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REGULAR ARTICLE

Advantages and limitations of clear-native PAGE

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Clear-native PAGE (CN-PAGE) separates acidic water-soluble and membrane proteins (p$I < 7$) in an acrylamide gradient gel, and usually has lower resolution than blue-native PAGE (BN-PAGE). The migration distance depends on the protein intrinsic charge, and on the pore size of the gradient gel. This complicates estimation of native masses and oligomerization states when compared to BN-PAGE, which uses negatively charged protein-bound Coomassie-dye to impose a charge shift on the proteins. Therefore, BN-PAGE rather than CN-PAGE is commonly used for standard analyses. However, CN-PAGE offers advantages whenever Coomassie-dye interferes with techniques required to further analyze the native complexes, *e.g.,* determination of catalytic activities, as shown here for mitochondrial ATP synthase, or efficient microscale separation of membrane protein complexes for fluorescence resonance energy transfer (FRET) analyses. CN-PAGE is milder than BN-PAGE. Especially the combination of digitonin and CN-PAGE can retain labile supramolecular assemblies of membrane protein complexes that are dissociated under the conditions of BN-PAGE. Enzymatically active oligomeric states of mitochondrial ATP synthase previously not detected using BN-PAGE were identified by CN-PAGE.

Keywords:
ATP synthase / Blue-native / Clear-native / Respiratory chain / Supercomplexes

1 Introduction

A basic version of clear-native PAGE (CN-PAGE), originally termed colorless native PAGE [1], has been described shortly after the development of blue-native PAGE (BN-PAGE [2]). It was mainly used as a reference for BN-PAGE in order to illustrate the important role of the negatively charged Coomassie-dye for the preferred BN-PAGE. Since no charged dye is used in CN-PAGE, the electrophoretic mobility of proteins in CN-PAGE (in contrast to the charge shift technique BN-PAGE) depends on the intrinsic charge of the proteins. Molecular masses and oligomeric states of proteins and complexes are not easily determined by CN-PAGE unless the proteins of interest have favorable physical parameters, especially p*I*s below a limit of 5.4. Also, the resolution of CN-PAGE was found to be considerably lower compared to BN-PAGE that has been established in many laboratories as an excellent technique in biochemical and clinical research. For these reasons, application of CN-PAGE for resolution of membrane protein complexes seemed to offer no advantages, except for studies requiring non-stained proteins. So far, it was not often used until neutral detergents for protein solubilization like Triton X-100 or dodecylmaltoside (DDM) were replaced by digitonin, which can retain intact supramolecular membrane protein assemblies [3–7]. We found that CN-PAGE allows for a considerably improved sensitivity of the in-gel ATP hydrolysis assay for yeast mitochondrial ATP synthase. Sensitivity was at least 10-times higher compared to the in-gel assay in BN-gels. In contrast to BN-PAGE, this activity was sensitive to an inhibitor of ATP synthase [7].

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Abbreviations: BN, blue-native; **CN**, clear-native; **complex I**, NADH dehydrogenase complex; **complex II**, succinate ubiquinone reductase; **complex III**, ubiquinol cytochrome *c* reductase; **complex IV**, cytochrome *c* oxidase; **complex V**, ATP synthase; **DDM**, dodecylmaltoside; **FRET**, fluorescence resonance energy transfer; **Mapp**, apparent mass

Although we were convinced that CN-PAGE could not be optimized to reach the high-resolution power of BN-PAGE, a potential advantage seemed to be the milder conditions of CN-PAGE, which might allow for isolation of further physiological protein assemblies previously not detected by BN-PAGE [8].

Therefore the focus of the present work was to critically evaluate the CN-PAGE protocol, especially with respect to the detergent digitonin that is one of the mildest detergents for retaining physiological membrane protein assemblies. One-dimensional CN-PAGE was used to separate native complexes and supercomplexes. The complexes contained in supercomplexes were preferably identified following 2-D BN-PAGE, and the protein subunits could optionally be identified by 3-D SDS-PAGE.

2 Materials and methods

2.1 Chemicals

The chemicals 6-aminohexanoic acid, imidazole, and digitonin (catalog number 37006, purity > 50%) were obtained from Fluka. Digitonin was used directly without recrystallization. Ferritin from horse spleen, acrylamide and bisacrylamide (the commercial 2 × crystallized products), and Coomassie blue G-250 (Serva Blue G) were purchased from Serva. All other chemicals were from Sigma.

2.2 Sample preparation for BN-PAGE and CN-PAGE

Rat heart (50 mg) was homogenized in 500 μL sucrose buffer (250 mM sucrose, 20 mM Na-phosphate, pH 7). 5 mg aliquots were sedimented by 10 min centrifugation at 10 000 × *g*, and the sediment was used immediately for protein solubilization or stored at −80°C. Pellets from 5 mg of heart were suspended with 35 μL solubilization buffer (50 mM NaCl, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7), and solubilized with 8 μL digitonin (20% stock in water). Following 15 min centrifugation at 100 000 × *g*, Coomassie blue G250-dye (8 μL from a 5% stock in 750 mM 6-aminohexanoic acid) or Ponceau S-dye (1 μL from a 0.1% stock in water) was added to the supernatant for BN-PAGE or CN-PAGE, respectively. The colored solutions were applied to 0.15 × 1 cm sample gel wells for BN-PAGE or CN-PAGE.

