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REVIEW

Native electrophoretic techniques to identify protein–protein interactions

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Permanent protein–protein interactions are commonly identified by co-purification of two or more protein components using techniques like co-immunoprecipitation, tandem affinity purification and native electrophoresis. Here we focus on blue-native electrophoresis, clear-native electrophoresis, high-resolution clear-native electrophoresis and associated techniques to identify stable membrane protein complexes and detergent-labile physiological supercomplexes. Hints for dynamic protein–protein interactions can be obtained using two-hybrid techniques but not from native electrophoresis and other protein isolation techniques except after covalent cross-linking of interacting proteins *in vivo* prior to protein separation.

Keywords:
Blue-native electrophoresis / Clear-native electrophoresis / Protein complex / Protein–protein interaction / Supercomplex / Technology

1 Introduction

Blue-native electrophoresis (BNE [1–3]), clear-native electrophoresis (CNE [2, 4]), and high-resolution CNE (hrCNE [5, 6]) are variants of the same basic technique (CNE) that differ essentially by the cathode buffers used (Table 1). They are used to isolate hydrophobic and also water-soluble protein associates, and are especially useful to isolate larger membrane protein complexes and supercomplexes, as exemplified by Fig. 1. Two extended recent reviews summarize the numerous special applications in different research fields [7, 8]. Here we focus on the history of the native electrophoretic techniques, and their usefulness to identify protein–protein interactions through isolation of moderately stable protein complexes. The ability to preserve detergent-stable and even some detergent-labile protein–protein interactions depends on the detergent and cathode buffer variant used. Even minor variation of the cathode buffer for CNE, BNE and hrCNE changes the separation principles and therefore affects resolution considerably.

CNE starts with solubilization of biological membranes by neutral detergents and removal of insoluble material by centrifugation. The supernatant at neutral pH is then directly applied to the gel and run at pH 7. Since proteins migrate according to their intrinsic p*I*, only acidic proteins can migrate to the anode in CNE. All proteins with p$I > 7$ migrate to the cathode and are lost. Another disadvantage of CNE is smearing observed with many membrane proteins. However, CNE offers special advantages for in-gel catalytic activity assays and detection of fluorescent-labeled proteins [4, 5]. CNE is milder than BNE and is currently the mildest electrophoretic technique to isolate supramolecular structures [4, 9, 10].

BNE has initially been described as a simple technique to isolate membrane protein complexes immediately from purified mitochondria [1]. Like in CNE, mild neutral detergents are used for membrane solubilization and bis-tris [1, 2] or imidazole buffers [3, 11, 12] are applied to stabilize pH to 7.0–7.5 in the native gels. The anionic dye Coomassie Brillant Blue G-250, abbreviated here as Coomassie-dye or ''the dye,'' was introduced in BNE to impose a negative charge shift on proteins upon binding to the protein surface [2]. This charge shift is especially important for hydrophobic membrane proteins, since mutual repulsion of negatively

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Abbreviations: BNE, blue-native electrophoresis; **CNE,** clear-native electrophoresis; **hrCNE,** high-resolution clear-native elelctrophoresis

Table 1. Cathode-buffers specifying various native electrophoresis systems

	CNE	BNE	hrCNE-1	hrCNE-2	hrCNE-3
Tricine (mM)	50	50	50	50	50
Imidazole (mM)	7.5	7.5	7.5	7.5	7.5
Dodecylmaltoside (%)	–	–	0.02	–	0.01
Deoxycholate (%)	–	–	0.05	0.05	0.05
Triton X-100 (%)	–	–	–	0.05	–
Coomassie dye (%)	–	0.02 (or 0.002)	–	–	–
pH[a)]	~7.0	~7.0	~7.0	~7.0	~7.0

CNE: cathode buffer for CNE does not contain detergent or dye. Proteins migrate according to their intrinsic charge in CNE. BNE: dark blue cathode buffer for BNE containing 0.02% Coomassie dye can be replaced during electrophoresis by a similar buffer with tenfold lower dye content. This buffer exchange makes migrating protein bands visible during the electrophoretic run. hrCNE1-3: mixed anionic micelles from neutral detergents (dodecylmaltoside or Triton X-100) and anionic detergent (deoxycholate) are added to the CNE buffer to induce a negative charge shift on proteins. Buffer for hrCNE-3 is mildest.
a) No pH correction except with tricine or with imidazole.

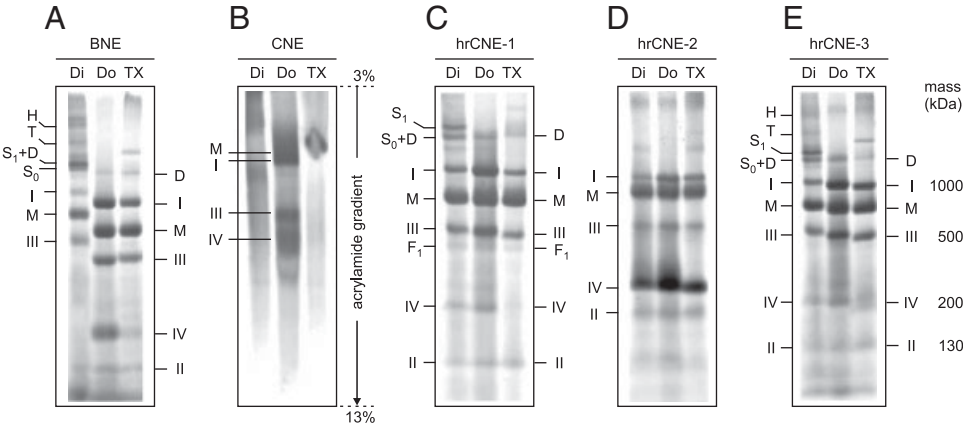

Figure 1. Comparative presentation of the native electrophoresis variants BNE (A), CNE (B), hrCNE-1 (C), hrCNE-2 (D) and hrCNE-3 (E), using bovine heart mitochondria solubilized by digitonin (Di), dodecylmaltoside (Do) and Triton-X-100 (TX). Acrylamide gradient gels (3–13% acrylamide) were used as indicated. I-IV, respiratory complexes I–IV. These complexes can also be used as native mass standards (masses indicated on the right side). M, D, T, H, monomeric, dimeric, tetrameric, hexameric forms of ATP synthase (complex V); F_1, catalytic part of ATP synthase. Using digitonin for solubilization of mitochondria (4 g/g protein), two supercomplexes (S_0 and S_1) of the respiratory chain containing complex I, complex III, and zero (0) or one (1) copy of complex IV were observed. They were optimally preserved in CN gels (B, lane Di) that, however, gave rise to extreme smearing of bands. Supercomplexes S_0 and S_1 and the oligomeric forms of ATP synthase (D, T, H) were also preserved in BN (A) and hrCNE-3 gels (E), and occasionally also after hrCNE-1 (C). Setting dodecylmaltoside/protein and Triton X-100/protein ratios to 2.4 and 3 g/g, respectively, no supercomplexes but the individual complexes were solubilized from mitochondrial membranes. BNE is the most robust and versatile technique and is therefore mostly used except for in-gel activity assays and detection of fluorescent proteins in 1-D native gels.

