213, 214] as constituents of the membrane subcomplex [196].

Moreover, combined with the information about the apparent masses of separated protein complexes in the 1-D gel, the stoichiometry of protein subunits can be assessed by means of densitometric staining indices in 2-D gels. The most elaborate example is the densitometric determination of the composition of the digitonin-solubilized respiratory supercomplexes $I_1III_2IV_{0-3}$ from bovine heart employing even 3-D BN/BN/SDS-PAGE of single supercomplexes with calibration by known amounts of chromatographically purified individual complexes I, $III_2$ and IV [145]. In conclusion, this illustrates the potential to accurately analyse the subunit composition of protein complexes, direct binding partners within a complex, and the location of subunits based on native gel approaches.

Besides conventional staining procedures, fluorescent CyDyes, which covalently bind to lysine residues by incubation of native membranes, were used for sensitive visualisation of thylakoid proteins [215] and differential in-gel electrophoresis (DIGE) of mitochondrial protein complexes [214] in 2-D-BN/SDS gels. Similarly, (4-iodobutyl)-triphenylphosphonium (IBTP) was used to derivatise accessible cysteine residues in rat liver mitochondria showing in 2-D-BN/SDS-PAGE of DDM extracts that thiols are exposed on the matrix faces of respiratory complexes I, II and IV [216]. It is important to monitor any effect of artificially induced covalent modifications on protein–protein interactions by control experiments.

### 4.4 Migration shift analysis in native PAGE

There are several options to scrutinise whether apparently comigrating proteins are actually part of a complex, the more so in case of complicated protein patterns. The most simple one to exclude coincidental comigration is to run gels with different polyacrylamide gradients which may increase the resolution of particular mass ranges and subtly alter the mobilities of proteins not forming a common complex as described for example by Farhoud *et al.* [59].

Significantly, there are important biochemical procedures which shift the mobilities of proteins. First, proteins not being part of the same complex may be distinctly influenced by the sample preparation like testing of several solubilization conditions, *e.g.* by differentially affecting two comigrating protein complexes leading to dissociation (and lower apparent mass in the 1-D native gel) of one and concomitantly retaining the other. This was shown in the case of two protein complexes from outer membrane proteins of the cyanobacterium *Anabaena sp.* comigrating after DDM-solubilization, but disrupting one by solubilization with either OG or FOS-choline [217].

Second, the application of suitable antibodies to the starting material before solubilization or directly to the protein mixture (detergent-extract) efficiently shifts the target protein to higher apparent masses or may even prevent the entering into the gel by the large extra mass of one or more bound antibody molecules. True complex components are coshifted whereas the mobilities of proteins not interacting with the antibody-recognising protein remain unaltered. This technique was first employed by Truscott *et al.* [218] to demonstrate that authentic ADP/ATP carrier (AAC) imported into yeast mitochondria is stably associated with the general import pore (GIP) complex in a specific mutant with impaired function of Tim10 and strongly affected import of AAC (see also Section 5.1). Antibody-shift BN-PAGE was particularly successful in detecting low-abundant protein complexes like mitochondrial translocation intermediates (*e.g.* [219–226]), which often are detectable only by use of radioactively labelled proteins (see Section 4.5), and was utilized to confirm the composition of other protein complexes [227–229].

Third, genetic approaches in tractable biological systems like yeast and cyanobacteria can provide clear demonstration of heterooligomeric protein complexes by analysing the deletion mutants each lacking one of the candidate genes. In case of complex formation, the lack of each *bona fide* subunit must lead either to disappearance of physically interacting proteins, *e.g.* by high turnover, or to migration shift of them, most likely to lower masses since subcomplexes or nonassembled proteins can accumulate. For example, disruption of either the two subunits of the yeast mitochondrial prohibitin complex (~1000 kDa), which is highly conserved in all eukaryotes, resulted in the absence of the complex [230], indicating the physical interaction of the two proteins. Likewise, the

 

assembly/stability of large multiprotein assemblies and accumulation of subcomplexes was tested by BN-PAGE analysis in various systems, *e.g.* of complex I in mitochondria from human patients with specific gene defects [231] and from some of the *Neurospora crassa* mutants each lacking a gene of a complex I subunit [232, 233]. Comparable studies revealed subcomplexes of OXPHOS complexes III–V including those from patients and mutants with defects in assembly factors (*e.g.* [234–239]). In some cases, the formation of aberrant aggregates *in vivo* from remnant subunits of protein complexes may be also conceivable.

Importantly, phenotypes of mutants with deleted or site-specifically mutated genes can offer direct hints to specific functions of the affected proteins, and likewise, genetic interactions recognisable by the phenotypes of mutants with different combinations of several mutated genes suggest physical interactions of the respective proteins.

