High Resolution Clear Native Electrophoresis for In-gel Functional Assays and Fluorescence Studies of Membrane Protein Complexes*

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Clear native electrophoresis and blue native electrophoresis are microscale techniques for the isolation of membrane protein complexes. The Coomassie Blue G-250 dye, used in blue native electrophoresis, interferes with in-gel fluorescence detection and in-gel catalytic activity assays. This problem can be overcome by omitting the dye in clear native electrophoresis. However, clear native electrophoresis suffers from enhanced protein aggregation and broadening of protein bands during electrophoresis and therefore has been used rarely. To preserve the advantages of both electrophoresis techniques we substituted Coomassie dye in the cathode buffer of blue native electrophoresis by non-colored mixtures of anionic and neutral detergents. Like Coomassie dye, these mixed micelles imposed a charge shift on the membrane proteins to enhance their anodic migration and improved membrane protein solubility during electrophoresis. This improved clear native electrophoresis offers a high resolution of membrane protein complexes comparable to that of blue native electrophoresis. We demonstrate the superiority of high resolution clear native electrophoresis for in-gel catalytic activity assays of mitochondrial complexes I–V. We present the first in-gel histochemical staining protocol for respiratory complex III. Moreover we demonstrate the special advantages of high resolution clear native electrophoresis for in-gel detection of fluorescent labeled proteins labeled by reactive fluorescent dyes and tagged by fluorescent proteins. The advantages of high resolution clear native electrophoresis make this technique superior for functional proteomics analyses. ***Molecular & Cellular Proteomics 6:1215–1225, 2007.***

Blue native electrophoresis (BNE)[1] has been recognized as an efficient microscale electrophoretic technique to separate and characterize membrane proteins and complexes in the mass range of 100 kDa to 10 MDa (1, 2). It has been used in various clinical and biochemical fields (3). Clear native electrophoresis (CNE; Refs. 2 and 4) in contrast has only recently been recognized as a valuable means to isolate and functionally investigate supramolecular protein assemblies (5), *e.g.* for fluorescence resonance energy transfer analyses of dimeric and oligomeric mitochondrial ATP synthase (6, 7) and also for stromal proteome analysis of chloroplasts (8). The basic separation principles and the performance of BNE and CNE are markedly different, although the experimental setups are largely identical except for the addition of Coomassie Blue G-250 dye (denoted as Coomassie dye in the following) to sample and cathode buffer for BNE.

The most important component in BNE is the anionic Coomassie dye. Following solubilization of biological membranes and centrifugation, Coomassie dye is added to the supernatant. Binding of a large number of dye molecules to the protein surfaces imposes a charge shift on the proteins that causes even basic proteins to migrate to the anode at pH 7.5 during BNE (1). Proteins are pulled to the electrophoresis front by their excess negative charges. However, they are not separated according to the charge/mass ratio but according to the protein size by the decreasing pore size of acrylamide gradient gels. Protein migration gradually ceases with the running distance until a size-specific pore size limit for each specific protein is reached. The tendency of membrane proteins to aggregate is reduced in BNE due to the negative surface charge imposed by the anionic Coomassie dye. Effectively covering hydrophobic surfaces with anionic dye confers the behavior of water-soluble proteins to membrane proteins. Therefore, no detergent must be added to blue native gels, and detergent-sensitive proteins are kept in a native state. Blue stained native protein bands are detected already during BNE, facilitating excision of bands, electroelution, and electroblotting.

CNE does not use Coomassie dye and therefore lacks the

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[1] The abbreviations used are: BNE, blue native electrophoresis; Coomassie dye, Coomassie Blue G-250; CNE, clear native electrophoresis; hrCNE, high resolution clear native electrophoresis; DOC, deoxycholate; DDM or dodecylmaltoside, dodecyl-β-D-maltoside; TX, Triton X-100; NHS, *N*-hydroxysuccinimide; NTB, nitrotetrazolium blue; YFP, yellow fluorescent protein; complex I, NADH:ubiquinone reductase; complex II, succinate:ubiquinone reductase; complex III, ubiquinol:cytochrome *c* reductase; complex IV, cytochrome *c* oxidase; complex V, ATP synthase; 1D, one-dimensional; 2D, two-dimensional; EYFP, enhanced YFP; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

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advantages of the charge shift technique BNE (4). It is limited to the separation of acidic proteins with a pI below the pH of the gels and is often characterized by protein aggregation during electrophoresis and by a significantly lower resolution as compared with BNE. However, it offers clear advantages for in-gel catalytic activity assays and detection of fluorescent labels. Moreover CNE is even milder than BNE and very useful to retain physiological protein assemblies, *e.g.* multimeric states of mitochondrial ATP synthase (4, 5). Because CNE is ideal to preserve physiological oligomeric states, CNE has even been preferred to BNE in a recent proteomics study (8). We asked whether non-colored anionic compounds, especially anionic detergents, might be useful to substitute for Coomassie dye in the cathode buffer to preserve the advantages of BNE and CNE as well. Such non-denaturing solubilization of membranes and electrophoresis of membrane protein complexes in the presence of low concentrations of the anionic detergent SDS was pioneered by Reinman and Thornber (9), and because many of the analyzed photosynthetic complexes were green, the separations became known as "green gels." The method was extended to membranes from photosynthetic bacteria also (10) and has later been modified manyfold (11–13). Instead of the commonly used anionic detergent SDS in green gels, here we used mixed micelles of the anionic detergent sodium deoxycholate (DOC) and various mild non-ionic detergents to supplement the cathode buffer used previously in CNE (4). This single modification of the cathode buffer of CNE kept membrane proteins solubilized, imposed a negative charge shift on proteins, and increased resolution considerably. The modified CNE was named high resolution CNE (hrCNE) to discriminate between the two different techniques. In fact, the term hrCNE is used for three variants that differ by the specific mixture of anionic and neutral detergents that is added to the cathode buffer to impose a charge shift on membrane proteins. The mildness of hrCNE can be tailored to match the stability of the protein complexes of interest by varying the detergent used for solubilization and by selecting the appropriate cathode buffer.

Using the mild non-ionic detergent dodecyl-β-D-maltoside (designated here as dodecylmaltoside or DDM) to solubilize mitochondria, five complexes (complexes I–V) of mitochondrial oxidative phosphorylation are usually solubilized as individual membrane protein complexes, namely NADH:ubiquinone reductase (complex I), succinate:ubiquinone reductase (complex II), ubiquinol:cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), and the ATP synthase (complex V). DDM is a mild detergent, but it cannot preserve some labile hydrophobic protein-protein interactions that tie individual mitochondrial complexes together to form respiratory supercomplexes, also called respirasomes.

Digitonin, which is one of the mildest detergents known so far, is used instead of DDM for membrane solubilization whenever isolation of labile physiological protein assemblies is desired. Digitonin has been used to isolate supramolecular associations of multiprotein complexes, thereby identifying physiological protein-protein interactions without using chemical cross-linking (14–16). Fine tuning of the protein assemblies is then achieved by the choice of one of three cathode buffer variants for hrCNE.