charged protein surfaces reduces protein aggregation considerably. Furthermore, hydrophobic proteins with bound dye obtain solubility properties comparable to water-soluble proteins. No detergent is required to keep membrane proteins solubilized when they migrate through detergent-free gels. All Coomassie-binding membrane proteins migrate to the anode in BNE independent of the protein intrinsic p*I*. The dye seems to bind also to cationic amino acid residues on the surface of water-soluble proteins, as exemplified with cytochrome *c* (p*I* 10.7). Cytochrome *c* binds the dye in excess and migrates to the anode at the running pH 7.5 of BN gels [5]. Many other water-soluble proteins with p$I > 7$ do not bind the dye. They show cathodic migration and are lost with the cathode buffer. A disadvantageous aspect of the dye is noticed in the presence of neutral detergents, as used for the initial protein solubilization: mixed anionic dye/detergent micelles are formed in this situation that can dissociate detergent-labile subunits from multiprotein complexes and may disassemble supramolecular structures. Another disadvantage of the dye is interference with fluorimetric and catalytic activity assays in BN gels, suggesting the use of clear-native gels in these cases, especially hrCNE [5, 6]. hrCNE was developed to preserve the advantages of BNE and CNE and to eliminate the disadvantages which has proven successful in some but clearly not in all aspects.

hrCNE is closer to BNE than to CNE, since hrCNE is a charge shift technique and resolution is comparable to BNE. Instead of the Coomassie-dye, non-colored mixed micelles of neutral and anionic detergents are used here to induce the charge shift on proteins. There are three variants, hrCNE1–3, differing in the composition of the mixed anionic detergent micelles (Table 1) which modulates the mildness of the electrophoretic system. All hrCNE variants offer advantages similar to CNE for in-gel fluorescent and catalytic activity assays. However, hrCNE may promote dissociation of labile subunits from protein complexes and therefore has to be considered less mild than CNE and even harsher than BNE.

Overall, BNE is the most robust variant that is preferable to hrCNE and CNE except for analyses of fluorescent-labeled proteins or in-gel catalytic activity assays. CNE, the mildest variant, is specifically useful for the analysis of physiological supramolecular structures provided the limitations in resolution are acceptable.

2 Development of techniques associated with BNE and CNE

The basic task of native electrophoresis is isolation of proteins and protein complexes on the microgram scale for a number of general purposes that were developed step by step as outlined in Sections 2.1–2.7. As surveyed recently [7], these general tools have been applied in many specific research areas, for example, to study protein import, dynamics of proteasomes, biogenesis and assembly of membrane protein complexes, and to explore mitochondrial alterations in apoptosis, carcinogenesis, neurodegenerative disorders and mitochondrial encephalomyopathies.

2.1 1-D BN and 2-D BN/SDS gels and blots, and recovery of proteins from BN gels

BNE, originally named blue-native PAGE (BN-PAGE), has been first described in 1991 as a method to isolate membrane protein complexes immediately from isolated biological membranes [1]. Also described were associated techniques like extraction of native membrane protein complexes from the gel, handling of 1-D BN gel-strips for 2-D tricine-SDS-PAGE (Fig. 2A), and electroblotting of 1-D BN and 2-D SDS gels. Native protein extracts have been used for catalytic activity measurements [1] and are currently used for advanced structural investigations (see Section 2.7). Electroblotting of 1-D BN and 2-D BN/SDS gels and immunological detection of proteins by specific antibodies became widely used techniques, especially when low abundant proteins are to be analyzed or the available protein amounts are limiting, *e.g.* when analyzing patient biopsy specimens or cultured human cells [7].

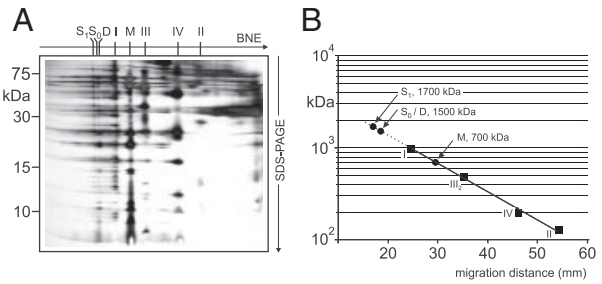

Figure 2. Use of 2-D BN/SDS-PAGE for native mass estimation of membrane protein complexes. Assignment of complexes as in Fig. 1. (A) 2-D BN/SDS-PAGE of bovine mitochondrial complexes solubilized by digitonin and separated by BNE similar to Fig. 1A, lane Di. (B) Bovine respiratory complexes I–IV were used as mass standards (black squares, masses indicated in Fig. 1, right side) and plotted *versus* their migration distances in BNE (arrow on top of Fig. 2A). Estimated masses for monomeric complex V (M), dimeric complex V (D), and supercomplexes S_0 and S_1 were 700 kDa (M), 1500 kDa (D), 1500 kDa (S_0) and 1700 kDa (S_1), respectively.

2.2 Use of BNE to estimate mass and oligomeric state of native proteins, and to isolate highly pure protein from chromatographically pre-enriched fractions

The potential of BNE to determine native molecular masses and oligomeric states of protein complexes was recognized in 1994 [2] and applied for the first time to identify chloroplast b_6f complex as a structural and functional dimer [13]. Mass determination by BNE, as exemplified by Fig. 2B, requires measurement of the migration distances of the protein(s) of interest and of some mass standards in the same BN gel. These migration distances must not be taken immediately from 1-D BN gels (Fig. 1) but can also be derived from 2-D BN/SDS gels or blots (Fig. 2A). From the vast number of applications to estimate native protein mass and oligomeric state by BNE it became apparent that mass determination, especially of smaller membrane proteins, requires additional information, especially on protein-bound Coomassie-dye, to obtain reliable data [7, 3, 14].