Instead of total heart homogenates, also purified mitochondria were used. Rat heart mitochondria were prepared according to Jakobus and Saks [9] but without using BSA and trypsin. Crude mitochondria were further purified on a sucrose step gradient as described for yeast mitochondria [10] except that the layers contained 15, 23, 32, 37, 47, 55, and 60% sucrose. The band on top of the 47% sucrose layer contained the purified mitochondria. Aliquots containing 250 μg mitochondrial protein were sedimented by 10 min centrifugation at 10 000 × *g*, and solubilized by adding 40 μL solubilization buffer (50 mM NaCl, 50 mì imidazole, 2 mM 6-aminohexanoic acid, 1 mì EDTA, pH 7), and 2.5, 5.0, and 10 μL digitonin (20% stock in water) to set digitonin/protein ratios of 2, 4, and 8 g/g, respectively. Following 15 min centrifugation at 100 000 × *g*, Coomassie-dye (2.5, 5 and 10 μL of a 5% Coomassie-stock in 750 mM 6-aminohexanoic acid) or Ponceau S-dye (uniformly 1 μL from a 0.1% stock in water for all three samples) was added to the three supernatants for BN-PAGE or CN-PAGE, respectively. The total supernatants were applied to 0.15 × 1 cm sample gel wells for BN-PAGE or CN-PAGE.

2.3 Electrophoretic techniques and gels

For CN-PAGE and BN-PAGE [11] the same buffers and running conditions were used, except that for CN-PAGE the applied sample and the cathode buffer contained no Coomassie-dye, and 0.025% digitonin was included in linear 3 to 13% acrylamide gradient gels. It should be noted that membrane solubilization conditions, as described in 2.2, and the gel buffers used here for CN-PAGE, as described for BN-PAGE [11], differ from the original papers on BN-PAGE and CN-PAGE [2, 1]. BN-PAGE, 2-D BN-PAGE (with 0.02% dodecylmaltoside added to the cathode buffer), and 3-D SDS-PAGE were performed as described [11, 12, 8], except that linear 4 to 13% acrylamide gradient gels were used for BN-PAGE, and linear 5 to 13% acrylamide gradient gels for 2-D BN-PAGE.

2.4 In-gel quantification of protein and catalytic activity

Densitometric in-gel quantification of Coomassie G250-stained proteins and of lead-phosphate precipitates generated during in-gel ATP hydrolysis assays [13] was performed using a Personal Densitometer (Molecular Dynamics) as described [14]. The in-gel ATPase assay [13] was modified: 1) CN-gels were used instead of BN-gels to allow for a rapid oligomycin-sensitive assay. 2) CN-gels were pre-incubated for 3 h in 270 mM glycine, 35 mM Tris, pH 8.4. This increases ATPase rates 2–4 fold (see 3.5) and permits complete inhibition of ATP synthase in a parallel assay supplemented with oligomycin (5 μg/mL). 3) The ATP hydrolysis assay in 270 mM glycine, 35 mM Tris, 8 mM ATP, 14 mM MgSO4, 0.2% $Pb(NO_3)_2$, pH 8.4, had to be stopped after 5–10 min by 30 min incubation in 50% methanol, 50% water, followed by 30 min incubation in water. 4) Following densitometry the lead-phosphate precipitates were dissolved by overnight incubation in 50% methanol, 10% acetic acid, and the gel was restained for 60 min with 0.025% Coomassie G-250 dye in 10% acetic acid. For background destaining 10% acetic acid was used, and finally the gel was transferred to water. Coomassie-staining of native and SDS-gels [12] and silver-staining [15] were used as described.

3 Results and discussion

3.1 General differences between CN-PAGE and BN-PAGE

Characteristics of BN-PAGE and CN-PAGE using dodecylmaltoside-solubilized mammalian mitochondrial complexes have been described [1]. Some results that help to interpret the results of CN-PAGE following protein-solubilization with a different detergent, namely digitonin, are briefly summarized here. 1) It was concluded that CN-PAGE can be used for native mass determination only if the p*I* of a protein is low (p$I < 5.4$). 2) The electrophoretic mobility of the mitochondrial membrane protein complexes studied was reduced compared to BN-PAGE (Fig. 7B in [1]). However, all proteins migrated to the anode, indicating that the p*I* values of all mitochondrial complexes were below 7.5 which is the running pH in BN- and CN-gels. 3) NADH dehydrogenase complex (complex I) and ubiquinol cytochrome *c* reductase (complex III) showed moderately reduced electrophoretic mobility. ATP synthase (complex V) was stronger retarded than complexes I and III, but less than the severely retarded cytochrome *c* oxidase (complex IV) which is isolated in monomeric form using the detergents dodecylmaltoside or Triton X-100. All together, these effects caused coinciding or not well separated bands of complexes I and V in CN-PAGE, and complex IV migrated close to the band of complex III (Fig. 2 in [1]). 4) As described in the following, the assignment of bands in CN-PAGE for individual dodecylmaltoside-solubilized complexes (dodecylmaltoside/CN-PAGE) was simple compared to digitonin/CN-PAGE using the milder detergent digitonin.