### 4.5 Analysis of different assembly stages in native PAGE using radiolabelled proteins

The radiolabelling of proteins facilitates a very sensitive detection of proteins separated in gels by autoradiography which has been vastly used in combination with BN-PAGE to monitor different assembly steps of protein complexes like the time-dependent incorporation of newly synthesized proteins into the OXPHOS complexes [240–242]. Specifically, the assembly process of mitochondrial ATP synthase [241] and complex IV (cytochrome oxidase) [242] *via* distinct subcomplexes could be visualized by metabolic labelling with [$^{35}S$]methionine of cultured human cells in pulse-chase experiments with 2-D BN/SDS-PAGE. Likewise, the intermediate assembly stages of photosystem II (PSII) in cyanobacteria [40, 243–250], *C. reinhardtii* [215] as well as *Arabidopsis* and spinach leaves [251–253] were investigated in this manner after *in vivo* radiolabelling experiments, which provides superior resolution than fractionation of protein complexes with the more circumstantial density-gradient centrifugation [254].

Most often employed has been the *in vitro* synthesis of radiolabelled proteins to perform in organello assembly assays with BN-PAGE. This became an essential approach to elucidate the import of proteins into mitochondria (see Section 5.1) by detection of radiolabelled, imported precursor proteins like those of carrier protein monomers and subunits of the translocases of inner and outer membrane, respectively, and even enabled the identification of intermediates during the import and assembly pathways into oligomers or multiprotein complexes (*e.g.* [138–142, 218, 220–222, 255–262]). Notably, the *in vitro* studies of imported precursor proteins allowed the modulation of the applied conditions like ionic strength, temperature and membrane potential, leading for example to arrested stages of the import process, to analyse protein complex formation in a time-dependent manner. Similarly, the intermediate interaction of *in vitro* imported radiolabelled twin-arginine-containing precursor proteins with the receptor complex (cpTatC–Hcf106 complex) of the thylakoid membrane [263, 264] as well as the assembly of the cpTatC–Hcf106 complex itself was shown by BN-PAGE utilizing radiolabelled subunits [265].

## 5 Analysis of protein complexes in organelles and prokaryotes

### 5.1 The protein import machinery of mitochondria

BN-PAGE is inextricably connected with the ongoing elucidation of the post-translational import process of nuclearly encoded proteins into mitochondria and related pathways like the assembly of outer membrane proteins [266–268]. Indeed, the predominant share of BN-PAGE papers published up to date in the highest-ranking journals like *Nature*, *Science* and *Cell* deals with this issue (*e.g.* [220, 221, 224, 259, 269–273]). Furthermore, dozens of other excellent reports employing BN-PAGE are dedicated to it (*e.g.* [218, 219, 222, 223, 225, 226, 255–258, 260–262]).

Significantly, digitonin became the standard detergent to solubilise protein complexes of the mitochondrial protein import machinery from isolated mitochondria and/or mitochondrial outer membranes, after it was observed that digitonin treatment of *N. crassa* mitochondria enabled the extraction of the intact translocase of the outer membrane of mitochondria (TOM), whereas Triton X-100 induced the dissociation of some loosely attached subunits [274]. Later, digitonin was proved to efficiently solubilise translocase of the inner membrane of mitochondria (TIM) and TOM from yeast mitochondria, but to be more gentle than Triton X-100, DDM or OG [275–278]. Already in the mid-1990s, BN-PAGE was adopted to separate two digitonin-solubilized yeast TOM complexes [279] and soon emerged as a prime tool to analyze mitochondrial protein complexes involved in protein import in yeast (*e.g.* [255]), *N. crassa* (*e.g.* [280–283]), plants [160, 284, 285] and mammals (*e.g.* [286–288]). Even distinct protein complexes of imported proteins as intermediates at different kinetic stages like the TOM-TIM-preprotein supercomplex [255, 289], which are transient *in vivo*,

 

could be detected by BN-PAGE in combination with biochemical and genetical manipulation of the import processes (see Sections 4.4 and 4.5). Such experiments significantly contributed to deduce consecutive events of protein translocation and subsequent protein complex formation in mitochondria.

The translocons of the inner envelope membrane (Tic) [161, 290] and of the outer envelope membrane (Toc) of pea chloroplasts [291] were analyzed by BN-PAGE, too, after solubilization with decylmaltoside (DM) [161, 290] and digitonin or Triton X-100 [291]. Furthermore, protein complexes of the peroxisomal protein import machinery were separated by BN-PAGE of digitonin-solubilized rat liver peroxisomes [229, 292].