Using BNE for final purification of chromatographically pre-purified membrane protein complexes often dissociated subunits from complexes and caused irreversible denaturation. Presumably this effect was due to mixed anionic micelles that formed from the neutral detergent present in the isolation buffer and the added anionic dye [2]. The deleterious effect could largely be prevented by abstaining from adding Coomassie-dye to the protein sample before applying it to the native gel, as exemplified with the isolation of highly pure respiratory complex III [12, 15].

CNE, originally named colorless-native PAGE (CN-PAGE) was introduced in 1994 [2] as a ''second choice'' electrophoresis variant just to demonstrate the beneficial effects of the Coomassie-dye in BNE or the considerably lower resolution when the dye is missing. The benefits of

CNE for the isolation of supramolecular structures like oligomeric ATP synthase were only recognized much later analyzing yeast [9, 10] and mammalian mitochondria [4].

2.3 Protein quantification on 1-D BN and 2-D BN/SDS gels for clinical applications

First applications of BNE in clinical diagnostics were described in 1995, specifically the identification of deficiencies of respiratory chain complexes and ATP synthase in mitochondrial disorders by quantitative analyses of 2-D BN/SDS gels [16–19]. Also postmortem analyses of brain tissue from patients with Alzheimer's and Parkinson's disease revealed characteristic alterations [7, 20, 21].

2.4 Amino-terminal sequencing of proteins on 2-D BN/SDS gels

Some of the earliest proteomic studies were based on amino-terminal protein sequencing by Edman degradation, *e.g.* the analysis of isoforms of subunits of cytochrome *c* oxidase (respiratory complex IV) that change in the developing rat heart [22], and the characterization of the subunits of respiratory complexes in plant mitochondria and chloroplasts [23, 24]. Since highly abundant proteins were studied, samples as small as 5 mg heart muscle were sufficient. Use of mass spectrometric techniques, as summarized in [7], now allows approaching also low abundant proteins. In contrast to blots of 2-D BN/SDS gels on polyvinylidendifluoride membranes that were preferred for Edman degradation, bands excised from BN gels or protein spots from 2-D BN/SDS gels are now mostly used for mass spectrometric studies.

2.5 Detection of fluorescence-labeled proteins on 2-D BN/SDS and 1-D hrCN gels

Pre-labeling of protein samples by reactive fluorescent dyes or fusion with fluorescent proteins before application to native gels has been used only a few times so far [5, 25–31]. Since Coomassie-dye quenches fluorescence by around 95% [5], 1-D BN gels are not immediately suitable for fluorescence detection. However, fluorescence detection is possible after removal of Coomassie-dye by 2-D SDS gels, as shown first for proteins marked by reactive cyanine dyes [25], and also for GFP-labeled proteins [27]. DIGE employs pre-labeling of protein samples by fluorescent cyanine dyes (*e.g.* CyDyes) before separation by 2-D IEF/SDS gels [32, 33]. Similarly, novel BN-DIGE techniques use protein-labeling by CyDyes before separation by 2-D BN/SDS gels [31]. hrCNE gels can be used immediately to identify fluorescence-labeled proteins in the first native dimension [5].

2.6 In-gel catalytic activity assays for clinical and biochemical research

Surprisingly late, in 1996/1997, in-gel catalytic activity assays for mitochondrial oxidative phosphorylation enzymes have been introduced for BN and CN gels [34, 35],] although some experience with histological staining and even with enzyme assays in non-denaturing gels [36] had been available for a while. Oxidative phosphorylation complexes from biopsies of patients with mitochondrial disorders, from mouse models, and also from plants could then be analyzed semi-quantitatively in 1-D native gels [37–41]. Performance of in-gel activity assays is even better using hrCNE gels as exemplified by Fig. 3A–G. Other enzyme assays for malate- and oxaloacetate-producing enzymes, for NADP-dependent dehydrogenases, for NAD-kinases, and for dihydrolipoamide-dehydrogenase diaphorase indicate the versatility of BNE/enzyme assay combinations [41–44]. Catalytic activity assays for mitochondrial oxidative phosphorylation complexes are optimal in CN gels with respect to signal intensity and sensitivity to inhibitors [4]. hrCN gels are preferred over BN gels because Coomassie-dye in BN gels interferes with many catalytic activity assays [5, 6].

2.7 Advanced structural investigations using protein extracts from BN gels

Blue protein bands extracted by electroelution or by diffusion from the native gel have been used for catalytic activity measurements [1], for the generation of polyclonal antibodies and for proteomic investigations [15], for 2-D crystallization and atomic force microscopy of the c-ring of ATP synthase [45, 46], and for electron microscopic single particle analysis [47, 48]. Most electron microscopic single particle analyses, however, used chromatography or density gradient centrifugation for the separation of proteins in the presence of detergents. In these cases, BNE was used just as a tool to identify and select the fractions containing the desired supramolecular structures [49–57]. LILBID mass spectrometry is a novel technique for the mass spectrometric analysis of large complexes by the whole subunits and not by their fragments [58]. This novel mass spectrometric technique was found useful also for the analysis of protein extracts from BN gels, as exemplified by the subunit analysis of extracted respiratory complex I that revealed a number of previously incorrectly assigned protein maturation sites [59].

3 Identification of protein–protein interactions

Two-hybrid techniques that were pioneered by Fields and Song [60] are important techniques to identify labile or