Knowing the migration properties of the individual complexes in dodecylmaltoside/CN-PAGE, and also the migration properties of the digitonin-solubilized respiratory chain supercomplexes and dimeric ATP synthase in BN-PAGE (Fig. 1A, upper panel and [8]), helped to assign also the diffuse bands in digitonin/CN-PAGE (Fig. 1B, upper panel). However, the most important technique for the identification of the constituent complexes in supercomplexes separated by CN-PAGE was a 2-D resolution (native in both dimensions) by 2-D BN-PAGE (Fig. 1, lower panels). This

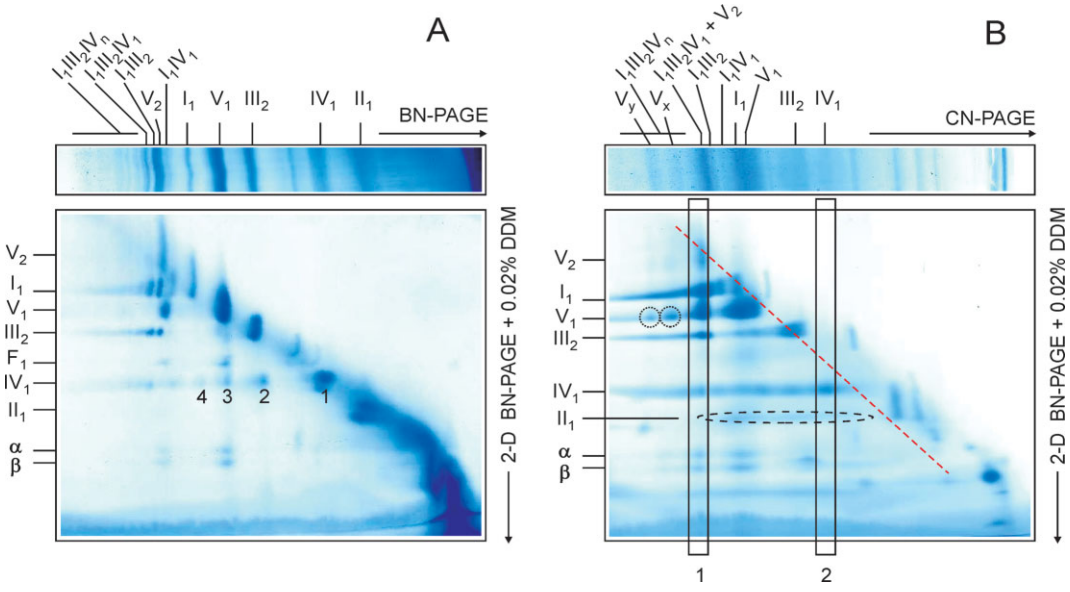

Figure 1. Two-dimensional native resolution of rat heart mitochondrial complexes. Digitonin-solubilized mitochondrial complexes from rat heart homogenates were separated by 1-D BN-PAGE (A, upper panel), and 1-D CN-PAGE (B, upper panel). The 1-D separated respiratory chain supercomplexes were dissociated into the individual complexes by 2-D BN-PAGE, using dodecylmaltoside (DDM) in the cathode buffer (lower panels). I_1, II_1, IV_1, V_1, and F_1 indicate the individual monomeric complexes I, II, IV, V, and the catalytic F_1-domain of complex V, respectively. V_2 and III_2, dimeric complexes V and III. Identified supercomplexes were: I_1IV_1, an associate of monomeric complexes I and IV; I_1III_2, the core structure of all larger supercomplexes containing monomeric complex I and dimeric complex III; $I_1III_2IV_1$ and $I_1III_2IV_n$ are supercomplexes that contain also one or n copies of complex IV (n = 1–4 for bovine heart [8]). The α and β subunits of complex V (α, β) dissociated from the holo-complex during 2-D BN-PAGE. (A, lower panel) Numbers 1–4 were assigned to complex IV monomer (1), and to complex IV that was dissociated from dimeric complex IV (2), tetrameric complex IV (4), and a III_2IV_1-supercomplex (3; cf. 3.2). (B) V_x and V_y mark oligomeric forms of complex V that were dissociated into monomeric complex V (V_1, circles in 2-D gel). Complex II was detected as an extended band (II_1, ellipsoid in 2-D gel). The dashed red line crossing monomeric complex I and dimeric complex III was used to visualize the lower electrophoretic mobilities (location below the red line) of complexes V and IV in 1-D CN-PAGE (cf. 3.1 and 3.2). Gel strips (boxed and marked 1 and 2) were excised from the 2-D gel, and used for separation of the subunits of complexes by 3-D SDS-PAGE (Fig. 2).