## 5.2 OXPHOS supercomplexes in mitochondria

It is a well-described result that digitonin-treatment of isolated mitochondria or outer mitochondrial membranes is able to efficiently solubilize intact protein assemblies such as the TOM and TIM complexes which are in many cases more susceptible against other detergents like DDM or Triton X-100 leading to dissociation of weak protein–protein interactions (see Section 5.1). Thus, it was obvious to extend the analysis of digitonin-solubilized mitochondrial protein complexes, in particular to examine the five OXPHOS complexes located in the inner membrane [5, 293]. In 1997, essentially all of the digitonin-solubilized yeast ATP synthase was found to elute in gel filtration analysis with an apparent mass $>$850 kDa suggesting a dimeric state [294]. Later, digitonin extracts of yeast mitochondria were examined by BN-PAGE recovering about half of the nearly quantitatively extracted ATP synthase as dimers (apparent mass $\sim$1000 kDa) [144]. In the same study, similar results were obtained after solubilization with Triton X-100 at low detergent/protein ratios. Notably, three specific $F_O$-subunits only occurring in the yeast ATP synthase dimer were detected, whose absence in mitochondria of deletion mutants had more or less profound effects on the assembly/stability of the dimers diminishing the yield after detergent treatment [144]. Even higher ATP synthase oligomers after solubilization of yeast mitochondria with low digitonin/protein ratios $\leq$ 2 g/g remained intact during BN-PAGE analysis [295]. Significant progress has been achieved to elucidate the molecular basis of the ATP synthase dimerisation/oligomerisation in yeast by various biochemical and genetical approaches, *e.g.* investigation of mutants with deletion of entire subunits or parts of them (truncated subunits), and subsequent analysis of mitochondrial digitonin extracts by BN-PAGE [107, 144, 239, 295–304] and CN-PAGE [107, 113–121] monitoring the oligomeric state of ATP synthases. Herewith, the subunits involved in direct interactions between ATP synthases, which are located in the membrane-embedded $F_O$-subcomplex, and even their critical amino acid sequences could be specified. Concomitantly, besides ATP synthase oligomers high yields of stoichiometric supercomplexes ($III_2IV_1$ and $III_2IV_2$) of the yeast respiratory complexes III and IV were separated and identified from digitonin extracts applied to BN-PAGE [107, 145, 149, 174, 305–308] and CN-PAGE [107, 111]. Likewise, the same approach resulted in the detection of ATP synthase dimers and specific supercomplexes of complexes I, III and IV preserved by digitonin solubilization of mitochondria from bovine heart [60, 110, 145, 146, 159] and other mammalian tissues [104, 110, 112, 156, 235, 309, 310], the filamentous fungus *Podospora anserina* [108], and higher plants [109, 147, 148, 311]. Analysis by the more gentle CN-PAGE was reported to improve the yields of preserved OXPHOS supercomplexes from fungi and mammals [107, 108, 110, 112, 120], but not from higher plants [109].

Strikingly, the preparation of the mitochondrial starting material appears to influence the proportion of OXPHOS supercomplexes retained after extraction with digitonin and analysis by BN- and CN-PAGE. In detail, Krause *et al.* [110] investigated digitonin-solubilized mitochondria, which were isolated from fresh bovine heart, and detected by BN-PAGE significantly higher amounts of respiratory supercomplexes $I_1III_2IV_{0-4}$ (1500–2300 kDa) and ATP synthase dimers ($V_2$) than reported before [145, 146]. In addition, even supercomplexes $I_xIII_yIV_z$ with higher apparent masses than that of $I_1III_2IV_4$ (2300 kDa) as well as higher oligomers of ATP synthase ($V_3$ and $V_4$) were found (Fig. 3A) [110]. The application to CN-PAGE retained nearly all of the complexes I, III and IV as supercomplexes and $\sim$80% of total ATP synthase as dimers and higher oligomers (Fig. 3B) [110]. A similar pattern of OXPHOS supercomplexes, but in contrast to [110] a significant proportion of individual complexes III and IV not assembled into supercomplexes, was obtained by Wittig and Schägger [112] who analysed density-gradient purified rat heart mitochondria by CN-PAGE. In the recent study of Hunzinger *et al.* [60], the BN-PAGE data of [110] were confirmed, analysing mitochondria solubilized with digitonin in imidazole-buffered 50 mM NaCl essentially like in [112] and isolated from tissue, which was derived from another bovine heart than that used by Krause *et al.* [110] and stored for 6 days at $-$80°C after slaughtering. At least in case of the bovine heart used as source for mitochondria by Krause *et al.* [110], an extended storage time (25 months) of the tissue aliquot before processing significantly decreased the yields of digitonin-solubilized OXPHOS supercomplexes obtained by BN-PAGE (unpublished observation) which are very similar to the

 