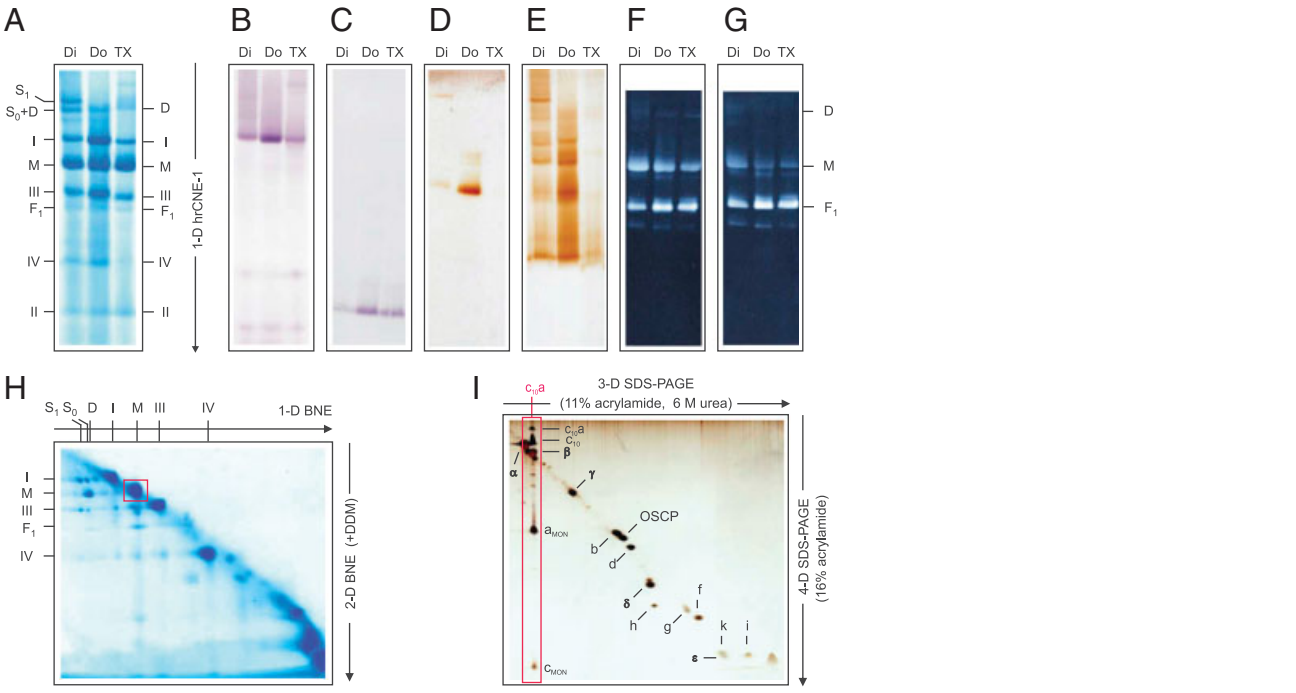

Figure 3. Multidimensional gels to identify and characterize protein–protein interactions. Assignment of complexes as in Fig. 1. (A–G) Detection of bovine mitochondrial complexes in 1-D hrCNE-1 gels. (A) Coomassie-stained hrCNE-1 gel, also shown in Fig. 1C. In-gel activity assays for respiratory complex I (B), complex II (C), complex III (D) and complex IV (E). In-gel ATPase assay of complex V in the absence (F) or in the presence (G) of oligomycin, an inhibitor of fully assembled ATP synthase. (H) 2-D BN/BN gel of solubilized bovine mitochondria using 1-D BNE for separation of supercomplexes and 2-D modified BNE (with 0.02% dodecylmaltoside added to the cathode buffer) for the release of individual complexes. Respiratory supercomplexes S_0 and S_1 released the constituent complexes I, III and IV. Dimeric complex V (D) dissociated into the monomeric complexes (M), and released also small amounts of the catalytic F_1 domain. Spots of individual complexes, *e.g.* complex V (boxed red), can be further analyzed by dSDS-PAGE, *i.e.* by 3-D and 4-D SDS gels, as exemplified in (I). (I) Complex V from yeast, cut out from a 2-D BN/BN gel similar to figure part H, was first resolved by 3-D tricine-SDS-PAGE. All subunits of complex V, except subunits c and a, migrated as individual bands in the 3-D SDS gel, since only one spot each for the indicated individual subunits was revealed after the 4-D separation. Subunits c and a comigrated quantitatively as a c_{10}a-complex together with the ß-subunit in the 3-D SDS gel (marked c_{10}a). Under the acidic transfer conditions used for 4-D tricine-SDS-PAGE, the c_{10}a-complex dissociated largely into c_{10}-ring and the individual subunits c and a (boxed red).

dynamic protein–protein interactions although the number of false positive and also false negative results is high. All other techniques that are commonly used to identify protein–protein interactions like the tandem affinity purification technology pioneered by Rigaut *et al.* [61], or co-immunoprecipitation, or native electrophoretic techniques like BNE, can only identify permanent interactions, except if *in vivo* cross-linking is used prior to protein isolation. The individual protein constituents in the isolated sample are eventually identified preferentially by mass spectrometric techniques.

3.1 Isolation of stable protein complexes by native electrophoresis

The requirements to isolate permanent or stable protein associations by native electrophoresis are very similar to the requirements for chromatographic preparations. The most important factors are ionic strength, pH and the choice of detergent for membrane protein complexes.

"Salting in" effects favoring protein solubilization can be observed already at low ionic strength, say 50 mM NaCl. Such low ionic strength conditions are often required to support membrane protein solubilization by detergents and also to keep some water-soluble proteins in solution. Higher salt concentrations, say >500 mM, are largely avoided during protein isolation protocols, since high ionic strength favors dissociation of protein–protein and protein–nucleic acid interactions. High salt concentrations in the samples for native electrophoresis are also avoided, since this would cause extreme stacking of proteins in the sample well resulting in protein aggregation, especially of membrane proteins. All native electrophoresis variants discussed here (BNE, CNE and hrCNE) rely on two alternative low ionic strength buffers for protein solubilization: solubilization buffer A contains 50 mM NaCl, 50 mM imidazole/HCl, pH 7, and solubilization buffer B contains 500 mM

6-aminohexanoic acid, 25 mM imidazole/HCl, pH 7 buffer [3]. 6-aminohexanoic acid, a zwitterionic compound, supports membrane protein solubilization similar to NaCl but unlike NaCl it seems not to promote dissociation of protein–protein contacts.

The pH of all native gels is kept in the neutral or slightly alkaline range which seems to be tolerated by most protein complexes. Mild neutral detergents like Triton X-100 or dodecylmaltoside are commonly used to solubilize membrane proteins and complexes for chromatographic purifications and also for native electrophoresis, as exemplified in Fig. 1. As a general rule, detergent/protein ratios around 2–3 g/g are chosen in order to avoid extensive delipidation and denaturation.

Water-soluble proteins and complexes do not require detergents for solubility and commonly do not even bind detergent so that essentially no risk of denaturation of water-soluble proteins by detergents exists. Also they are less prone to protein aggregation compared to membrane proteins. Therefore, water-soluble proteins mostly give rise to sharp bands in CNE compared to the diffuse and broadened bands of membrane proteins. We must, however, keep in mind that CNE is generally limited to the separation of acidic proteins with p*I* 7 if tricine/imidazole [3] or tricine/bis-tris buffers [1, 2] are used, or to proteins with p*I* 8 if an alternative glycine/tris-buffer is used to expand the useful pH range [62]. This means that all stronger basic water-soluble and membrane proteins are lost using CNE.