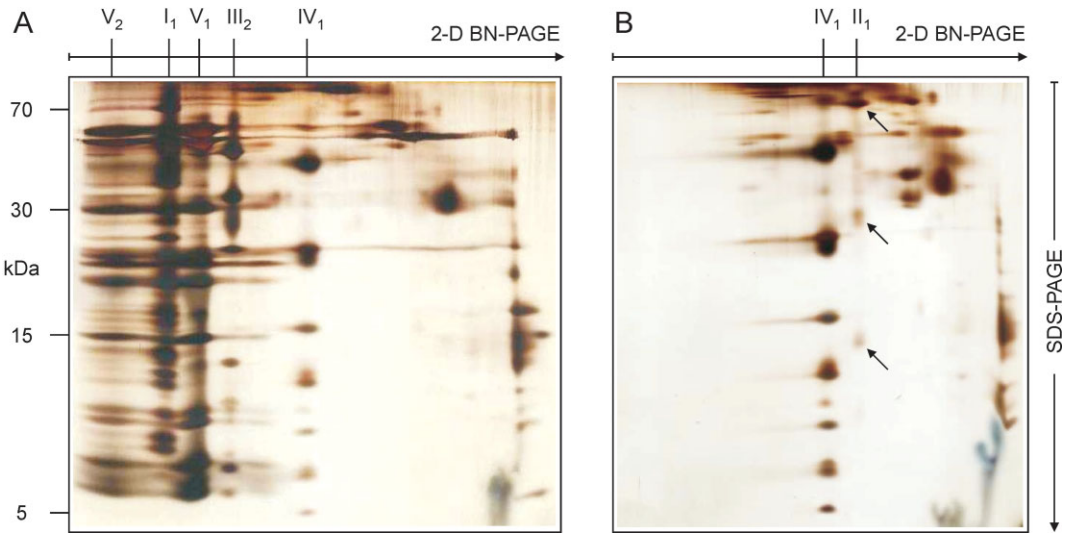

Figure 2. 3-D SDS-PAGE identifying the constituent proteins of supercomplexes. Assignment of complexes as in Fig. 1. (A) Gel strip 1 from the 2-D native gel (Fig. 1B, lower panel) was analyzed. The characteristic protein subunit patterns of complexes indicated the presence of complexes I, III, and IV in the supercomplex analyzed, and large amounts of dimeric complex V comigrating with this supercomplex in CN-PAGE. (B) Analysis of gel strip 2 from the 2-D native gel (Fig. 1B, lower panel) identified complexes IV and II (three complex II subunits marked by arrows).

2-D BN-PAGE using dodecylmaltoside in the cathode buffer caused dissociation of supramolecular structures into the individual complexes. The protein subunits and the characteristic polypeptide patterns of the complexes could be identified after resolution of individual lanes, as exemplified in Fig. 2.

Comparing 1-D digitonin/BN-PAGE using 4 to 13% acrylamide gradient gels and 1-D digitonin/CN-PAGE using 3 to 13% gels (Fig. 1, upper panels), indicated a considerably reduced electrophoretic migration in CN-PAGE for the specific membrane protein complexes studied. The lower resolution power of CN-PAGE compared to BN-PAGE, was immediately apparent. Complex I and all supramolecular assemblies containing complexes I and III migrated closer to monomeric complex V, and complex IV was closer to complex III, as expected from the results using dodecylmaltoside/CN-PAGE [1].

3.2 Oligomeric states of cytochrome *c* oxidase

Starting with digitonin/BN-PAGE, several spots for complex IV could be identified after 2-D BN-PAGE (numbered 1, 2, 3, and 4 in Fig. 1A, lower panel). Corresponding apparent masses in 1-D BN-PAGE were around 200, 450, 700, and 900 kDa, respectively, using monomeric complex II (M_r 130), dimeric complex III (M_r 500), and monomeric complex I (M_r 1000) for calibration. Since complex IV seemed to be the only dissociation product from the supramolecular assemblies in 1-D BN-PAGE, complex IV spots 1, 2, and 4 were assigned to monomeric, dimeric, and tetrameric complex IV in 1-D BN-PAGE. Some complex IV (no. 3) was detected in a column of spots below monomeric complex V, suggesting that in 1-D BN-PAGE either trimeric complex IV (M_r 600) or an assembly of dimeric complex III with monomeric complex IV (III_2IV_1, M_r 700) comigrated with monomeric complex V (apparent mass (M_{app}) around 700 kDa). Three-dimensional SDS-PAGE revealed the presence of complexes V, IV, and III (not shown). This seemed to support the assumption, that spot no. 3 in 2-D BN-PAGE originated from a III_2IV_1-complex but the additional existence of trimeric complex IV in 1-D BN-PAGE cannot be excluded. Previously, the oligomeric forms of complex IV were not clearly detected in bovine heart mitochondria, and may indicate a higher stability of oligomeric complex IV in rat heart. Further spots for complex IV were detected after dissociation of supercomplexes (below the supercomplexes assigned in 1-D BN-PAGE). These supercomplexes have been assigned and characterized previously for bovine heart mitochondria, with the exception of the I_1IV_1-complex that has not been observed directly but only under slightly different conditions [8]. This latter complex contained monomeric complex I and presumably monomeric complex IV, since an apparent mass of around 1.2 MDa was determined using supercomplex I_1III_2 (1.5 MDa), monomeric complex I (1.0 MDa), and dimeric complex III (500 kDa) for calibration.

Starting with digitonin/CN-PAGE followed by 2-D BN-PAGE (Fig. 1B) individual complex IV was found as an extended band with some locally increased staining intensities that could not be assigned reliably to specific oligomeric states of complex IV. However, we addressed the principal question whether or not complex IV is retained as a dimer under the mild conditions of digitonin/CN-PAGE.