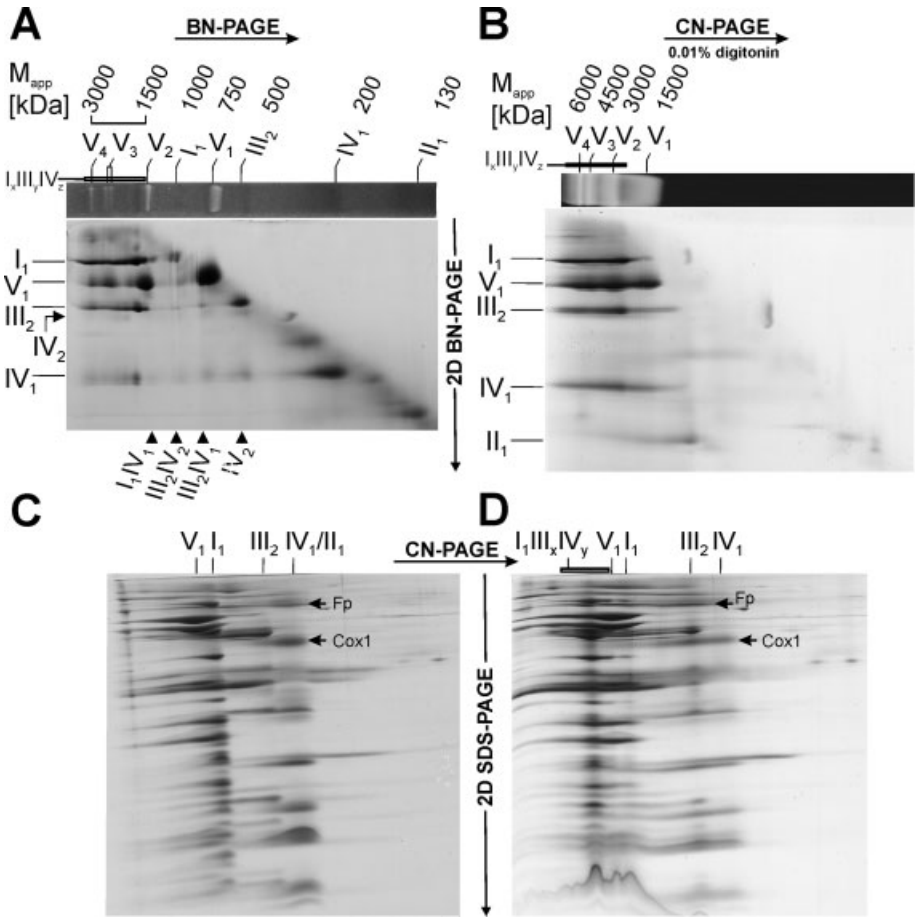


**Figure 3.** Respiratory supercomplexes and ATP synthase oligomers from bovine heart mitochondria isolated from fresh tissue (A and B), or from tissue of the same heart stored for 25 months at −80°C (C and D) analyzed in the 1-D by (A) BN-PAGE (4–13%), (B) CN-PAGE (3–16%) with 0.01% digitonin present in the gel or (C and D) CN-PAGE (3–16%). 2-D gels are CBB-stained. (A and B) Solubilization with digitonin/protein = 4 g/g, (C) solubilization with DM/protein = 1.2 g/g, (D) solubilization with digitonin/protein = 3 g/g, each in 150 mM potassium acetate, 30 mM HEPES-NaOH (pH 7.4), 10% glycerol, 0.5 mM Pefabloc SC®. (A and B) Separation of monomeric (V1) and oligomeric ($V_{2-4}$) ATP synthases all displaying ATP hydrolase activity (upper panels). 2-D BN-PAGE with 0.02% DDM in the cathode buffer to dissociate OXPHOS supercomplexes into their individual complexes (lower panels). (C and D) 2-D SDS-PAGE to resolve the subunits of the OXPHOS complexes and their supercomplexes. Besides the ATP synthases ($V_{1-4}$) the individual respiratory complexes I–IV as well as the respiratory supercomplexes $I_xIII_yIV_z$, $I_1III_xIV_y$, $I_1IV_1$, $III_2IV_2$, $III_2IV_1$ and $IV_2$ are indicated. Flavoprotein (Fp) of complex II and the Cox1 subunit of complex IV are marked by arrows. Note that digitonin-solubilized complex II migrates in CN-PAGE (B and D) as an extended band, whereas most of DM-solubilized complex II comigrates with individual complex IV (C). (B) Note that the apparent molar masses of ATP synthases ($V_{1-4}$) approximately correspond to the two-fold of the respective molar masses obtained under BN-PAGE conditions. A and B slightly modified from [110], with permission.