BNE and hrCNE enhance the solubility and anodic migration of membrane proteins, since hydrophobic proteins bind the anionic Coomassie-dye (in BNE) or mixed anionic/neutral detergent micelles (in hrCNE) whereas many water-soluble proteins do not bind these anionic compounds and therefore no improvement compared to CNE is observed for these proteins using BNE or hrCNE. Inspite of this uncertainty with water-soluble proteins, many water-soluble proteins and complexes have been successfully separated by BNE, *e.g.* [63–69]. Separations of acidic water-soluble proteins by CNE, of Coomassie-binding and basic water-soluble proteins by BNE, and of basic water-soluble proteins that were capable to bind anionic detergent micelles have already been described in the original methodical papers [2, 5]. A very interesting comparison of the distribution of proteasome subtypes using BNE and CNE has been presented recently [70].

Presumably all separations of membrane protein complexes by BNE performed before 1998 are examples for the separation/isolation of (detergent-)stable complexes, with the exception of a paper by Grandier-Vazeille and Guerin [34] that anticipated several features necessary for the isolation of supercomplexes. Further milestones in the isolation of detergent-labile associations of membrane protein complexes were the isolation of dimeric ATP synthase from yeast in 1998 [71] and the isolation of respiratory supercomplexes from yeast and mammals in 2000 [72, 73].

3.2 Conservation of detergent-labile protein–protein interactions

Only a few alternatives are currently available to preserve detergent-labile interactions in supramolecular structures of membrane protein complexes: (i) if common neutral detergents like Triton X-100 or dodecylmaltoside are used, as described for the isolation of stable complexes by BNE, the detergent/protein ratio must be kept low and close to the solubilization-limits, as exemplified by the isolation of dimeric ATP synthase from yeast [71]. (ii) Neutral detergents milder than Triton X-100 or dodecylmaltoside can be used, for example, digitonin, as exemplified in Fig. 1. The digitonin/protein ratio is less critical for the isolation of supra-structures. Reducing the digitonin/protein ratio to near the solubilization-limits offers ideal conditions to isolate very large assemblies like respiratory supercomplexes by BNE [72]. Another very mild detergent, BigCHAP, has recently been introduced to isolate oligomeric ATP synthase [52]. (iii) In addition to detergent effects on the stability of supramolecular structures, also the choice of the native electrophoresis variant can have remarkable effects. Separating the same sample by BNE and CNE revealed that respiratory supercomplexes from yeast mutants deficient in cardiolipin synthase are stable in CN gels but dissociate into the individual complexes III and IV in BN gels [9]. The milder conditions in CNE compared to BNE have also been demonstrated recently for mammalian ATP synthase [4, 74]. However, since membrane protein complexes often migrate as very broad bands in CN gels, BNE remains the method of choice to isolate more stable supramolecular assemblies. Dissociation of supercomplexes by detergents is partly explained by breaking of hydrophobic protein–protein interactions [9] but also lipids like cardiolipin in general [9] and one specific cardiolipin molecule at the interface of respiratory complexes III and IV [75] seem to be important components to glue yeast respiratory complexes III and IV together into respirasomes [9, 75, 76]. Also minor modifications of the cardiolipin structure, as induced by mutations in the yeast and human taffazin genes, affect respirasome stability in yeast [77] and in patients suffering from the devastating Barth syndrome [7, 78].

3.3 2-D native gels, native in both dimensions, reduce sample complexity and reveal loosely associated proteins

In principle, detergent-labile and non-permanently associated proteins can be identified using *in vivo* cross-linking prior to protein solubilization by detergents and separation by BNE. A simpler alternative to identify interacting/associated proteins is isolation of supramolecular assemblies under mildest conditions, *i.e.* using low ionic strength, neutral pH, mildest detergents and mildest electrophoresis variants. However, the complexity of the supramolecular

structure may cause analytical problems if the sample contains too many protein components or if the mass differences between subunits are too small. Therefore, supramolecular assemblies from mammalian mitochondria separated by 1-D BNE containing around 70 subunits were dissociated into the individual complexes by modified BNE in a second native dimension gel [72], as shown in Fig. 3H. The properties of modified 2-D BNE of course must be harsher than of 1-D BNE to release individual complexes from the supercomplexes. This was achieved by adding detergent (0.02% dodecylmaltoside) to the Coomassie-containing cathode buffer for 2-D BNE. The released complexes aligned as columns of spots below the position of supercomplexes in the 1-D BN gel (Fig. 3H). Densitometric quantification of the released complexes from bovine supercomplexes was used to determine the stoichiometric ratio of the $I_1III_2IV_{0\text{-}4}$-supercomplexes containing monomeric complex I, dimeric complex III and a varying copy number (0–4) of complex IV [72]. Application of 2-D BN/BN gels to the proteomic analysis of mammalian ATP synthase revealed two novel associated proteins, AGP and MLQ [79]. Recently, also the combination of 1-D BNE and 2-D hrCNE to 2-D BN/hrCN systems was used to characterize the low abundant respiratory supercomplexes in the yeast *Yarrowia lipolytica* [80]. 2-D native electrophoresis using the 2-D BN/hrCN combination was found ideal for fluorescent and functional assays of mitochondrial complexes [81].

3.4 Identification of interactions between highly hydrophobic proteins under SDS-PAGE conditions

The identification of protein–protein interactions under SDS-PAGE conditions is restricted to highly hydrophobic proteins and the few suitable proteins must be found empirically. These limitations seem not really encouraging but no useful alternative techniques are currently available.

Highly hydrophobic subunit *c* from mitochondrial ATP synthases is known to form oligomeric rings in the native enzyme. In some organisms the oligomeric rings resist unfolding by SDS so that c_{10}-rings can be observed in SDS-gels provided that the applied sample is not heated in SDS solution. Very recently, we realized that c-rings from yeast ATP synthase remained almost quantitatively associated with another neighboring hydrophobic protein, the a-subunit, in 2-D BN/SDS gels [10]. Further identification of a dimeric form of the c_{10}a-complex $(c_{10}a)_2$ and of dimeric subunit a (a_2) suggested that the two c-rings in dimeric ATP synthase are linked together *via* subunit a-dimers [10]. The subunit c-a association is easily identified by a 2-D electrophoretic technique known as dSDS-PAGE [82] as exemplified in Fig. 3I. For this purpose, monomeric ATP synthase is isolated by 2-D BN/BN gels similar to Fig. 3H. Individual spots, *e.g.* the ATP synthase (boxed red), are then cut out from the gel and separated by dSDS-PAGE, *i.e.* by two special orthogonal SDS-gels (Fig. 3I). The c_{10}a-complex, the c_{10}-ring, and released individual subunits c and a, are identified as columns of protein spots in the dSDS gel (boxed red in Fig. 3I).