Following 1D hrCNE, proteins of interest can optionally be extracted in native state or electroblotted on inert membranes in SDS-denatured state, *e.g.* for immunodetection. 1D hrCNE gels can also be used immediately for in-gel catalytic activity assays and for detection and quantification of proteins previously tagged with fluorescent labels like fluorescent proteins or reactive fluorescent dyes. Individual subunits can be detected and quantified by subsequent resolution with SDS-PAGE in a second dimension.

EXPERIMENTAL PROCEDURES

Materials—Coomassie Blue G-250 (Serva Blue G) was purchased from Serva. Dodecyl-β-D-maltoside was purchased from Glycon (Luckenwalde, Germany). Digitonin (catalog number 37006, >50% purity, used without recrystallization) and Triton X-100 were purchased from Fluka. Sodium deoxycholate was purchased from Merck. *N*-Hydroxysuccinimide (NHS)-rhodamine and NHS-fluorescein were purchased from Pierce. Cy monoreactive NHS esters Cy3, Cy3.5, Cy5, and Cy5.5 and CyDye DIGE Fluor Cy2, Cy3, and Cy5 were purchased from GE Healthcare. All other chemicals were purchased from Sigma. *Yarrowia lipolytica* yeast strain Δnugm GH1 (Δ*nugm*::*URA3 Mat*A *ndh2i ura3*-302 *his*-1 *xpr2*-322) was complemented with pEYFP30 (basic vector pEYFP from BD Biosciences Clontech) to assemble a 30 kDa-EYFP fusion protein in complex I instead of the wild-type 30-kDa subunit of complex I (17). The yeast strain was grown in YPD medium (yeast extract, bacto peptone, D-glucose) supplemented with hygromycin B (100 mg/liter). Bovine heart mitochondria (18) and mitochondria from the yeast *Y. lipolytica* were prepared as described previously (19).

Fluorescent Labeling—NHS dyes were dissolved in dimethylformamide (10 μg/μl), and aliquots were stored in liquid nitrogen for several weeks without detectable decay. For standard labeling, 10-μl aliquots of NHS dyes were added to 10 mg of mitochondria (suspended in 1 ml of 250 mM sucrose, 20 mM sodium phosphate, pH 7.5) and gently shaken for 1 h in the dark at room temperature. 20-fold diluted dye was used if reduced labeling was desired. Labeled mitochondria were sedimented by 10-min centrifugation (10,000 × *g* at 4 °C) and stored frozen at −80 °C.

Acrylamide Gel Preparation—Identical gels were used for CNE, hrCNE, and BNE. 5–13% acrylamide gradient gels were used for separation of DDM-solubilized respiratory complexes, and 4–13% acrylamide gradient gels were used for separation of digitonin-solubilized respiratory complexes and supercomplexes. Gels were prepared as described previously (3).

Sample Preparation for Native Electrophoresis—Solubilization of biological membranes on ice was identical for CNE, hrCNE, and BNE (3). Briefly 4-mg aliquots (an amount sufficient to load 20 gel wells (0.5 × 0.15 cm)) of sedimented (10 min at 20,000 × *g*) bovine heart mitochondria were vortexed with 400 μl of buffer (50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.0) and solubilized by adding 100 μl of DDM (10%), corresponding to a DDM/protein ratio of 2.5 (g/g), or by adding 40–120 μl of digitonin (20%), corresponding to digitonin/protein ratios ranging from 2–6 (g/g). Following centrifugation (15 min at 100,000 × *g*) the supernatant was divided into three equal parts. The supernatant for BNE (around 160 μl) was supplemented with 20 μl of 50% glycerol and with the

following amounts of Coomassie Blue G-250 dye from a 5% suspension in 500 mM 6-aminohexanoic acid. Using DDM and digitonin for solubilization, 8 and 20 μl, respectively, from the 5% Coomassie dye suspension were added to the supernatant to set a detergent/Coomassie ratio of 8 (g/g). The supernatants for CNE and hrCNE (around 160 μl each) were supplemented with 20 μl of 50% glycerol, 0.1% Ponceau S. The red dye Ponceau S does not bind to proteins under the prevailing conditions and was added here to mark the running front during electrophoresis. Equal sample volumes (20 μl) were applied to multiple 0.5 × 0.15-cm gel wells for BNE, CNE, and hrCNE. The actual protein load for each lane (around 75 μg of protein) was approximately half of the initial protein amount (150 μg of sedimented mitochondrial protein). *Y. lipolytica* mitochondria were solubilized like bovine heart mitochondria except that DDM/protein and digitonin/protein ratios of 1.6 and 3 (g/g), respectively, were used.

Buffers for Native Electrophoresis—The same anode buffer (25 mM imidazole/HCl, pH 7.0) was used for hrCNE, CNE, and BNE. Cathode buffer for CNE (50 mM Tricine, 7.5 mM imidazole, pH 7.0) was supplemented with various additives for BNE and hrCNE. As summarized in Table I, the anionic Coomassie dye (0.02 or 0.002%) was added for BNE, and the anionic detergent DOC (0.05%) was added for all three hrCNE variants. Non-ionic detergent was also added to generate mixed micelles and to make the hrCNE variants functional. Depending on the desired mildness of the electrophoresis, one of three different additions was optionally used. For the variants hrCNE-1 and hrCNE-2, 0.02% DDM and 0.05% Triton X-100, respectively, were added to the cathode buffers. These cathode buffers, named DDM buffer and TX buffer, are sufficiently mild to keep individual respiratory complexes functional, but they did not preserve large physiological assemblies. To isolate physiological supramolecular assemblies, digitonin was used for membrane solubilization, and low DDM amounts (0.01%) were added to the cathode buffer for hrCNE-3. This 0.01% DDM buffer turned out to be considerably milder than the commonly used 0.02% DDM buffer. Special features of the cathode buffers for hrCNE-1, hrCNE-2, and hrCNE-3 with respect to resolution and in-gel activity assays are discussed with Figs. 1 and 2.

Electrophoresis Conditions—Electrophoresis conditions for CNE and hrCNE gels were the same as applied for BNE (3). Gels were run in the cold (4–7 °C), and the initial voltage was set to 100 V. When the sample had entered the sample gel, the voltage was raised to 500 V with the current limited to 15 mA (for 0.15 × 14 × 14-cm gels). The electrophoresis was stopped after 3–4 h when the sharp line of the red Ponceau S dye approached the gel front.

In-gel Catalytic Activity Assays—The in-gel assays followed the principles described by Zerbetto *et al.* (20) with the following modifications. The nitrotetrazolium blue (NTB) concentration in the complex II assay was matched with the NTB concentration in the complex I assay (2.5 mg/ml), many components that were not helpful for a specific assay (like EDTA and cyanide in the complex II assay and catalase and sucrose in the complex IV assay) were omitted, a specific preincubation buffer was applied to increase the sensitivity of the complex V assay (4), and two complexes (complexes I + II or complexes III + IV) were optionally assayed in consecutive steps using the same gel strip. The assays were performed at 20–25 °C. All assays except the complex V assay (stopped by 50% methanol) were stopped using 50% methanol, 10% acetic acid fixing solution.