results of Schägger and Pfeiffer [145, 146]. Notably, the CN-PAGE analysis of this digitonin extract reveals a distribution of individual OXPHOS complexes and supercomplexes comparable to that under BN-PAGE conditions (Fig. 3D) in contrast to the results obtained with mitochondria from fresh bovine heart [60, 110], even though no detergents were added to the CN gel representing the mildest variant. Moreover, DM extracts of the same mitochondria analyzed under identical conditions resulted exclusively in separated individual OXPHOS complexes (Fig. 3C) in agreement with the results of DDM extracts from Schägger *et al.* [48]. This is an important outcome, since all these data imply that CN-PAGE (and likewise BN-PAGE) does not trigger any significant aggregation of digitonin-solubilized OXPHOS complexes even without detergents present in the gel. In fact, although the precise reason is not known, it has to be expected that the quality/integrity of mitochondria rather declines than improves by increasing storage times of the source tissue, thus seemingly affecting susceptible protein–protein interactions during subsequent detergent treatment and reducing the proportion of detergent-stable OXPHOS supercomplexes. Taken together, it is suggested that digitonin treatment of bovine heart mitochondria does not induce the artificial gluing of incompletely solubilized OXPHOS complexes as sometimes assumed, but that detergent-extracted supercomplexes reflect rather the *in vivo* situation.

 

In line with this, the electrophoretic results of digitonin-solubilized OXPHOS supercomplexes are now corroborated by single particle structures of the plant supercomplex $I_1III_2$ [312], the ATP synthase dimers from bovine heart [313] and the colorless alga *Polytomella* [314], which were fractionated by sucrose-density-gradient centrifugation, as well as of the enzymatically active bovine heart supercomplexes $I_1III_2$ and $I_1III_2IV_1$ purified by BN-PAGE and electroelution [159].

The high performance of digitonin to maintain rather weak protein–protein interactions was also exploited to investigate the non-OXPHOS proteins of mitochondrial fractions from five rat organs separated by 2-D BN/SDS-PAGE leading to the mass spectrometric identification of 92 non-OXPHOS proteins mostly being constituents of known protein complexes [156] (Table 2). Hitherto, comparable studies examined DDM-solubilized mitochondria from rat heart and liver [315], human heart [98] and plants [73, 316, 317]. Beyond the digitonin-solubilized OXPHOS supercomplexes and other complexes described in Section 5.1, specific protein complexes from mitochondrial digitonin extracts of yeast [58, 138–142, 174, 318–321], mammals [57, 138, 142, 322, 323], fishes [143], plants [324] and trypanosomes [325] were analyzed by BN-PAGE.

### 5.3 Photophosphorylation complexes and supercomplexes in chloroplasts and cyanobacteria

The first use of 2-D BN/SDS-PAGE to investigate the thylakoid membrane of spinach and tobacco chloroplasts adopted the original solubilization protocol for mitochondria [47, 48] with the detergents DDM and Triton X-100 which allowed the analysis of the subunit composition of all protein complexes involved in photophosphorylation (photosystem I (PSI) and PSII, $b_6f$, $CF_OF_1$-ATP synthase, light-harvesting complexes LHC and subcomplexes [6, 326]) and the soluble ribulose bisphosphate carboxylase/oxygenase (RubisCO) from the stroma in a single gel [327]. Afterwards, BN-PAGE was employed in numerous studies to separate the thylakoid protein complexes from isolated chloroplasts or thylakoids of higher plants (*e.g.* [62, 109, 200–205, 251–253, 263–265, 328–340]) and the alga *C. reinhardtii* [62, 124, 129, 215, 341] as well as from cyanobacteria [40, 62, 206, 243–250, 342–346], which employed solubilization with DDM [40, 200–206, 215, 243–253, 328–335, 340–346], DM [62, 109], *n*-dodecanoylsucrose [109] and digitonin [109, 124, 129, 263–265, 336–339]. Importantly, besides the individual complexes various photophosphorylation supercomplexes involving the PSI and PSII as well as light-harvesting complex I (LHCI) and light-harvesting complex II (LHCII), respectively, could be detected in detergent-extracts of higher plants, *C. reinhardtii*, and cyanobacteria (*e.g.* [109, 124, 129, 329, 330, 333, 339, 340]), which are predominantly in agreement with structural data obtained by electron microscopy (EM) studies [326]. However, the separation of a small proportion of plant PSI as apparent oligomers in BN-PAGE of digitonin extracts [109, 339] was suggested to represent artifacts according to EM studies [347].