Complex IV was clearly located below the dashed red diagonal in 2-D BN-PAGE (Fig. 1B, lower panel) that crossed the spots for monomeric complex I and dimeric complex III. Complexes I and III were used as markers, since they did not change their oligomeric state during 1-D CN-PAGE and 2-D BN-PAGE. Monomeric complex V and monomeric complex IV were found below that diagonal. In the 2-D electrophoresis system 1-D BN-PAGE/2-D BN-PAGE this position would clearly indicate that the size/oligomeric state of a supramolecular structure has been altered upon transition from 1-D to 2-D separation. However, since it is known that the electrophoretic mobilities of complexes V and IV in CN-PAGE are more strongly reduced than the mobilities of complexes I and III, as discussed in 3.1, we conclude that there is no evidence for retention of a dimeric state of complex IV during digitonin/CN-PAGE.

3.3 Identification of respiratory chain supercomplexes

Further locally increased concentrations of complex IV in the extended complex IV band after 1-D CN-PAGE and 2-D BN-PAGE (Fig. 1B) were assigned to respiratory chain supercomplexes, since the patterns of complexes were similar to 1-D BN-PAGE followed by 2-D BN-PAGE (Fig. 1A). The identity of the individual complexes was also determined by 3-D SDS-PAGE (Fig. 2A), as exemplified for the supercomplex that comigrated with dimeric complex V in 1-D CN-PAGE (lane 1 in Fig. 1B). The resolution of supercomplexes in CN-PAGE was considerably lower than in BN-PAGE. However, the amounts of retained supercomplexes, especially those containing a higher copy number of complex IV ($I_1III_2IV_n$) seemed to be higher, since the same sample amounts were used for 1-D BN-PAGE and 1-D CN-PAGE. Plotting the migration distances in CN-PAGE for supercomplex I_1III_2, complex I, and dimeric complex III versus the masses known from BN-PAGE (1500, 1000, and 500 kDa, respectively) on a logarithmic scale resulted in a straight calibration line (Fig. 3A), suggesting that the p*I* values of complexes I and III are very similar. Therefore, an estimation of the apparent masses and an estimation of the number of complex IV copies contained in supercomplexes was possible using this calibration line. The values, 1.25 MDa for the I_1IV_1-complex, and 1.65 MDa for the $I_1III_2IV_1$-complex were in good agreement with those obtained by BN-PAGE.

3.4 Oligomeric forms of ATP synthase

In digitonin/CN-PAGE two bands assigned V_x and V_y were detected that were not observed in digitonin/BN-PAGE (Fig. 1, upper panels). These two bands were dissociated by 2-D BN-PAGE into monomeric complex V (circles), and therefore represent higher oligomeric states of complex V that are present in addition to the previously described monomeric and dimeric forms identified also in digitonin/BN-PAGE [3–8, 16–21]. We asked whether the monomeric and oligomeric forms were enzymatically active, since bovine dimeric ATP synthase has been described to represent an inactive storage form of ATP synthase, and oligomerization has been suggested to be involved in regulation of ATP synthase activity [22]. In yeast, however, the dimeric form was found active and required for mitochondrial cristae formation [7, 16–19]. Complex V from *Chlamydomonas reinhardtii* mitochondria has been shown to exist only as an active unusually stable dimer. No dissociation of monomers or F_1-domains was observed upon dodecylmaltoside treatment [23].

