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Blue Native Electrophoresis for Isolation of Membrane Protein Complexes in Enzymatically Active Form

Hermann Schägger and Gebhard von Jagow

Gustav-Embden Zentrum der Biologischen Chemie, Klinikum der Johann Wolfgang Goethe Universität, Theodor-Stern-Kai 7, Haus 25B, 6000 Frankfurt/M. 70, Federal Republic of Germany

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A discontinuous electrophoretic system for the isolation of membrane proteins from acrylamide gels has been developed using equipment for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Coomassie dyes were introduced to induce a charge shift on the proteins and aminocaproic acid served to improve solubilization of membrane proteins. Solubilized mitochondria or extracts of heart muscle tissue, lymphoblasts, yeast, and bacteria were applied to the gels. From cells containing mitochondria, all the multiprotein complexes of the oxidative phosphorylation system were separated within one gel. The complexes were resolved into the individual polypeptides by second-dimension Tricine–SDS–PAGE or extracted without SDS for functional studies. The recovery of all respiratory chain complexes was almost quantitative. The percentage recovery of functional activity depended on the respective protein complex studied and was zero for some complexes, but almost quantitative for others. The system is especially useful for small scale purposes, e.g., separation of radioactively labeled membrane proteins, N-terminal protein sequencing, preparation of proteins for immunization, and diagnostic studies of inborn neuromuscular diseases.

There are several buffer systems for native electrophoresis of water soluble proteins (1). Since the Laemmli system is the most commonly used SDS–PAGE[1] method (2), the same buffer system is often tried for separation of water soluble proteins simply by replacing SDS with Triton X-100. The methods for native electrophoresis of membrane proteins, however, although attempted many times in the past, suffered from too many drawbacks to be accepted as standard isolation procedures.

The published methods can be divided into two classes: (i) those relying on the proteins' own charge to determine the anodic or cathodic migration at a given pH, and those (ii) using charged but mild detergents to induce a charge shift on the proteins so that all proteins binding the detergent migrate in the same direction. The use of deoxycholate, Sarkosyl, or mixtures of Triton X-100 and deoxycholate (3–6) has been described.

Any new method generally applicable to membrane proteins when the isoelectric points are not known in advance must be a charge shift method. The system should work at a pH near 7.5 because functional mitochondrial complexes are usually prepared at pH 7–8 (7). Complex III of the respiratory chain especially was shown to be stable only between pH 6.2 and 8.0 (8). The most important problem in native electrophoresis of membrane proteins, that of membrane protein aggregation, should be minimized. Only then will it be possible to isolate proteins directly from crude membrane extracts.

We found two compounds to be most useful for inducing a charge shift on the membrane proteins. With Coomassie blue G, multiprotein complexes were optimally separated ("Blue Native PAGE") and with taurodeoxycholate, lower molecular mass proteins were resolved ("Native PAGE").

MATERIALS AND METHODS

Chemicals

Laurylmaltoside (dodecyl-β-D-maltoside) and DNAase I from bovine pancreas were obtained from Boehringer, Mannheim; 6-aminocaproic acid was from Fluka; octylglucoside and Mega 9 (nonanoyl-*N*-methylglucamide) were from Oxyl, Bobingen; Triton X-100, acrylamide, and bisacrylamide (the commercial 2×

[1] Abbreviation used: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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TABLE 1

Gel Composition and Polymerization Conditions

	Sample gel	Gradient separation gel	
	4% T	6% T	13% T
AB-mix	0.6 ml	2.5 ml	4.7 ml
Gel buffer (3x)	2.5 ml	7 ml	6 ml
Glycerol	—	—	3.6 g
APS (10%)	60 μl	90 μl	60 μl
Temed	6 μl	9 μl	6 μl
Total volume	7.5 ml	21 ml	18 ml

Note. APS (10%): Ammonium persulfate solution (10%), freshly prepared.

cryst. products), Amido Black 10 B, and all Coomassie dyes were from Serva, Heidelberg. All other chemicals were from Sigma, Munich.