Complexes I and II were optionally analyzed on separate gel strips or by consecutive NADH:NTB reductase and succinate:NTB reductase assays using the same gel strip as described here. Gel strips loaded with extracts from 150 μg of bovine heart mitochondria were assayed first for complex I activity in assay buffer (25 mg of NTB and 100 μl of NADH (10 mg/ml) added to 10 ml of 5 mM Tris/HCl, pH 7.4). After about 3–5 min the reaction was either stopped using the fixing solution, or the gel was immediately transferred to 5 mM Tris/HCl, pH 7.4, scanned for densitometric quantitation, and reused for the immediately following succinate:NTB reductase assay (complex II stain). The complex II assay buffer contained 200 μl of sodium succinate (1 M), 8 μl of phenazine methosulfate (250 mM dissolved in DMSO), and 25 mg of NTB in 10 ml of 5 mM Tris/HCl, pH 7.4. Around 10–30 min of incubation was required.

Complexes III and IV were optionally analyzed on separate gel strips or analyzed consecutively using the same gel strip as described here. Specific staining of individual complex III and of respiratory supercomplexes containing complex III was achieved by incubating the gel strip in complex III assay buffer (5 mg of diaminobenzidine dissolved in 10 ml of 50 mM sodium phosphate, pH 7.2). After about 60 min of staining, the gel was scanned for densitometric quantitation and then put back into the complex III assay buffer, and 100 μl of horse heart cytochrome *c* (5 mM) was finally added to start the complex IV assay. The complex IV assay is faster than the complex III assay and requires about 30 min of further incubation.

To quantify the ATP hydrolysis activity of holocomplex V and F_1 subcomplex, two almost identical gel strips from the same native gel were preincubated in parallel for two h in 35 mM Tris, 270 mM glycine, pH 8.3 (25 °C) containing or not containing the complex V inhibitor oligomycin (5 μg/ml). Following removal of the incubation solution, the gels were incubated in assay buffer (35 mM Tris, 270 mM glycine, 14 mM $MgSO_4$, 0.2% $Pb(NO_3)_2$, 8 mM ATP, pH 8.3, containing or not containing 5 μg/ml oligomycin). ATP hydrolysis correlated with the development of white lead phosphate precipitates. The reaction was stopped using 50% methanol for 30 min, and then the gel was transferred to water and scanned for densitometric quantification. Acidic fixing solution (50% methanol, 10% acetic acid) must not be used at that step because lead phosphate precipitates are dissolved in acidic solution. However, following scanning and quantification, the lead phosphate precipitates can be dissolved in acidic solution for restaining with Coomassie dye. The precipitates should be completely dissolved because residual precipitate would bind Coomassie dye rather strongly.

Densitometric Quantification—A Typhoon Trio laser scanner (GE Healthcare) was used to identify fluorescent samples and labeled protein subunits of complexes in 1D and 2D gels. The Bio-Rad ChemiDoc XRS system was used for densitometric quantification.

Semidry Electroblotting of CNE and hrCNE Gels—Native gels were incubated for 20 min in a 3-fold gel volume of buffer (300 mM Tris, 100 mM acetic acid, 1% SDS, pH 8.6) turning the gel several times. The gel was then placed between two glass plates and stored for 1 h at 20–25 °C to spread SDS in the gel evenly and to denature proteins. Proteins were then electroblotted from the gel onto PVDF membranes using SDS-free transfer buffer for both electrodes (150 mM Tris, 50 mM acetic acid, pH 8.6). The experimental setup was as follows. A PVDF membrane was wetted with methanol and then incubated in transfer buffer until the membrane was submerged in buffer. A 3-mm stack of Whatman chromatographic papers (17 CHR) was soaked with transfer buffer and placed on the lower electrode, which was the cathode in this arrangement. First the gel and then the wetted PVDF membrane were placed on top. Another 3-mm stack of chromatographic papers followed by the upper electrode (the anode) and finally a 5-kg load were put on top. The proteins were transferred preferentially overnight (or at least 6 h) with the current set to 0.4 mA/cm^2 (around 60 mA for a 12 × 12 cm gel) and the voltage limited to 15 V (the actual voltage during transfer was 4–5 V under the conditions set). A power supply with a minimal load resistance $<$30 ohms was required for the transfer under the low current and low voltage conditions.

Electroelution of Proteins from hrCNE Gels—H-shaped electroelutor vessels were built according to Hunkapillar *et al.* (21) or commercially available from C.B.S. Scientific Co. (Del Mar, CA). The cathodic and anodic arms of the vessels were sealed with low cutoff (2-kDa) dialysis membranes. The vessels were placed in the electroelutor

apparatus, and the anodic and cathodic compartments of the apparatus were filled with electrode buffer (25 mM Tricine, 3.75 mM imidazole, pH 7.0). Protein bands from clear native gels can be identified by various techniques, for example by Coomassie staining of side strips from the same preparative gel, by fluorescent labeling of proteins, or by use of colored proteins. Here we used the reddish colored respiratory complex III. Bands of native protein complexes were cut out of the hrCNE gel and squeezed through syringes to mash the gel, and the gel debris were injected into the cathodic compartment of the electroelutor vessel. The gel debris were covered with transfer buffer supplemented with 0.02% DDM and 0.05% DOC. The anodic arm of the electroelutor vessel and the horizontal tube connecting anodic and cathodic arms were then filled with detergent-free transfer buffer, and electroelution was started (500 V and the current limited to 5 mA per vessel to avoid damage in case false buffer was used erroneously). The actual current per vessel was 2 mA. Electroelution was stopped after 4 h. The electroeluted protein was concentrated on the dialysis membrane of the anodic arm. Non-colored solutions were removed from the electroelutor vessel using a Pasteur pipette, leaving the concentrated electroeluted protein in a volume smaller than 0.5 ml in the anodic arm. The recovery of bovine heart complex III extracted from preparative hrCNE gels was >60% (data not shown).

RESULTS AND DISCUSSION

Separation of Dodecylmaltoside-solubilized Membrane Proteins by hrCNE, CNE, and BNE—To compare the performance of the native electrophoresis variants and to emphasize their special advantages and limitations, identical aliquots of the same sample (bovine heart mitochondria solubilized by dodecylmaltoside) were applied to all gels/strips shown in Fig. 1. Differences between the native electrophoresis variants (Fig. 1, *A–D*) must therefore be attributed solely to the use of different cathode buffers that specify the otherwise identical electrophoresis variants hrCNE-1, hrCNE-2, CNE, and BNE. The *left panels* in Fig. 1, *A–D*, show fixed and Coomassie-stained 1D gels; *lanes* to the *right* show catalytic activity assays for complexes I–V (*CI–CV*).