Notably, digitonin fragmentation of thylakoids was an essential tool in the pioneering work to unravel the photophosphorylation apparatus in higher plant chloroplasts (*e.g.* [348–350]). Its property to differentially solubilise the thylakoid proteins, in particular the far better extraction of PSI than of PSII [349], contributed as a tool to discover the segregation of photophosphorylation complexes in the stroma thylakoids (PSI, $CF_OF_1$-ATP synthase, $b_6f$) and the tightly appressed grana thylakoids (PSII, LHCII, $b_6f$) of higher plants [350, 351]. The merely partial solubilization of higher plant thylakoids with digitonin is easily recognisable, since the extracted proteins display not much green colour due to protein-bound chlorophylls in contrast to the remaining green pellets indicating substantial amounts of detergent-resistant membranes with chlorophyll-containing proteins (*e.g.* [109, 263, 349]). These digitonin-resistant membranes are mainly grana thylakoids in which most of the PSII is located. As an explanation, the bulky, rigid structure of the steroid core of digitonin molecules was proposed to be unable to efficiently interact with the densely appressed grana membranes in contrast to other detergents like Triton X-100 or *p*-nonylphenoxy-polyethoxyethanol (Nonidet P-40) which extract significantly more thylakoid proteins [349]. In line with this, stroma and grana thylakoids can be efficiently separated for subsequent analyses by treatment with low concentrations of digitonin [352–354]. Nonetheless, photophosphorylation complexes and supercomplexes like PSII dimers associated with LHCII can be extracted with digitonin and detected in 2-D BN/SDS- and BN/BN-PAGE [339], but obviously not in quantitative yield as suggested by the authors who provided no precise data to support this claim [339], the more so as parallel BN gels with DDM extracts shown by Heinemeyer *et al.* [339] reveal far higher amounts of solubilized thylakoid membrane proteins. Most probably, the rather low amounts of digitonin-solubilized PSII supercomplexes [339] predominantly originate from the small proportion of PSII species located in the grana margins and/or the stroma thylakoids [355]. In line with this interpretation, the very recent survey of Danielsson *et al.* [340] reporting BN-PAGE analysis of DDM extracts from mechanically separated thylakoid membrane domains of spinach demonstrated that a small propor-

 

tion of total DDM-stable PSII-LHCII supercomplexes is located in the grana margins. It has to be anticipated that BN- and CN-PAGE studies employing digitonin and more efficient detergents like β-D-decylmaltoside (DM) [109], DDM (*e.g.* [334, 340]) and *n*-dodecanoylsucrose [109] appear to be highly suitable to analyze the physiologically relevant redistribution of protein complexes between the distinct thylakoid membrane areas (*e.g.* [352]) and/or conceivable alterations of the membrane architecture in higher plants as well as possible changes of the supramolecular organization of thylakoid membrane proteins under different growth conditions. Similarly, BN- and CN-PAGE analysis of digitonin extracts from mitochondria contaminated with chloroplasts of spinach green leaf facilitated the separation and identification of complex-IV-containing respiratory supercomplexes without major interference by thylakoid protein complexes, because only low amounts of stroma thylakoid membrane proteins were extracted [109].

In contrast to the situation in higher plants, the thylakoids of some green algae like *C. reinhardtii* as well as cyanobacterial membranes have no tightly stacked membrane areas [351]. Indeed, the digitonin treatment of thylakoids from *C. reinhardtii* proved to be efficient in analyzing the entire photophosphorylation system in quantitative yield by Rexroth *et al.* [124, 129]. Strikingly, this solubilization protocol led to the separation of afore unknown dimeric $CF_OF_1$-ATP synthases [124] in both BN- and CN-PAGE, but only after a more gentle isolation procedure of the thylakoids than employed before [129] suggesting that the thylakoids have suffered some subtle damage in the earlier study [129] leading to a reduced stability of these homo-supercomplexes. Likewise, the authors demonstrated by elegant BN-PAGE experiments analysing digitonin extracts with varying amounts of specific anions present during solubilization that the ratio of chloroplast ATP synthase dimers to monomers is strongly dependent on the concentration of phosphate or vanadate in the physiologically significant range up to 10 mM but not on the ionic strength of these anions *per se* [124]. A corresponding effect was also observed by inclusion of vanadate during isolation of thylakoids leading to significantly higher yields of $CF_OF_1$-ATP synthase monomers at the expense of the dimers. These results implied that $CF_OF_1$-ATP synthase dimerisation occurs under conditions with low phosphate [124]. In contrast, the mitochondrial ATP synthase as contaminant, and thus serving as an internal standard, was found under all conditions exclusively as dimer pointing to another mode of interaction between the two monomers [124]. In fact, the mitochondrial ATP synthase from *C. reinhardtii* and *Polytomella* was shown before in BN-PAGE to be exclusively retained in the dimeric state even with DDM solubilization [79–82].

Likewise, the digitonin treatment of cyanobacterial membranes should lead to very efficient extraction of photophosphorylation complexes and other membrane proteins and provide valuable results in combination with BN(CN)-PAGE analysis.