4 Perspectives

Searching the literature, we could not identify any publication using *in vivo* chemical cross-linking followed by BNE and mass spectrometry to identify non-permanent and dynamic protein–protein interactions. It seems understandable that even larger proteomics facilities hesitate to start such high throughput approaches, since cross-link products are expected to be of low abundance and non-uniformly linked. However, we expect that focussing on one or a few proteins of interest and using specific antibodies for the initial search of cross-link products will alleviate the scientific tasks and the interpretation of the results considerably. Perhaps, a newly emerging technique called blue-native DIGE [31] for the comparative analysis of protein complexes will also be useful to compare protein complexes with or without prior *in vivo* cross-linking.

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5 References

[1] Schägger, H., von Jagow, G., Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 1991, *199*, 223–231.

[2] Schägger, H., Cramer, W. A., von Jagow, G., Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal. Biochem.* 1994, *217*, 220–230.

[3] Wittig, I., Braun, H. P., Schägger, H., Blue-Native PAGE. *Nat. Protoc.* 2006, *1*, 416–428.

[4] Wittig, I., Schägger, H., Advantages and limitations of clear native polyacrylamide gel electrophoresis. *Proteomics* 2005, *5*, 4338–4346.

[5] Wittig, I., Karas, M., Schägger, H., High resolution clear-native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. *Mol. Cell. Proteomics* 2007, *6*, 1215–1225.

[6] Wittig, I., Carrozzo, R., Santorelli, F. M., Schägger, H., Functional assays in high resolution clear native gels to quantify mitochondrial complexes in human biopsies and cell-lines. *Electrophoresis* 2007, *28*, 3811–3820.

[7] Wittig, I., Schägger, H., Features and applications of blue-native and clear-native electrophoresis. *Proteomics* 2008, *8*, 3974–3990.

[8] Krause, F., Detection and analysis of protein-protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (Membrane) protein complexes and supercomplexes. *Electrophoresis* 2006, *27*, 2759–2781.

[9] Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C. *et al.,* Cardiolipin stabilizes respiratory chain supercomplexes. *J. Biol. Chem.* 2003, *278*, 52873–52880.

[10] Wittig, I., Stuart, R. A., Velours, J., Schägger, H., Characterization of domain-interfaces in monomeric and dimeric ATP synthase. *Mol. Cell. Proteomics* 2008, *7*, 995–1004.

[11] Wittig, I., Schägger, H., Electrophoretic methods to isolate protein complexes from mitochondria. *Methods Cell Biol.* 2007, *80*, 723–741.

[12] Schägger, H., in: Hunte, C., von Jagow, G., Schägger, H. (Eds.), *Membrane Protein Purification and Crystallization*, Academic Press, San Diego 2003, pp. 105–130.

[13] Huang, D., Everly, R. M., Cheng, R. H., Heymann, J. B. *et al.*, Characterization of the chloroplast cytochrome b_6f complex as a structural and functional dimer. *Biochemistry* 1994, *33*, 4401–4409.

[14] Heuberger, E. H., Veenhoff, L. M., Duurkens, R. H., Friesen, R. H., Poolman, B., Oligomeric state of membrane transport proteins analyzed with blue native electrophoresis and analytical ultracentrifugation. *J. Mol. Biol.* 2002, *317*, 591–600.

[15] Schägger, H., Native electrophoresis for isolation of mitochondrial oxidative phosphorylation protein complexes. *Methods Enzymol.* 1995, *260*, 190–202.

[16] Bentlage, H., De Coo, R., Ter Laak, H., Sengers, R., Human diseases with defects in oxidative phosphorylation: I. Decreased amounts of assembled oxidative phosphorylation complexes in mitochondrial encephalomyopathies. *Eur. J. Biochem.* 1995, *227*, 909–915.

[17] Klement, P., Nijtmans, L. G., van den Bogert, C., Houstek, J., Analysis of oxidative phosphorylation complexes in cultured human fibroblasts and amniocytes by blue-native-electrophoresis using mitoplasts isolated with the help of digitonin. *Anal. Biochem.* 1995, *231*, 218–224.

[18] Schägger, H., Bentlage, H., Ruitenbeek, W., Pfeiffer, K. *et al.,* Electrophoretic separation of multiprotein complexes from blood platelets and cell lines: Technique for the analysis of diseases with defects in oxidative phosphorylation. *Electrophoresis* 1996, *17*, 709–714.

[19] Schägger, H., Electrophoretic techniques for isolation and quantitation of oxidative phosphorylation complexes from human tissues. *Methods Enzymol.* 1996, *264*, 555–566.

[20] Schägger, H., Ohm, T., Human diseases with defects in oxidative phosphorylation: II. F1F0 ATP-synthase defects in Alzheimeŕs disease revealed by blue native polyacrylamide gel electrophoresis. *Eur. J. Biochem.* 1995, *227*, 916–921.

[21] Schägger, H., Quantification of oxidative phosphorylation enzymes after blue native electrophoresis and two-dimensional resolution: Normal complex I protein amounts in Parkinsoń s disease conflict with reduced catalytic activities. *Electrophoresis* 1995, *16*, 763–770.

[22] Schägger, H., Noack, H., Halangk, W., Brandt, U., von Jagow, G., Cytochrome c oxidase in developing rat heart: Enzymatic properties and aminoterminal sequences suggest identity of the fetal heart and the adult liver isoform. *Eur. J. Biochem.* 1995, *230*, 235–241.

[23] Jänsch, L., Kruft, V., Schmitz, U. K., Braun, H. P., New insights into the composition, molecular mass and stoichiometry of the protein complexes of plant mitochondria. *Plant J.* 1996, *9*, 357–368.

[24] Kügler, M., Jänsch, L., Kruft, V., Schmitz, U. K., Braun, H. P., Analysis of the chloroplast protein complexes by blue-native polyacrylamide gelelectrophoresis. *Photosynth. Res.* 1997, *53*, 35–44.

[25] Perales, M., Eubel, H., Heinemeyer, J., Colaneri, A. *et al.*, Disruption of a nuclear gene encoding a mitochondrial gamma carbonic anhydrase reduces complex I and supercomplex I+III2 levels and alters mitochondrial physiology in *Arabidopsis*. *J. Mol. Biol.* 2005, *350*, 263–277.

[26] Granvogl, B., Reisinger, V., Eichacker, L. A., Mapping the proteome of thylakoid membranes by de novo sequencing of intermembrane peptide domains. *Proteomics* 2006, *6*, 3681–3695.