Following the choice of DDM for membrane solubilization, one specific cathode buffer from Table I was chosen next for hrCNE, either the "DDM buffer" containing 0.02% DDM and 0.05% DOC for hrCNE-1 or the "TX buffer" containing 0.05% Triton X-100 and 0.05% DOC for hrCNE-2. These buffers caused considerably different migration of mitochondrial complexes that is immediately apparent from the Coomassie-stained gel strips (*left panels* in Fig. 1, *A* and *B*). For comparison with published protocols, CNE using the basic cathode buffer but without any additions (Fig. 1*C*) and BNE using the same cathode buffer but with added Coomassie dye (Fig. 1*D*) are also shown. The resolution of mitochondrial complexes by hrCNE-1 using DDM buffer (Fig. 1*A*) and hrCNE-2 using TX buffer (Fig. 1*B*) was comparable to the resolution of BNE (Fig. 1*D*) and clearly superior to CNE (Fig. 1*C*). The optional use of one of two different cathode buffers for hrCNE relates to variations in the detergent sensitivity of proteins. DDM can be milder than Triton X-100 for one specific complex but less mild for another one. This was exemplified by in-gel catalytic activity assays (marked *CI–CV+*) for bovine mitochondrial complexes I–V (Fig. 1).

The NADH:NTB reductase assay for holocomplex I and its hydrophilic catalytic parts was non-critical. This assay was not even disturbed by the Coomassie dye used for BNE (Fig. 1*D*). However, using hrCNE-2 (TX buffer), a second band just beneath the band of complex I (Fig. 1*B*) indicated a catalytically active subcomplex. This means that complex I was partly dissociated by Triton X-100. Because it was not dissociated by the DDM buffer used in hrCNE-1 (Fig. 1*A*) this buffer is preferred for the analysis of bovine complex I.

The complex II in-gel activity assay resembled the complex I assay except that succinate was used as electron donor instead of NADH. It was therefore possible to perform complex I and II assays sequentially using the same native gel strip as exemplified by Fig. 1, *B* and *D*. The complex II assay was rather insensitive in BNE gels (Fig. 1*D*) compared with CNE gels with very high signal intensity but low resolution of complex II (Fig. 1*C*). Both hrCNE gel types (Fig. 1, *A* and *B*) were found to be superior to CNE and BNE because signal intensities were acceptable and resolution of complex II was high.

Complex III was the only complex for which no in-gel activity assay was available so far. The novel protocol used diaminobenzidine to stain complex III heme specifically. It essentially followed the common complex IV staining protocol; however, cytochrome *c* was omitted from the assay. Selective staining of complex III was achieved with hrCNE-1 and CNE gels (Fig. 1, *A* and *C*) but not with BNE (Fig. 1*D*) and hrCNE-2 gels (Fig. 1*B*).

Specific in-gel complex IV staining indicated that complex IV was isolated essentially in monomeric form by BNE (Fig. 1*D*), by hrCNE-2 (Fig. 1*B*), and presumably also by CNE (Fig. 1*C*). In the latter case, the presence of complex IV in monomeric state was not immediately evident as discussed recently (4). However, multiple association states of complex IV, presumably monomers, dimers, and tetramers, were separated by hrCNE-1 (Fig. 1*A*). Comparing hrCNE-1 (Fig. 1*A*) and hrCNE-2 (Fig. 1*B*) suggested that DDM, in contrast to Triton X-100, either preserved physiological oligomeric states of complex IV, as proposed recently (4), or promoted artificial homoassociation of complex IV.

Complex V, also named F_1F_0 ATP synthase, was stained by an ATP hydrolysis assay that can identify holo-F_1F_0 complex and catalytically active F_1 subcomplex as well. In CNE gels that had not been incubated with the complex V inhibitor oligomycin (Fig. 1*C*, *CV−*), monomeric holocomplex V (*M*) and catalytically active F_1 subcomplex (*F_1*) were identified. In CNE gels that were preincubated with oligomycin (Fig. 1*C*, *CV+*), only the free F_1 domain was identified because this subcomplex does not contain the oligomycin sensitivity-conferring protein and therefore could not be inhibited. Full inhibition of monomeric complex V in turn indicated that this holocomplex V was stable during the 1-h assay because any dissociation of F_1 subcomplex would be detected by ATP hydrolysis and lead phosphate precipitation at the position of monomeric complex V. The strong band of F_1 subcomplex that was seen below holocomplex V either was a stable assembly interme-