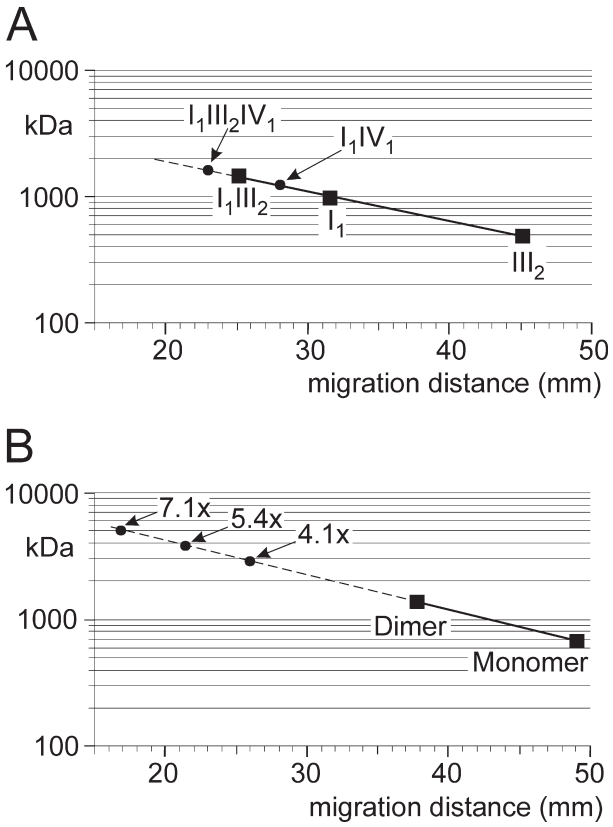

Figure 3. Estimation of native protein masses and oligomeric states in CN-gels. ■ marks protein complexes used for calibration, ● marks analyzed complexes. (A) In order to estimate the number of complex IV copies in supercomplexes separated in CN-gels we used the known masses for dimeric complex III (III_2, M_r 500), monomeric complex I (I_1, M_r 1000), and a supercomplex containing monomeric complex I, dimeric complex III (I_1III_2, M_r 1500), and the corresponding migration distances in CN-PAGE from Fig. 1B. Complex I_1IV_1 (M_{app} approximately 1.25 MDa) contained monomeric complex I and one copy of complex IV (M_r 200). Complex $I_1III_2IV_1$ (M_{app} approximately 1.65 MDa) contained monomeric complex I, dimeric complex III, and one copy of complex IV. (B) The copy number of complex V in the oligomers from Fig. 4A was estimated using the monomeric and dimeric forms for extrapolation. The bands next to the dimer showed 4.1, 5.4, and 7.1 times the apparent mass of the monomer, and were assigned as complex V tetramers, hexamers and octamers, respectively (cf. 3.4).

We analyzed the in-gel ATP hydrolysis activity of complex V in CN-PAGE after solubilization of mitochondria using variable amounts of digitonin (Fig. 4A, left panel), and found catalytic activity for the monomeric and oligomeric forms as well. Activities of all bands could virtually completely be inhibited by preincubation with oligomycin (Fig. 4A, central panel), indicating that holo-ATPase was retained during in-gel activity stain, and catalytic F_1-domains were not dissociated. The fastest migrating active band was identified as monomeric complex V after 2-D BN-PAGE (Fig. 4B). Using the masses of monomeric and dimeric ATP synthase (700 and 1400 kDa, as determined by 1-D BN-PAGE, Fig. 1A), and the migration distances in 1-D CN-PAGE (Fig. 4A, left panel) a straight calibration line on a semi-logarithmic plot was drawn for the estimation of oligomeric states (Fig. 3B). This seems to give a reasonable estimate of the native masses, if homo-oligomers with comparable p*I* values are analyzed. No hints on hetero-oligomers were found, *i.e.,* we could not detect any increased amounts of complexes I-IV comigrating with the oligomeric forms of complex V, except those that can be assigned to respiratory chain supercomplexes according to the results of BN-PAGE (Figs. 1 and 4B). Extrapolating the calibration line, the next bands above the dimer had 4.1, 5.4 and 7.1 times the mass of the monomer (Fig. 3B). Since no indications for a trimeric form were obtained, this suggested that the higher mass forms of complex V are assemblies of dimers, *i.e.,* tetramers, hexamers, and octamers. It seems not possible to assign a trimeric state to the band next to the dimer, although the calibration curve for the low acrylamide area in CN-gels might not be as linear as for the 5 to 18% acrylamide range analyzed previously (Fig. 7B in [1]), and the actual acrylamide gradient might not always be exactly linear.

Next we compared in-gel ATPase activities for digitonin/CN-PAGE and the corresponding complex V protein amounts in order to estimate specific ATPase activities. It was not possible to reuse the same gel after staining with Coomassie-dye for quantification (Fig. 4A, right panel), since a background of respiratory chain complexes and supercomplexes contaminated all complex V bands (cf. Fig. 1B). We quantified complex V after 2-D BN-PAGE (Fig. 4B). From previous work it is known that some complex V dimer is retained at low dodecylmaltoside [8]. The conditions for 2-D BN-PAGE resemble this low dodecylmaltoside situation, thus some complex V retained in dimeric form had also to be taken into account for quantification.