### 5.4 Protein complexes in endoplasmic reticulum (ER)

The steroid detergent digitonin played also an important role in ER research. It was used to solubilize microsomes preserving stable ribosome-translocon interactions [356–358] and to purify intact oligosaccharyltransferase (OST) complex [359–361], while other detergents such as Triton X-100 and diheptanoyl-*sn*-phosphatidylcholine disrupt the ribosome-translocon junction [358, 362, 363]. Moreover, digitonin-solubilized membrane protein complexes like the Sec61 complex could be reconstituted in a functional form into liposomes [364]. Using BN-PAGE, Wang and Dobberstein [362] analysed four different fractions of digitonin-solubilized rough microsomes from dog pancreas and could separate four ribosome-associated protein complexes including OST, translocating chain-associated membrane protein (TRAP), and Sec61. Other protein complexes characterised by BN-PAGE are the TRAP complex, the small ribosome-associated membrane protein (RAMP4), the signal recognition particle (SRP), and the SRP receptor (SR) [362]. However, the Sec61 complex was found dissociated in case of solubilization with Triton X-100. Similarly, digitonin-solubilized RAMP4 was partially detected as an oligomeric species, while RAMP4 solubilized with Triton X-100 migrated solely as monomer [362]. Under the BN-PAGE conditions used by the authors, SRP54 partially dissociated from the SRP [362]. In a recent proteomics approach by 2-D BN/SDS-PAGE, 32 distinct proteins from purified ribosome-associated membranes of dog pancreas solubilized with digitonin were identified mostly as part of six complexes [363] (Table 2). Notably, besides individual OST (~500 kDa) two stable supercomplexes of OST (~600 and ~700 kDa) were selectively released from ribosomes and separated. Both OST supercomplexes contained the heterotrimeric Sec61 complex and the larger one the heterotetrameric TRAP in addition as deduced from the combined subunit patterns [363]. Two distinct OST complexes [365, 366] were also demonstrated by BN-PAGE analysing yeast microsomes either solubilized with digitonin [365] or octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$/Nikkol) [366, 367], which differ only by a single subunit, *i.e.*, the presence of the Ost3 or the Ost6 subunit instead. These two subunits are dispensable for enzymatic activity [365, 366] but modulate the affinity towards different protein substrates [365]. Importantly, the digitonin-solubilized

 

yeast OST complexes [365] have essentially the same apparent mass like the digitonin-solubilized mammalian OST complex (~500 kDa) [362, 363] but the two-fold apparent mass of the $C_{12}E_8$-solubilized ones in BN-PAGE [366, 367], which might indicate a dimeric state preserved by digitonin but not by $C_{12}E_8$ [365]. Another study using a membrane-based yeast two-hybrid system also found that Ost3 and Ost6 are part of two different complexes strongly supporting the BN-PAGE data [368].

Furthermore, BN-PAGE of digitonin-solubilized yeast microsomes separated glycosylphosphatidylinositol (GPI) transamidase complexes in the range of ~430–650 kDa which did not interact with Sec61 and OST complexes required for ER insertion and *N*-glycosylation of GPI proteins, respectively [369, 370].

The BN-PAGE of DDM-solubilized ER membranes of the murine cell line WEH17.2 demonstrated that the anti-apoptotic protein Bcl-2, colocalized in mitochondria and ER, is nearly completely associated with the inositol 1,4,5-triphosphate ($InsP_3$) receptor, functioning as ligand-gated calcium channel on the ER, to form a high-molecular-weight complex [371]. These data were corroborated by coimmunoprecipitation and by functional studies demonstrating that Bcl-2 significantly inhibited $InsP_3$-mediated calcium release into the cytosol [371].

There are also BN-PAGE reports on human platelet microsomal protein complexes [155] (Table 2) and on plant ER protein complexes solubilized with OG [157, 158] or DDM [372], respectively.