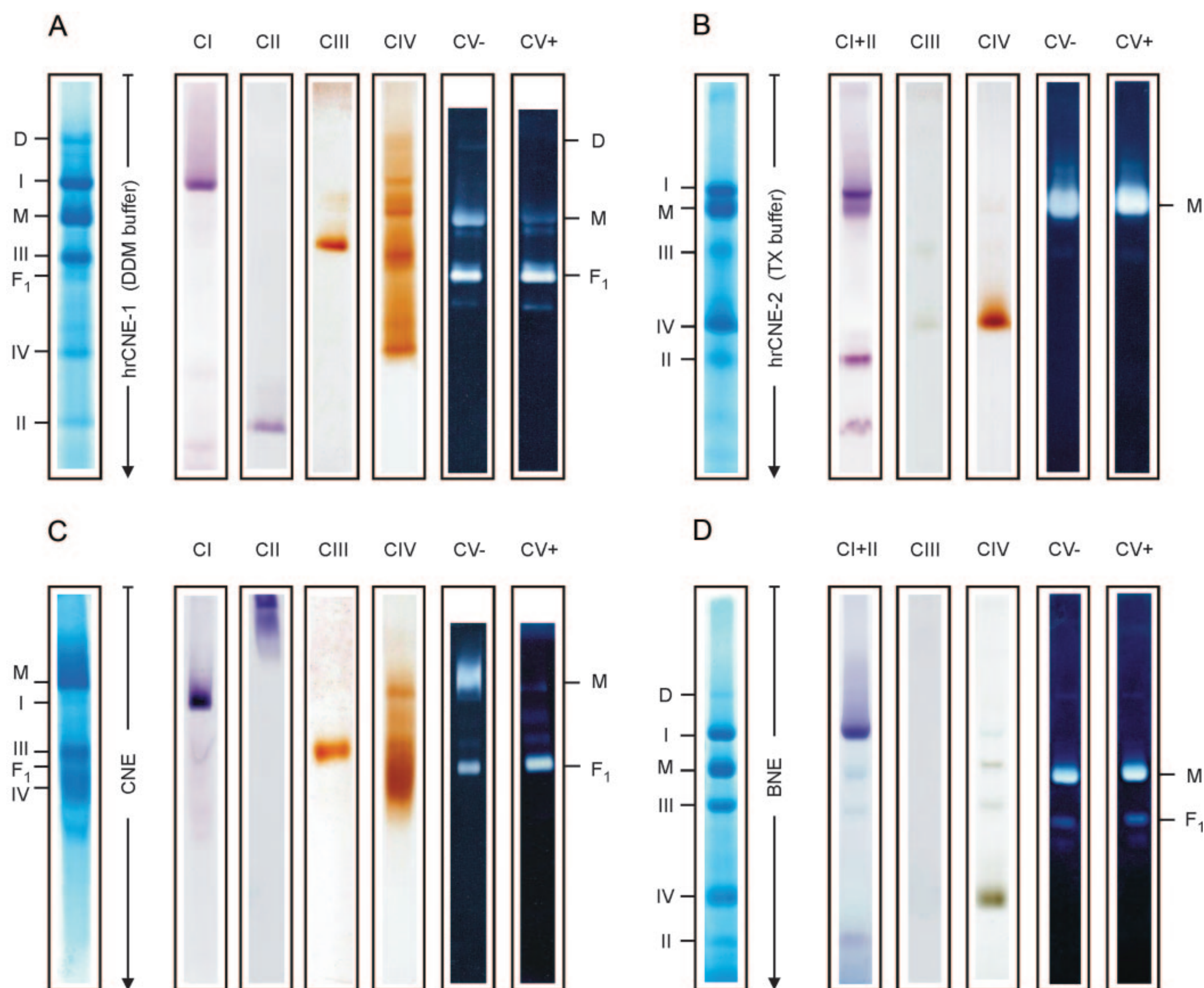

FIG. 1. **Specific features of native electrophoresis techniques using dodecylmaltoside-solubilized membrane protein complexes.** Bovine heart mitochondria were solubilized using a DDM/protein ratio of 2.5 (g/g). Identical aliquots were loaded on four identical acrylamide gradient gels but using four different cathode buffers (Table I) that specify the native electrophoresis system. *A*, hrCNE-1 using DDM buffer. *B*, hrCNE-2 using TX buffer. *C*, CNE using the basic cathode buffer. *D*, BNE using the basic buffer supplemented with Coomassie dye. *Left panels* show the Coomassie-stained native gel strips corresponding to the complex I–V (*CI–CV*) strips. *CI–CV*, in-gel catalytic activity assays for complexes I–V. *CI+II*, consecutive assays for complexes I and II using the same gel. *CV+* and *CV−*, ATP hydrolysis assays for complex V using (+) and not using (−) the inhibitor oligomycin. *I–V*, complexes I–V. *M* and *D*, monomeric and dimeric states, respectively, of complex V. F_1, F_1 subcomplex of complex V.

diate (22) or was dissociated from holocomplex V by the detergent DDM during membrane solubilization. Similarly complex V and F_1 domain were identified in BNE gels (Fig. 1*D*). However, in contrast to CNE, complex V was not oligomycin-sensitive following BNE (Fig. 1*D*, *CV+*). This is a serious disadvantage of BNE because the integrity of complex V cannot be tested by its sensitivity to oligomycin. The disadvantage of oligomycin insensitivity was also found for the hrCNE-2 variant using TX buffer (Fig. 1*B*) but not for hrCNE-1 (Fig. 1*A*), which at least partially retained oligomycin sensitivity.

All together resolution decreased in the order hrCNE-1 (DDM buffer), BNE, hrCNE-2 (TX buffer), and CNE. Variant hrCNE-2 (TX buffer, Fig. 1*B*) was preferred for the detection and quantification of complex IV. It yielded the highest ATP hydrolysis activity of complex V, but this activity was not oligomycin-sensitive. Variant hrCNE-1 (DDM buffer, Fig. 1*A*) was advantageous for complex I because no dissociation of complex I was detectable and for complex III, which was not detected when Triton X-100 (Fig. 1*B*) or Coomassie dye in BNE (Fig. 1*D*) was added to the cathode buffers.

Separation of Digitonin-solubilized Membrane Proteins by hrCNE and BNE—In an attempt to preserve physiological

TABLE I
Cathode buffers for use in CNE, BNE, and hrCNE variants 1–3

Buffers for hrCNE-1, hrCNE-2, and hrCNE-3 are usually abbreviated in the text as DDM buffer, TX buffer, and 0.01% DDM buffer, respectively.

	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
DDM (%)			0.02		0.01
DOC (%)			0.05	0.05	0.05
Triton X-100 (%)				0.05	
Coomassie Blue G-250 (%)		0.02 (0.002)[a]			
pH[b]	~7.0	~7.0	~7.0	~7.0	~7.0

[a] Cathode buffer for BNE containing 0.02% Coomassie dye is commonly replaced by a cathode buffer containing 0.002% Coomassie dye after one-third of the electrophoresis run.

[b] No pH correction except with Tricine or imidazole.

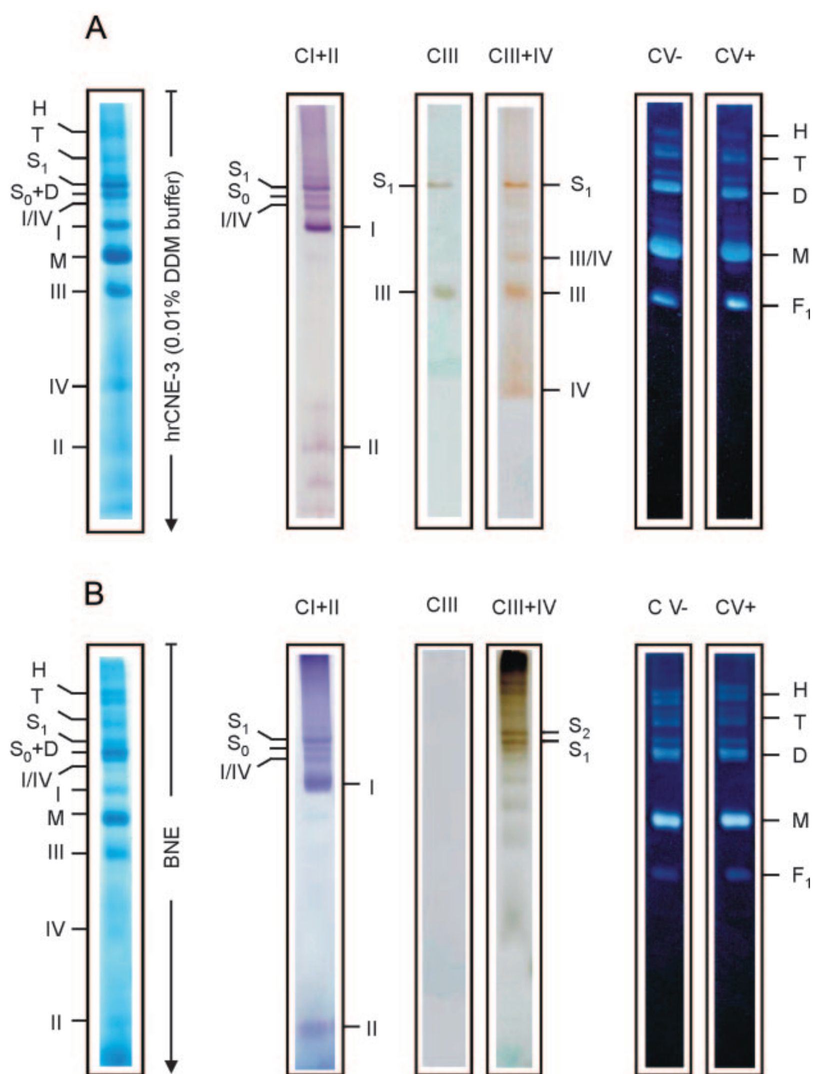

FIG. 2. **Digitonin-solubilized membrane protein supercomplexes are preserved using hrCNE and BNE.** Bovine heart mitochondria were solubilized using a digitonin/protein ratio of 2 (g/g). Identical aliquots were loaded on two identical acrylamide gradient gels. *A*, hrCNE-3 gel using 0.01% DDM buffer (Table I). *B*, BNE used as a reference gel. For assignment of complexes and in-gel assays, see Fig. 1. S_0, respiratory supercomplex containing monomeric complex I and dimeric complex III. S_1 and S_2, supercomplexes comprising the S_0 core complex and one (S_1) or two (S_2) copies of complex IV in addition. *I/IV*, association of complexes I and IV. *III/IV*, association of complexes III and IV. *CIII+IV*, consecutive in-gel stain of complexes III and IV in the same gel. *T* and *H*, tetrameric and hexameric states, respectively, of complex V.