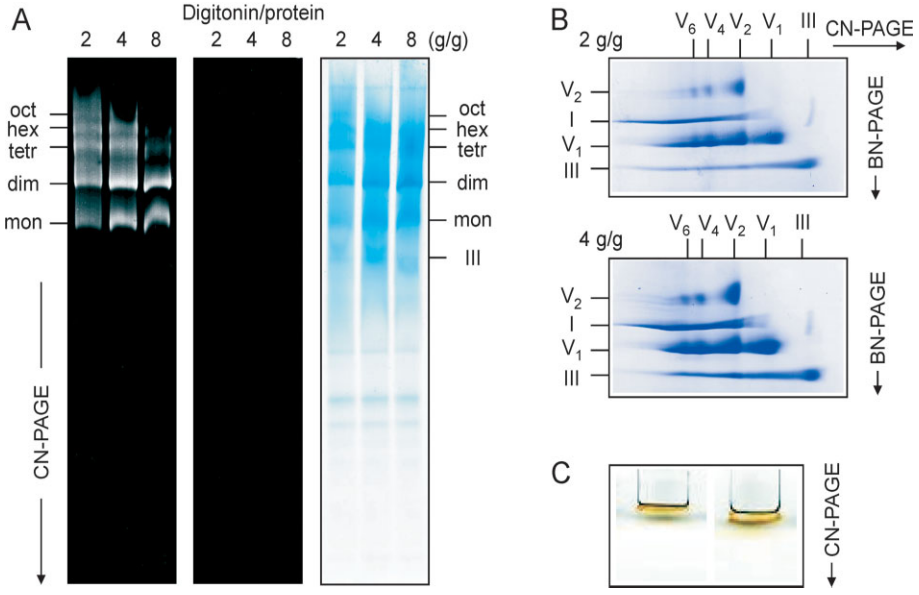

Figure 4. In-gel quantification of complex V protein amounts and ATP hydrolysis activity. Monomeric (mon) to hexameric (hex) states of complex V were also assigned V_1-V_6. (A) Rat heart mitochondria were solubilized setting various digitonin/protein ratios (2, 4, and 8 g/g) for CN-PAGE. The CN-gel was first analyzed for in-gel ATPase activity (A, left panel) revealing several oligomeric states of complex V as indicated. Another copy of the CN-gel was analyzed in parallel using the ATP synthase inhibitor oligomycin (A, central panel). Gel A, left panel, was then reused for Coomassie-staining (A, right panel). (B) Complex V amounts for specific oligomeric states from A, lanes 2 and 4, were quantified after 2-D BN-PAGE to separate the protein constituents of supercomplexes, namely complexes I, III, and IV. (C) Ferritin, a yellowish water-soluble 440 kDa protein with p*I* 4.2–4.6, was used to show the concentration of a dilute protein sample to a yellow band within the sample well for CN-PAGE (left side), before this concentrated band entered the CN-gel (right side).

Comparing in-gel ATPase activities for one specific lane (Fig. 4A, digitonin/protein ratio = 2 g/g) and the corresponding complex V protein amounts (Fig. 4B, upper panel), we found that the specific activities for the monomeric and higher oligomeric states were comparable (1.00, 0.68, 0.91, and 0.97, for the monomeric, dimeric, tertrameric, and hexameric state, respectively, when normalized to the monomer). Similar results were obtained for the lane using a digitonin/protein ratio of 4 g/g (the normalized specific activities were 1.00, 0.82, 0.98, and 0.88), and for the lane using a digitonin/protein ratio of 8 g/g (the normalized specific activities were 1.00, 0.72, 0.81, and 0.33). With the exception of the last value (detergent effects discussed below), all data suggested comparable and oligomycin-sensitive ATP hydrolysis activities for the monomeric and the oligomeric forms. This is in agreement with our previous analysis of yeast complex V dimers [7] but in stark contrast to a previously reported inactive dimeric state of complex V from bovine mitochondria ([22], discussed in 3.5).

Although there is ample evidence for the existence and the functional role of dimeric complex V, caution has to be applied before postulating a physiological significance of the higher supramolecular assemblies of complex V observed by digitonin/CN-PAGE. In order to test if CN-PAGE can favor protein aggregation we analyzed the initial migration behavior of a dilute sample of horse spleen ferritin within the sample gel well. Ferritin was chosen because of its yellowish color, which is helpful for detection during CN-PAGE. Ferritin (M_r 440; p*I* 4.2 to 4.5) that migrates in monomeric and dimeric form in CN-PAGE and BN-PAGE as well [1], was found to be considerably concentrated in the sample well before entering the 3% acrylamide gel (Fig. 4C). Considering that a similar accumulation can occur also with the membrane protein complex V, it seems possible that artificial association may occur, since concentrated membrane proteins in general have a tendency to aggregate and, in contrast to BN-PAGE, no protection by introducing surface charges on individual particles is provided. However, there are also arguments in favor of a physiological association of the dimers. 1) The observed associations are specific for complex V. Associations mediated by protein bound lipid should cause unspecific aggregations and precipitations of other membrane proteins as well. 2) Oligomeric forms of yeast complex V have been identified also using BN-PAGE [16–19]. 3) Electron microscopic studies with *Paramecium multimicronucleatum* mitochondria revealed associations of complex V dimers on tubular cristae, and Allen *et al.* [24, 25] proposed that associated ATP synthases have the potential to form a rigid arc with a functional role for budding of the inner mitochondrial membrane. The question whether or not physiological associations of complex V dimers exist in mammalian mitochondria cannot be answered definitively at present, and will have to be addressed by independent techniques.

3.5 Detergent-dependence of in-gel ATPase activity

Although comparison of in-lane specific ATPase activities gave reasonable results, we found that inter-lane comparison of specific activities did not make sense, since specific activities depended on the digitonin/protein ratio used for solubilization. The averaged specific ATPase activities for lanes 2 and 4 using digitonin/protein ratios of 2 and 4 g/g were 2.7 and 1.7 fold higher than for lane 8 using a ratio of 8 g/g (Fig. 4A). This led us to analyze detergent effects on the in-gel ATPase assay (Fig. 5).