[27] Metz, S., Wiesinger, M., Vogt, M., Lauks, H. *et al.*, Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP: fused receptor domains act as high affinity cytokine-binding proteins. *J. Biol. Chem.* 2007, *282*, 1238–1248.

[28] Reisinger, V., Eichacker, L. A., How to analyze protein complexes by 2D blue native SDS-PAGE. *Proteomics* 2007, *7*, Suppl 1, 6–16.

[29] Gillardon, F., Rist, W., Kussmaul, L., Vogel, J. *et al.*, Proteomic and functional alterations in brain mitochondria from Tg2576 mice occur before amyloid plaque deposition. *Proteomics* 2007, *7*, 605–616.

[30] Dani, D., Dencher, N. A., Native-DIGE: a new look at the mitochondrial membrane proteome. *Biotechnol. J.* 2008, *3*, 817–822.

[31] Heinemeyer, J., Scheibe, B., Schmitz, U. K., Braun, H.-P., Blue native DIGE as a tool for comparative analyses of protein complexes. *J. Proteomics* 2009, *72*, 539–544.

[32] Unlü, M., Morgan, M. E., Minden, J. S., Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997, *18*, 2071–2077.

[33] Tonge, R., Shaw, J., Middleton, B., Rowlinson, R. *et al.*, Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* 2001, *1*, 377–396.

[34] Grandier-Vazeille, X., Guerin, M., Separation by blue native and colorless native polyacrylamide gel electrophoresis of the oxidative phosphorylation complexes of yeast mitochondria solubilized by different detergents: specific staining of the different complexes. *Anal. Biochem.* 1996, *242*, 248–254.

[35] Zerbetto, E., Vergani, L., Dabbeni-Sala, F., Quantification of muscle mitochondrial oxidative phosphorylation enzymes

via histochemical staining of blue native polyacrylamide gels. *Electrophoresis* 1997, *18*, 2059–2064.

[36] Kuonen, D. R., Roberts, P. J., Cottingham, I. R., Purification and analysis of mitochondrial membrane proteins on nondenaturing gradient polyacrylamide gels. *Anal. Biochem.* 1986, *153*, 221–226.

[37] Jung, C., Higgins, C. M., Xu, Z., Measuring the quantity and activity of mitochondrial electron transport chain complexes in tissues of central nervous system using blue native polyacrylamide gel electrophoresis. *Anal. Biochem.* 2000, *286*, 214–223.

[38] Van Coster, R., Smet, J., George, E., De Meirleir, L. *et al.*, Blue native polyacrylamide gel electrophoresis: a powerful tool in diagnosis of oxidative phosphorylation defects. *Pediatr. Res.* 2001, *50*, 658–665.

[39] Jung, C., Higgins, C. M., Xu, Z., A quantitative histochemical assay for activities of mitochondrial electron transport chain complexes in mouse spinal cord sections. *J. Neurosci. Methods* 2002, *114*, 165–172.

[40] Sabar, M., Balk, J., Leaver, C. J., Histochemical staining and quantification of plant mitochondrial respiratory chain complexes using blue-native polyacrylamide gel electrophoresis. *Plant J.* 2005, *44*, 893–901.

[41] Singh, R., Chenier, D., Beriault, R., Mailloux, R. *et al.*, Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate-producing enzymes. *J. Biochem. Biophys. Methods* 2005, *64*, 189–199.

[42] Beriault, R., Chenier, D., Singh, R., Middaugh, J. *et al.*, Detection and purification of glucose 6-phosphate dehydrogenase, malic enzyme, and NADP-dependent isocitrate dehydrogenase by blue native polyacrylamide gel electrophoresis. *Electrophoresis* 2005, *26*, 2892–2897.

[43] Mailloux, R. J., Singh, R., Appanna, V. D., In-gel activity staining of oxidized nicotinamide adenine dinucleotide kinase by blue native polyacrylamide gel electrophoresis. *Anal. Biochem.* 2006, *359*, 210–215.

[44] Yan, L. J., Yang, S. H., Shu, H., Prokai, L., Forster, M. J., Histochemical staining and quantification of dihydrolipoamide dehydrogenase diaphorase activity using blue native PAGE. *Electrophoresis* 2007, *28*, 1036–1045.

[45] Poetsch, A., Neff, D., Seelert, H., Schägger, H., Dencher, N. A., Dye removal, catalytic activity and 2D-crystallization of chloroplast H^+-ATP Synthase purified by blue native electrophoresis. *Biochim. Biophys. Acta* 2000, *1466*, 339–349.

[46] Seelert, H., Dencher, N. A., Müller, D. J., Fourteen protomers compose the oligomer III of the proton-rotor in spinach chloroplast ATP synthase. *J. Mol. Biol.* 2003, *333*, 337–344.

[47] Schäfer, E., Seelert, H., Reifschneider, N. H., Krause, F., Dencher, N. A., Vonck, J., Architecture of active mammalian respiratory chain supercomplexes. *J. Biol. Chem.* 2006, *281*, 15370–15375.

[48] Schäfer, E., Dencher, N. A., Vonck, J., Parcej, D. N., Three-dimensional structure of the respiratory chain supercomplex $I_1III_2IV_1$ from bovine heart mitochondria. *Biochemistry* 2007, *46*, 12579–12585.

[49] Dudkina, N. V., Heinemeyer, J., Keegstra, W., Boekema, E. J., Braun, H.-P., Structure of dimeric ATP synthase from mitochondria: an angular association of monomers induces the strong curvature of the inner membrane. *FEBS Lett.* 2005, *579*, 5769–5772.

[50] Minauro-Sanmiguel, F., Wilkens, S., Garcia, J. J., Structure of dimeric mitochondrial ATP synthase: novel F_O bridging features and the structural basis of mitochondrial cristae biogenesis. *Proc. Natl. Acad. Sci.* USA 2005, *102*, 12356–12358.

[51] Dudkina, N. V., Sunderhaus, S., Braun, H. P., Boekema, E. J., Characterization of dimeric ATP synthase and cristae membrane ultrastructure from *Saccharomyces* and *Polytomella* mitochondria. *FEBS Lett.* 2006, *580*, 3427–3432.

[52] Thomas, D., Bron, P., Weimann, T., Dautant, A. *et al.*, Supramolecular organization of the yeast F_1F_O-ATP synthase. *Biol. Cell* 2008, *100*, 591–601.

[53] Heinemeyer, J., Braun, H. P., Boekema, E. J., Kouril, R., A structural model of the cytochrome c reductase/oxidase supercomplex from yeast mitochondria. *J. Biol. Chem.* 2007, *282*, 12240–12248.