Stock Solutions for Native and Blue Native PAGE

Blue Native PAGE refers to electrophoresis performed with Serva blue G in cathode buffer A and Native PAGE refers to the method using the noncolored cathode buffer B. The composition of acrylamide–bisacrylamide mixtures and of gels is described by two parameters, %T and %C, according to Hjerten (9). The %T value represents the total concentration of both monomers (acrylamide and bisacrylamide) in grams per 100 ml and %C is the percentage (by weight) of the crosslinker relative to the total monomer.

Cathode buffer A (for Blue Native PAGE; for separation of membrane multiprotein complexes): 50 mM Tricine, 15 mM Bistris, 0.02% Serva blue G, pH 7.0 (4°C).

Cathode buffer B (for Native PAGE; for small membrane proteins): 50 mM Tricine, 15 mM Bistris, 0.05% Na-taurodeoxycholate, 0.05% Triton X-100, pH 7.0 (4°C).

Anode buffer: 50 mM Bistris adjusted with HCl to pH 7.0 (4°C).

Gel buffer (3×): 1.5 M 6-aminocaproic acid, 150 mM Bistris adjusted with HCl to pH 7.0 (4°C).

AB-mix (acrylamide–bisacrylamide mixture): The 49.5% T, 3% C mixture contains 48 g acrylamide/100 ml and 1.5 g bisacrylamide/100 ml.

Gel Preparation

Separating gels were cast at 4°C and maintained at room temperature for polymerization. Separating gels with linear 6–13% acrylamide gradient gels (Table 1) were suitable for protein complexes in the molecular mass range from 10^6 Da to 10^5 Da. For resolution of smaller proteins, 7–16.5% gradient gels were used. The sample gel was cast at room temperature. After removal of the combs, the gels were overlayed with gel buffer (1×) and stored at 4°C.

Sample Preparation and Protein Load

Membrane proteins were preferentially solubilized with laurylmaltoside at a concentration sufficient to bring the proteins of interest into the 100,000*g* supernatant. (Triton X-100, Chaps, Mega 9, octylglucoside, Brij 35, and Tween 20 may also be used, depending on the solubility and stability of the proteins of interest.) Protein solubilization is supported by the presence of 6-aminocaproic acid (Fig. 1).

Starting from muscle tissue, there is no need to prepare mitochondria. Ten milligrams (wet weight) of bovine heart muscle (ca. 1 mg of total protein) was homogenized in 1 ml of buffer (440 mM sucrose, 50 mM K-phosphate, 2 mM EDTA, 0.1 mM PMSF, pH 7.2). Broken cells and mitochondria were sedimented at 20,000*g*. The supernatant, containing cytoplasmic proteins, was discarded. The sediment was extracted by adding 40 μl of 750 mM aminocaproic acid, 50 mM Bistris/HCl, pH 7.0, and 5 μl laurylmaltoside (10%). After a 30-min centrifugation at 100,000*g* the supernatant was supplemented with either Serva blue G (Blue Native PAGE) or taurodeoxycholate (Native PAGE). When large membrane protein complexes were to be separated using Blue Native PAGE, 2.5 μl of a 5% solution/suspension of Serva blue G in 500 mM aminocaproic acid was added so that the concentration of the dye was $\frac{1}{4}$ of