associations of membrane protein complexes, the commonly used mild detergent digitonin was chosen for membrane solubilization. The optimal cathode buffer for the especially mild hrCNE-3 variant was 0.01% DDM buffer (cathode buffer containing 0.01% DDM and 0.05% DOC). The commonly used DDM buffer containing 0.02% DDM and 0.05% DOC dissociated almost all supramolecular associations into individual complexes similar to Fig. 1*A*. It should be noted that the

cathode buffer with a higher ratio of neutral/anionic detergent unexpectedly had stronger dissociating properties.

The resolution of identical mitochondrial digitonin extracts by BNE and hrCNE-3 (0.01% DDM buffer) was comparable (Fig. 2). Oligomeric forms of complex V and various respiratory supercomplexes were identified in both gel types. Respiratory supercomplexes were tentatively assigned according to in-gel activity assays as shown in Fig. 2 and according to 2D SDS gels (not shown).

Catalytic activity assays for complexes I and II were performed consecutively using the same gel (Fig. 2, *A* and *B*, *CI+II lanes*). The NADH:NTB reductase assay for complex I revealed a very similar pattern of complex I-containing bands in BNE and hrCNE gels. Supercomplexes S_0 and S_1 contained monomeric complex I, dimeric complex III, and zero (S_0) or one (S_1) copy of complex IV according to detailed previous analyses (14). A further band (*I/IV*) not containing complex III presumably was an association of complexes I and IV.

The novel in-gel assay for complex III revealed dimeric complex III and supercomplex S_1 in the hrCNE gel (Fig. 2*A*, *lane CIII*) but not in the BNE gel (Fig. 2*B*, *lane CIII*). Apparently the assay was disturbed by the Coomassie dye used for BNE. Subsequent addition of cytochrome *c* to start the complex IV assay led to the detection of two supercomplexes in the BNE gel that were tentatively assigned as supercomplexes S_1 and S_2 containing one (S_1) or two (S_2) copies of complex IV. Monomeric complex IV, an association of complexes III and IV, and supercomplex S_1 were detected in the hrCNE-3 gel.

The complex V assay revealed several oligomeric forms of complex V assigned to monomeric, dimeric, tetrameric, and hexameric states according to previous studies (4). Partial dissociation of these oligomeric forms may explain the multiplicity of observed bands. In contrast to the oligomycin-sensitive monomeric complex V in hrCNE-1 (Fig. 1*A*, *M*) that was prepared under less mild conditions (DDM for solubilization and 0.02% DDM cathode buffer), the oligomeric complex V forms in the hrCNE-3 gel were not or only partially sensitive to the inhibitor oligomycin. We propose that some digitonin from solubilization is still bound to the complexes and interferes with oligomycin. All together digitonin solubilization for hrCNE-3 is useful to isolate physiological associations of membrane protein complexes similar to BNE and can be used as an alternative technique to BNE to study supramolecular structures.

In-gel Analyses of Fluorescent Dye-labeled Proteins and Complexes—Fluorescent proteins are well established tools in cell biology and ideally suited to study protein topology and dynamics in cells (23–25). Another class of low mass fluorescent markers, the reactive fluorescent dyes, has been used to analyze differential protein expression by differential gel electrophoresis (26–28). Initial attempts to use the two classes of fluorescent labels for protein detection following BNE were characterized by considerable fluorescence quenching due to the Coomassie dye used in BNE. This suggested using hrCNE instead of BNE for protein separation. Here we labeled proteins in mitochondrial membranes with seven different fluorescent dyes to compare fluorescence intensities in BNE and hrCNE gels and to demonstrate the advantages of hrCNE.

Fluorescent labeled mitochondrial membranes were solubilized using the detergent dodecylmaltoside, and identical samples were loaded on hrCNE-1 and BNE gels (Fig. 3). The separated mitochondrial complexes were assigned on Coomassie-stained gel strips (*left panels*). For immediate comparison of the fluorescence intensities on hrCNE-1 and BNE gels, the gel strips were scanned side by side to guarantee identical settings and picture adjustments. The fluorescence intensities were considerably higher on the hrCNE gel (Fig. 3*A*) compared with the BNE gel (Fig. 3*B*). Using the fluorescent signal intensities of the complex V bands for densitometric quantification, the signals were about 10- or 20-fold higher on the hrCNE gel compared with the BNE gel, *i.e.* Coomassie dye in BNE quenched 90–95% of the fluorescence intensities.

Labeled subunits of complexes can be identified in 2D hrCNE/SDS gels as exemplified in Fig. 3*C* using a Cy3-labeled gel strip from 1D hrCNE (similar to *lane Cy3* in Fig. 3*A*). 1D BNE gel strips containing fluorescent labeled separated complexes can also be used for 2D BNE/SDS-PAGE as has been shown recently (28) because Coomassie dye is removed during the second dimension SDS-PAGE and can no longer contribute to fluorescence quenching. Therefore, 2D BNE/SDS and 2D hrCNE/SDS gels show comparable results despite the considerable advantages of 1D hrCNE compared with 1D BNE.

In-gel Analysis of Yellow Fluorescent Protein (YFP)-tagged Proteins and Complexes—Using fluorescent protein tags instead of reactive fluorescent dyes, genetic work can be immediately linked to protein chemical analysis. Here we analyzed mitochondria from a *Y. lipolytica* yeast strain expressing YFP fused to the 30-kDa subunit of respiratory complex I (30 kDa-YFP). Equal amounts of DDM- and digitonin-solubilized *Yarrowia* mitochondria were loaded on hrCNE-1 and BNE gels (Fig. 3*D*, *left* and *right panels*, respectively). Following electrophoresis the native gels were scanned for fluorescence intensity of the 30 kDa-YFP fusion protein, which was normally assembled into respiratory complex I. The fluorescent signal of the 30 kDa-YFP fusion protein was hardly detectable in the 1D BNE gel due to about 90% quenching by Coomassie dye (Fig. 3*D*). Loading controls using 2D BN/SDS and 2D hrCNE/SDS gels confirmed comparable loading (data not shown).