[54] Bultema, J. B., Braun, H. P., Boekema, E. J., Kouril, R., Megacomplex organization of the oxidative phosphorylation system by structural analysis of respiratory supercomplexes from potato. *Biochim. Biophys. Acta* 2009, *1787*, 60–67.

[55] Dudkina, N. V., Eubel, H., Keegstra, W., Boekema, E. J., Braun, H. P., Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. *Proc. Natl. Acad. Sci. USA* 2005, *102*, 3225–3229.

[56] Peters, K., Dudkina, N. V., Jänsch, L., Braun, H. P., Boekema, E. J., A structural investigation of complex I and $I+III_2$ supercomplex from *Zea mays* at 11-13 A resolution: assignment of the carbonic anhydrase domain and evidence for structural heterogeneity within complex I. *Biochim. Biophys. Acta* 2008, *1777*, 84–93.

[57] Dudkina, N. V., Sunderhaus, S., Boekema, E. J., Braun, H. P., The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. *J. Bioenerg. Biomembr.* 2008, *40*, 419–424.

[58] Morgner, N., Kleinschroth, T., Barth, H. D., Ludwig, B., Brutschy, B., A novel approach to analyze membrane proteins by laser mass spectrometry: from protein subunits to the integral complex. *J. Am. Soc. Mass Spectrom.* 2007, *18*, 1429–1438.

[59] Morgner, N., Zickermann, V., Kerscher, S., Wittig, I. *et al.*, Subunit mass fingerprinting of mitochondrial complex I. *Biochim. Biophys. Acta* 2008, *1777*, 1384–1391.

[60] Fields, S., Song, O., A novel genetic system to detect protein-protein interactions. *Nature* 1989, *340*, 245–246.

[61] Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M. *et al.*, A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* 1999, *17*, 1030–1032.

[62] Wittig, I., Schägger, H., Electrophoretic methods to isolate protein complexes from mitochondria. *Methods Cell Biol.* 2007, *80*, 723–741.

[63] Krause-Buchholz, U., Schöbel, K., Lauffer, S., Rödel, G., *Saccharomyces cerevisiae* translational activator Cbs1p is

associated with translationally active mitochondrial ribosomes. *Biol. Chem.* 2005, *386*, 407–415.

[64] Focke, M., Gieringer, E., Schwan, S., Jänsch, L. *et al.*, Fatty acid biosynthesis in mitochondria of grasses: malonyl-coenzyme A is generated by a mitochondrial-localized acetyl-coenzyme A carboxylase. *Plant Physiol.* 2003, *133*, 875–884.

[65] Jiang, H., Rao, K. S., Yee, V. C., Kraus, J. P., Characterization of four variant forms of human propionyl-CoA carboxylase expressed in *Escherichia coli*. *J. Biol. Chem.* 2005, *280*, 27719–27727.

[66] Lasserre, J. P., Beyne, E., Pyndiah, S., Lapaillerie, D., A complexomic study of *Escherichia coli* using two-dimensional blue native/SDS polyacrylamide gel electrophoresis. *Electrophoresis* 2006, *27*, 3306–3321.

[67] Miyagi, Y., Matsumura, Y., Sagami, H., Human geranylgeranyl diphosphate synthase is an octamer in solution. *J. Biochem. (Tokyo)* 2007, *142*, 377–381.

[68] Mailloux, R. J., Singh, R., Appanna, V. D., In-gel activity staining of oxidized nicotinamide adenine dinucleotide kinase by blue native polyacrylamide gel electrophoresis. *Anal. Biochem.* 2006, *359*, 210–215.

[69] Singh, R., Chenier, D., Beriault, R., Mailloux, R., Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate-producing enzymes. *J. Biochem. Biophys. Methods* 2005, *64*, 189–199.

[70] Shibatani, T., Carlson, E. J., Larabee, F., McCormack, A. L., Global organization and function of mammalian cytosolic proteasome pools: Implications for PA28 and 19S regulatory complexes. *Mol. Biol. Cell* 2006, *17*, 4962–4971.

[71] Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., Schägger, H., Yeast F_1F_0-ATP synthase exists as a dimer: identification of three dimer specific subunits. *EMBO J.* 1998, *17*, 7170–7178.

[72] Schägger, H., Pfeiffer, K., Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J.* 2000, *19*, 1777–1783.

[73] Cruciat, C. M., Brunner, S., Baumann, F., Neupert, W., Stuart, R. A., The cytochrome bc_1 and cytochrome c oxidase complexes associate to form a single supracomplex in yeast mitochondria. *J. Biol. Chem.* 2000, *275*, 18093–18098.

[74] Krause, F., Reifschneider, N. H., Goto, S., Dencher, N. A., Active oligomeric ATP synthases in mammalian mitochondria. *Biochem. Biophys. Res. Commun.* 2005, *329*, 583–590.

[75] Wenz, T., Hielscher, R., Hellwig, P., Schägger, H., Richers, S., Hunte, C., Role of phospholipids in respiratory cytochrome bc_1 complex catalysis and supercomplex formation. *Biochim. Biophys. Acta* 2009, *1787*, 609–616.

[76] Zhang, M., Mileykovskaya, E., Dowhan, W., Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. *J. Biol. Chem.* 2005, *280*, 29403–29408.

[77] Brandner, K., Mick, D. U., Frazier, A. E., Taylor, R. D. *et al.,* Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth Syndrome. *Mol. Biol. Cell* 2005, *16*, 5202–5214.

[78] McKenzie, M., Lazarou, M., Thorburn, D. R., Ryan, M. T., Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. *J. Mol. Biol.* 2006, *361*, 462–469.

[79] Meyer, B., Wittig, I., Trifilieff, E., Karas, M., Schägger, H., Identification of two proteins associated with mammalian ATP synthase. *Mol. Cell. Proteomics* 2007, *6*, 1690–1699.

[80] Nübel, E., Wittig, I., Kerscher, S., Brandt, U., Schägger, H., Two-dimensional native electrophoretic analysis of respiratory supercomplexes from *Yarrowia lipolytica*. *Proteomics* 2009, *in* press.

[81] Wumaier, Z., Nübel, E., Wittig, I., Schägger, H., Two-dimensional native electrophoresis for functional assays of mitochondrial complexes. *Methods Enzymol.* 2009, in press

[82] Rais, I., Karas, M., Schägger, H., Two-dimensional electrophoresis for the isolation of integral membrane proteins and mass spectrometric identification. *Proteomics* 2004, *4*, 2567–2571.