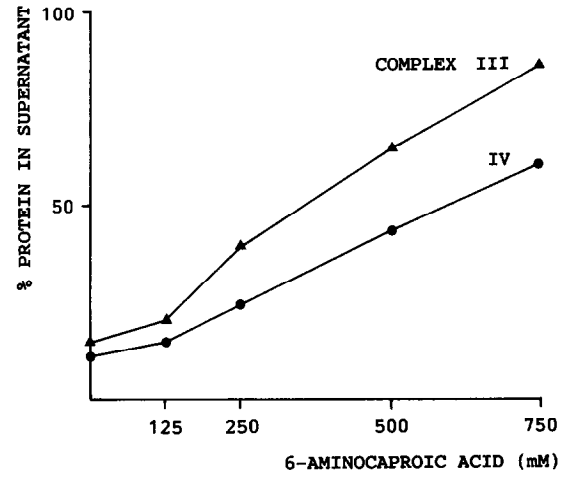

FIG. 1. Solubilization of membrane proteins by Triton X-100 and aminocaproic acid. Of mitochondrial membrane proteins, only the solubilization of complexes III and IV was monitored because these complexes could easily be quantitated photometrically. Bovine heart mitochondria were treated with Triton X-100 (3%) in the presence of varying concentrations of aminocaproic acid. The protein concentration before centrifugation was 13 mg/ml. In the presence of 750 mM aminocaproic acid the same percentage of the membrane protein complexes III and IV was found to be solubilized as in the presence of 500 mM NaCl.

the laurylmaltoside concentration. When small membrane proteins were to be separated using Native PAGE, 2.5 μl of taurodeoxycholate (2%) was added. The taurodeoxycholate concentration was thus 1/10 of the laurylmaltoside concentration. Serva blue G was added to one reference sample; 25 μl of the protein solution was applied to 1-cm pockets in 1.6-mm gels. The electrophoresis tank already contained the appropriate buffer.

Starting from isolated, sedimented mitochondria (0.4 mg of total protein) the membrane proteins were extracted by addition of 40 μl of the buffer containing 0.75 M aminocaproic acid, 50 mM Bistris/HCl, pH 7.0, and 7.5 μl of laurylmaltoside (10%). Ultracentrifugation and supplementation with Serva blue G or taurodeoxycholate were performed as described above.

Starting from yeast cells (*Saccharomyces cerevisiae*) or bacterial cells (*Escherichia coli*) some additional steps were necessary. Mitochondrial membranes from yeast and bacterial membranes were prepared by breaking the cells in a french press (SLM AMINCO) at 23,000 psi, removing the cell walls, etc., at 6000*g* (20 min), and centrifugation at 200,000*g* (2 h). The membranes were suspended in a more than 10-fold volume of 5 mM $MgCl_2$, 10 mM Bistris/HCl, pH 7.0, with 1 mM PMSF (stock: 0.5 M in DMSO). After addition of 0.2 mg/ml DNase I the suspension was kept for 1 h at 25°C before the membranes were centrifuged at 100,000*g* (1 h). With *E. coli* the DNase treatment/centrifugation was repeated once more. The sediments after centrifugation could be solubilized like intact mitochondria as described above. Without DNase treatment/centrifugation the proteins did not enter the gel. We assume that DNA dramatically reduces the pore size on top of the stacking gel.

The minimum amount of mammalian cells necessary for detection of the protein subunits of the complexes decides whether the method will be applicable in clinical research where only limited quantities of muscle tissue from biopsies are available. Starting from bovine heart muscle, a minimum of 5 mg muscle tissue was necessary to allow an identification of the individual protein subunits by staining with Coomassie (see below). Starting from Molt-4 lymphoblasts, 20–30 mg of cells (wet weight) was necessary (not shown). When the amount of protein available was not limiting, e.g., when bovine heart mitochondria were solubilized, the maximal load to a 3-mm gel was 10 mg of extracted protein representing ca. 200–600 μg of each multiprotein complex.

Electrophoresis Conditions

Analytical (1.6 mm) and preparative (3 mm) gels with a total length of 14 cm were run at 4–7°C in a vertical electrophoresis apparatus according to Studier (10). Electrophoresis was started at 100 V until the protein sample was within the stacking gel. Voltage was then set to 500 V with the current limited to 15 mA. The running times were 3–6 h. Alternatively, gradient gels from 7% T to 16.5% T could also be run at 200 V overnight. The running pH during electrophoresis was 7.5.