Migration of Water-soluble Proteins in hrCNE—Following the analysis of membrane protein complexes we asked whether the anionic detergent deoxycholate can also bind to water-soluble proteins during hrCNE, thereby imposing a charge shift on the proteins and pulling the water-soluble proteins to the anode irrespective of the intrinsic pI of the protein. If no charge shift were imposed, neutral or basic water-soluble proteins would not enter the hrCNE gels. Therefore, the migration in hrCNE gels of several water-soluble

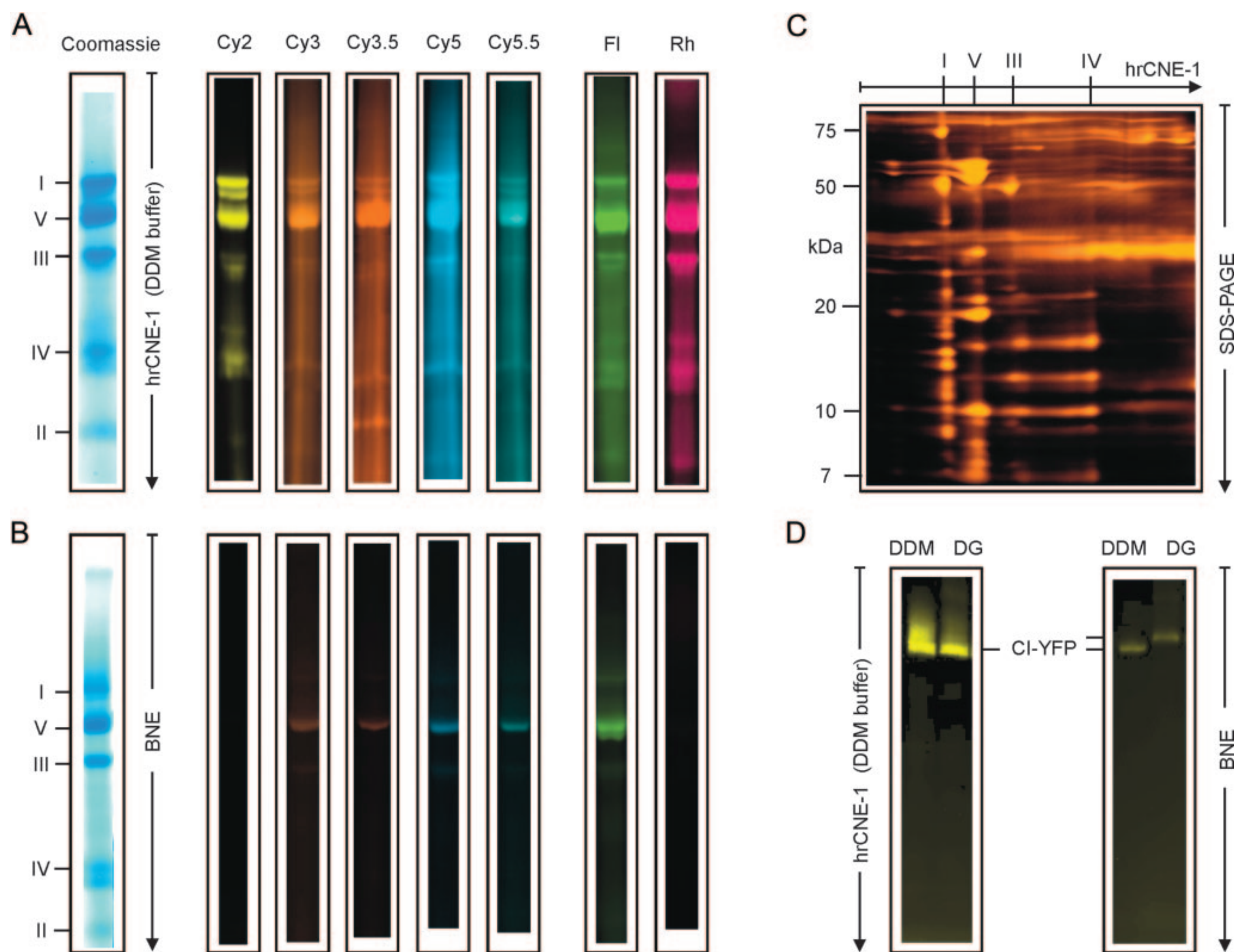

FIG. 3. **Fluorescent signal intensities in hrCNE and BNE gels.** Bovine heart mitochondria were labeled with NHS fluorescent dyes (Cy2, Cy3, Cy3.5, Cy5, and Cy5.5), fluorescein (*Fl*), and rhodamine (*Rh*). Labeled mitochondrial membranes were solubilized using a DDM/protein ratio of 2.5 (g/g), and equal amounts were loaded on native gels for hrCNE-1 **(*A*)** and BNE **(*B*)**. Migration distances of mitochondrial complexes I-V (*I–V*) were assigned on Coomassie-stained gel strips (*left panels*). Corresponding hrCNE and BNE gel strips (Cy2, Cy3, Cy3.5, Cy5, Cy5.5, fluorescein, and rhodamine) were scanned side by side for direct comparison of the fluorescent signals and are shown as pseudocolors. The signals were around 10–20-fold higher in hrCNE (*upper panels*) compared with BNE (*lower panels*). *C*, 2D hrCNE/SDS gel using a Cy3-labeled gel strip, similar to *strip Cy3* in *A*, to identify the subunits of mitochondrial complexes. *D*, identification of YFP-tagged respiratory complex I (*CI-YFP*) following separation by hrCNE-1 (*left panel*) or BNE (*right panel*). Mitochondria from the yeast strain *Y. lipolytica* were solubilized by DDM and digitonin (*DG*) using detergent/protein ratios of 1.6 and 3 (g/g), respectively, and equal amounts were loaded onto the gel wells. The YFP fluorescence signals were around 10-fold higher in hrCNE compared with BNE, which suffered from considerable fluorescence quenching by Coomassie dye.

proteins (Table II, protein numbers 2–10) was analyzed and compared with the migration in BNE (Fig. 4). The selected marker proteins have previously been found useful for molecular mass determination by BNE because at least one of two prerequisites to guarantee sufficient electrophoretic mobility was met: the isoelectric point of the protein was not higher than pI = 5.5 (Table II, protein numbers 2–4 and 7) or a charge shift was induced on proteins with pI > 5.5 by binding a high number of Coomassie dye molecules (Table II, proteins numbers 5, 6, and 8–10). Membrane proteins (protein numbers 1a–1e) and acidic proteins (protein numbers 2–4 and 7) showed normal size-dependent anodic migration in BNE, hrCNE-1, and hrCNE-2 gels, but only one or two basic proteins (protein numbers 8 and 10) showed size-proportional migration in hrCNE-2 and hrCNE-1 gels, respectively. The electrophoretic mobility of other basic proteins (protein numbers 6 and 9) was very low and clearly independent of the protein size indicating that no sufficient charge shift by binding deoxycholate had occurred. Therefore, the utility of hrCNE for a given water-soluble protein has to be tested empirically. With the exception of acidic marker proteins, hrCNE seems even less suited for mass determination of native water-soluble proteins than BNE.