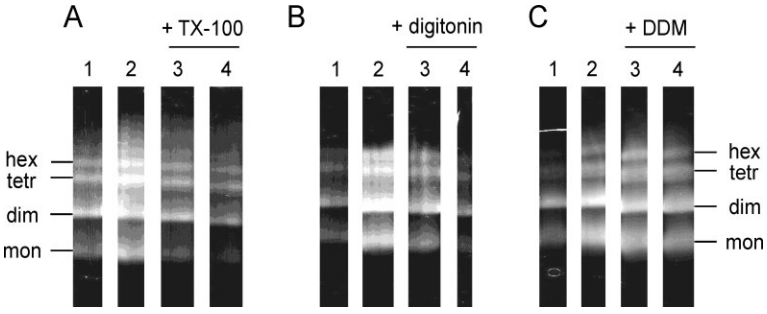

Figure 5. Detergent-sensitivity of the in-gel ATP hydrolysis assay. We analyzed 4 strips (1–4) each from three different 1-D CN-gels using digitonin/protein ratios of (A) 2 g/g, (B) 4 g/g, and (C) 8 g/g. All gel strips 1 were assayed directly in assay buffer (tris/glycine buffer supplemented with ATP, Mg_2SO_4, and $Pb(NO_3)_2$), whereas all gel strips 2 were preincubated for 3 h in non-supplemented tris/glycine buffer, before this buffer was replaced by the assay buffer. Strips 2 are the detergent-free references for the detergent-treated strips 3 and 4. (A) 0.025% and 0.05% Triton X-100 (TX-100) was added to incubation and assay buffers for gel strips 3 and 4, respectively, which reduced activities compared to the reference strip 2. Similarly, (B) digitonin and (C) dodecylmaltoside (DDM) was used for gel strips 3 and 4 (0.025% and 0.05% detergent, respectively).

We analyzed four strips each taken from three different 1-D CN-gels (Fig. 5A–C). Digitonin/protein ratios of 2, 4, and 8 g/g were used for CN-gels A, B, and C, respectively. Gel strips 1 were assayed directly in tris/glycine buffer supplemented with ATP, Mg_2SO_4, and $Pb(NO_3)_2$, whereas gel strips 2 were preincubated for 3 h in tris/glycine buffer, before this buffer was removed and replaced by the assay buffer. Preincubation enhanced the assay sensitivity 2 to 4 fold. This suggested that an inhibiting compound in the CN-gel strip, potentially digitonin, might diffuse out of the gel during preincubation. Therefore, we added 0.025% and 0.05% digitonin to incubation and assay buffers for gel strips 3 and 4 (Fig. 5B), and measured 61 and 26% residual ATPase activities, as compared to reference strip 2. We conclude that digitonin, although being an ideal detergent for retaining supramolecular structures, interferes with the in-gel ATPase assay. The significantly reduced specific activity for the band of hexameric complex V (Fig. 4A, lane 8) points to the presence of digitonin near the top of the CN-gel. Digitonin near the top of CN-gels also impairs the resolution of low mass subunits in 2D SDS-gels, and causes an uneven running front. The extent of this interference correlates with the amount of digitonin used for solubilization (2-D SDS-gels not shown).

We next asked whether this inhibition is specific for the detergent digitonin. With gel strips 3 and 4 in Fig. A we added 0 025 and 0.05% Triton X-100 which reduced the activities to 52 and 33%, respectively. However, when adding 0 025 and 0.05% dodecylmaltoside to gel strips 3 and 4 (Fig. 5C), activities were not at all or only marginally affected (108 and 78% residual activity, respectively, as compared to control gel strip 2).

We conclude that Triton X-100 also inhibits the in-gel ATPase assay. Since Tomasetig *et al.* [22] used Triton X-100 for separation of monomeric and dimeric bovine complex V, local Triton concentrations may explain their inability to identify active complex V dimers. An alternative explanation, that we consider more likely, concerns the assay conditions.

The assays used here for CN-PAGE had to be stopped after 10 min and were fully oligomycin-sensitive. In contrast, the BN-gels as used by Tomasetig *et al.* required overnight incubation in the assay buffer and the assay was not sensitive to the inhibitor oligomycin. Therefore it seems possible that Coomassie-dye in BN-gels inhibits ATPase activity of the holo-ATP synthase, and these authors actually measured the ATPase activity of F_1-domains possibly dissociated from a less stable monomer [20] but not from a more stable dimer under the conditions of their extremely extended assays.

3.6 Identification of succinate ubiquinone dehydrogenase

Complex II or succinate ubiquinone dehydrogenase has been shown to migrate as an extremely broad band in dodecylmaltoside/CN-PAGE. A similar result was obtained here using digitonin/CN-PAGE. A rather extended band for complex II could be detected after 2-D BN-PAGE (Fig. 1B, dashed ellipsoid). The identity of complex II was confirmed by 3-D SDS-PAGE as exemplified in Fig. 2B (three of the four subunits are marked by arrows).

4 Concluding remarks

For standard analyses, BN-PAGE rather than CN-PAGE should be used, since the resolution is higher, and estimations of molecular masses/oligomeric states are easily obtained. However, CN-PAGE offers advantages whenever Coomassie-dye would interfere with further analytical techniques, *e.g.,* with determination of catalytic activities as shown here. CN-PAGE using dodecylmaltoside and digitonin as well has also been described as a very efficient microscale separation technique for FRET analyses [3–6].

CN-PAGE works under milder conditions than BN-PAGE, since the negatively charged Coomassie-dye in the presence of a neutral detergent, as used in BN-PAGE, can mimic some properties of an anionic detergent, and can cause dissociation of proteins from membrane protein complexes, as exemplified in Fig. 1 with a partial dissociation of the catalytic F_1-domain and individual α and β subunits from holo-complex V. The mild conditions, especially of digitonin/CN-PAGE, have been used to retain supramolecular assemblies of respiratory chain complexes that were dissociated under the harsher conditions of BN-PAGE [7]. As described here, oligomeric states of mitochondrial ATP synthase previously not detected using BN-PAGE were identified by digitonin/CN-PAGE. Therefore we think that CN-PAGE is an interesting tool for functional proteomic approaches.

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