### 5.5 Protein complexes in prokaryotes

Protein complexes of various prokaryotes have been investigated by BN-PAGE. The corresponding studies of protein complexes from photosynthetically active bacteria [40, 62, 206, 217, 243–250, 342–346] were described above. Interestingly, the hitherto most successful proteomics approach based on 2-D BN/SDS-PAGE in terms of identified proteins investigated DDM- and digitonin-solubilized membrane proteins as well as soluble proteins from the methanogenic archaeon *M. thermautotrophicus* [59] (see Sections 4.3 and 4.4 and Table 2), of which many were detected as homo- or heterooligomeric complexes. Notably, the solubilization with digitonin retained protein supercomplexes which were disrupted into the individual complexes after DDM solubilization [59]. Likewise, digitonin solubilization of membranes from the Gram-negative soil bacterium *Paracoccus denitrificans* facilitated the separation of specific respiratory supercomplexes of complexes I, III and IV by BN-PAGE [373] which have another stoichiometry as the homologous mitochondrial respiratory supercomplexes (see Section 5.2). In the case of *Escherichia coli*, DDM-solubilized outer and inner membranes were analyzed by 2-D BN/SDS-PAGE leading to the separation of many protein complexes while digitonin solubilization was reported to result in less efficient membrane protein extraction [137]. Nevertheless, digitonin solubilization of *E. coli* membranes was successful in separating distinct twin-arginine translocation complexes by BN-PAGE [374, 375]. Furthermore, the DDM-solubilized respiratory complex I of *E. coli* [376, 377] as well as various protein complexes of *Agrobacterium* strains solubilized with DDM [378, 379] or digitonin [380] were characterised after BN-PAGE separation. The subunit composition of the $Na^+$-$F_OF_1$-ATP Synthase (~590 kDa) of *Acetobacterium woodii* was determined with 2-D BN/SDS-PAGE after solubilization with various detergents leading to consistent results [381].

In another proteome-wide approach, 2-D BN/SDS-PAGE was used to separate protein complexes from sucrose-gradient fractionated cell extracts of the phenol-degrading *Pseudomonas sp.* strain phDV1 grown either on glucose or phenol as sole carbon source, which facilitated the detection and identification of soluble protein complexes involved in phenol degradation [382] (Table 2).

## 6 Limitations and conclusions

The use of BN-PAGE as a separation tool has been tremendously successful in the last 10 years or so in detecting and analysing soluble and membrane protein complexes from all kind of biological starting material. Of special value for functional proteomics is the robust performance of BN-PAGE which can be done in every laboratory without the need of very expensive and highly sophisticated equipment. Besides the versatility of the employed method, the reliability and accuracy of identifying protein–protein interactions are essential requirements. It appears that the detection of false-positives (artificial, nonphysiological protein complexes) is a pitfall which occurs less frequently than the outcome that true protein–protein interactions escape detection because of detergent-solubilization and BN-PAGE conditions, notably the presence of anionic Coomassie dye, which may induce the disruption of weak interactions. Indeed, there is only one report [347] providing evidence by alternative approaches that BN-PAGE might produce false-positives, namely the small amounts of higher plant PSI oligomers obtained from digitonin-solubilized chloroplasts [109, 339] as described in Section 5.3. Nonetheless, the possibility of detecting artificially aggregated protein complexes has to be taken into account. On the other hand, the detection of very weak interactions like those of

 

transient protein complexes by BN-PAGE and the milder but less versatile CN-PAGE is probably not possible in many cases. Thus, the proper application of BN-PAGE as a rather unbiased method in terms of amenable proteins can provide superior results in combination with parallel approaches based on other principles which corroborate the detection of protein–protein interactions.

The sample preparation, *i.e.* the appropriate solubilization conditions and even the origin and isolation procedure of the starting material, is without doubt the essential bottleneck to get optimal results during subsequent biochemical analysis. Importantly, as summarised in Table 1, essentially all gentle detergents appear to be compatible with BN-PAGE. This allows a thorough screening of various detergents to test the efficiency of membrane protein extraction and the preservation of certain protein complexes. In fact, the survey of protein–protein interactions using different conditions (*e.g.* various detergents and parrallel analysis with the milder CN-PAGE) provides a better evaluation of their physiological relevance. In addition, such different sample preparation and electrophoresis conditions may provide the detection of more protein complexes and individual proteins than only one protocol may offer. For example, the differential extractability/stability of membrane protein complexes by solubilization with different detergents (*e.g.* [109, 217]) (see also Sections 4.4 and 5.1–5.5) as well as different mobilities of some protein species in BN- and CN-PAGE (see also Section 4.1) may lead to identification of certain protein complexes which comigrate with other protein species or do not enter the gel in case of other conditions. This strategy increases also the chance to detect less abundant protein complexes.

Taken together, in line with the discussion throughout this review, soluble protein extracts as well as detergent-extracts of membranous samples like organelles should always be analyzed by BN-PAGE and CN-PAGE experiments in parallel, the more so since CN-PAGE is likewise easy to perform. This author affirms the recommendation of Schägger [50, 51] to test always the run of detergent-extracts without addition of large extra amounts of CBB dye before BN-PAGE. Similarly, mixtures of soluble proteins as well as prepurified membrane proteins containing only low amounts of lipid-detergent micelles should be analysed with diluted cathode buffer (*e.g.* 0.002% Coomassie G-250). In the future, the analysis of posttranslational modifications like protein phosphorylation of protein species separated by BN- and CN-PAGE [*e.g.* 243, 383–395] is expected to significantly increase.

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