Conclusions—High resolution clear native electrophoresis

TABLE II
Native membrane and water-soluble proteins analyzed on BNE and hrCNE gels

Protein	No. in Fig. 4	Source	pI	Molecular mass
				kDa
Membrane protein complexes				
Respiratory complex I	1a	Bovine heart		1000
ATP synthase (complex V)	1b	Bovine heart		~700
Respiratory complex III	1c	Bovine heart		500
Respiratory complex IV	1d	Bovine heart		200
Respiratory complex II	1e	Bovine heart		130
Water-soluble proteins with pI ≤ 5.5				
Thyroglobulin	2	Bovine thyroidea	5.5	669
Apoferritin (dimer)	3d	Equine spleen	4.2–4.5	886
Apoferritin (monomer)	3m	Equine spleen	4.2–4.5	443
β -Amylase	4	Sweet potato	5.2	~200
Albumin (dimer)	7d	Bovine serum	4.9	132
Albumin (monomer)	7m	Bovine serum	4.9	66
Water-soluble proteins with pI > 5.5				
L-Lactate dehydrogenase	5	Rat muscle	8.6	140
Conalbumin	6d	Chicken egg	5.9	156
Conalbumin	6m	Chicken egg	5.9	78
Carbonic anhydrase II	8	Bovine ery.[a]	5.9	29
Trypsinogen	9	Bovine pancreas	9.3	24
Cytochrome *c*	10	Equine spleen	10.7	12

[a] Erythrocytes.

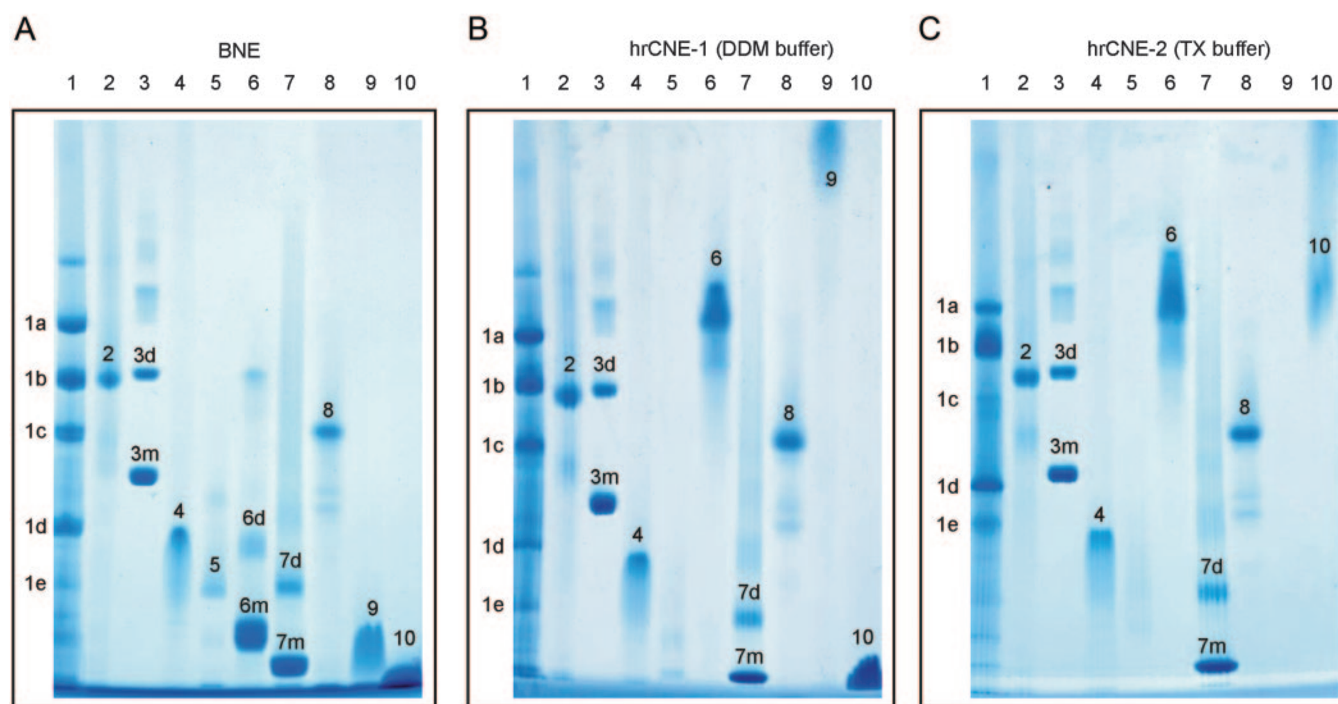

FIG. 4. **Separation of water-soluble and membrane proteins by native electrophoresis variants.** Membrane protein complexes from dodecylmaltoside-solubilized mitochondria (Table II, protein numbers 1a–1e) and several water-soluble proteins (Table II, protein numbers 2–10) were separated by BNE (*A*), hrCNE-1 (*B*), and hrCNE-2 (*C*). Assignments *m* and *d* indicate monomeric and dimeric states, respectively, of the specific protein.

variants were developed that offer high resolution comparable to blue native electrophoresis and that have considerable advantages with regard to in-gel catalytic activity and fluorescence assays. Therefore, hrCNE seems to be preferable to BNE for functional proteomics studies. In the present study, we exemplified the principles of the novel technique using isolated mitochondria from bovine heart and the yeast *Y. lipolytica*. Numerous further applications seem conceivable.

Currently we are using hrCNE for the functional analysis and quantitation of mitochondrial complexes in human mitochondrial disorders.[2] Green fluorescent protein- and red fluorescent protein-tagged mitochondrial complex I has been used for studies of mitochondrial fusion processes (29). A prerequisite for this work was to verify successful incorporation of the tagged protein subunit into the holocomplex as previously performed by 2D BNE/SDS gels (29). In the future, verification of successful incorporation will be considerably facilitated because this can now be analyzed immediately in 1D hrCNE gels. Another advantage of hrCNE gels is the high sensitivity of fluorescent labeling of proteins by reactive dyes. The sensitivity can approach or exceed the sensitivity of silver staining and, in contrast to silver stain, is well suited for densitometric quantification. Therefore, reactive dye protein labeling seems especially useful for quantitative analyses of low protein amounts, *e.g.* for quantification of mitochondrial complexes in cultured cells (also of patients with mitochondrial disorders), and to substitute for immunological detection on Western blots or for detection of very low protein amounts by radioactive labeling. Sequential labeling of proteins by a number of different reactive dyes at different stages of solubilization (membrane, supercomplex, individual complex, subcomplex, and individual subunit) is another interesting approach. The (in)accessibility of the reactive dye to a specific protein at a certain step can point to hidden/accessible protein surfaces that may help to characterize the interfaces of complexes in supercomplexes and to investigate large physiological supramolecular structures in biological membranes, a very active field in functional proteomics (4, 7, 14, 28–36).

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