Native Extraction of Membrane Protein Complexes from Gels

Native extraction from Blue Native Gels was performed at 4°C with an electroelutor/concentrator made according to Hunkapiller *et al.* (11). (A commercial apparatus is available from CBS Scientific Co., Del Mar, CA.) The H-shaped elution chamber is essentially composed of two vertical tubes connected by a horizontal tube. The lower ends of each vertical tube, sealed with dialysis membranes (cutoff value of 2 kDa, Reichelt GmbH, Heidelberg, FRG), dip into the electrode buffer (25 mM Tricine, 7.5 mM Bistris, pH 7.0) in the anodic and cathodic compartments of the electrophoresis tank.

The blue protein bands were excised from the gel with razor blades and squeezed through a syringe into the cathodic arm of the elutor chamber. Then both arms of the chamber were filled with electrode buffer, also filling the horizontal tube connecting both arms. Extraction was performed at 200 V overnight. The extracted protein collected as a thin blue layer on the anodic dialysis membrane. The extraction appeared almost quantitative, as tested by reextraction of the gel pieces in the presence of SDS (12).

Protein determination according to Bradford (13) with correction for Serva blue G present in the sample was only possible after partial or complete removal of excess Serva blue G during electrophoresis (see below). Complete removal of excess Serva blue G, however, led to partial aggregation of the electroeluted proteins.

Removal of Excess Serva Blue G during Blue Native PAGE

Usually Blue Native PAGE was performed with cathode buffer A throughout the whole run. For electroblotting of the native proteins, however, cathode buffer A was removed after about one-third of the whole run and the electrophoresis was continued with a similar cathode buffer containing no dye (50 mM Tricine, 15 mM Bistris pH 7.0). Protein bound dye thereby was not removed and the protein pattern was not changed.

For retaining solubility of proteins after native extraction, excess Serva blue G was partially removed by changing to a cathode buffer containing only 0.005% Serva blue G.

For visualization of faint protein bands, when the protein load was low, this cathode buffer with reduced Serva blue G concentration was used from the beginning.

Electroblotting of Blue Native Gels

Blotting of blue native gels should only be performed after removal of excess dye, since free Serva blue G also would bind strongly to Immobilon membranes and therefore more layers of Immobilon membranes would be necessary to trap all proteins.

A 3-mm stack of papers was soaked with electrode buffer (50 mM Tricine, 15 mM Bistris pH 7.0) and placed on the lower electrode (Anode). Several sheets of Immobilon wetted with methanol and soaked in electrode buffer were transferred to the paper stack followed by the gel. A 3-mm stack of papers soaked with electrode buffer was placed on top. A 5-kg load was placed on the cathode. The transfer at 20 V was stopped after 1 h. Background destaining, if necessary, was achieved with 25% methanol, 10% acetic acid.

Denaturing Tricine–SDS–PAGE for Second Dimension

Tricine–SDS–PAGE was performed as described in (14) with the following supplementation: A 1-cm lane of the first-dimension gel with the protein complexes under study was excised, dipped into 1% mercaptoethanol for a few seconds, and placed on a glass plate at the usual position for stacking gels. After positioning the spacers and covering with the second glass plate, the gel stuck between the plates and the system was brought into a vertical position. Then the separating gel mixture followed by some water was poured in through the gaps left between the gel strip and the spacers. After polymerization the stacking gel mixture was poured in, so that the strip of gel was surrounded, but not covered, by the stacking gel solution. After polymerization and mounting of the gel in the electrophoresis chamber, the cathode buffer was introduced. Solution I (4% SDS, 10% glycerol, 2% mercaptoethanol, 0.03% Serva blue G, 50 mM Tris adjusted to pH 7 with HCl) underlayed the cathode buffer so that a 1- to 2-mm layer covered the strip of the first-dimension gel. Electrophoresis was then started.

Electroblotting of SDS–Gels, Staining

The semidry electroblotting method is a modification of the procedure described by Kyhse-Anderson (15). The modification was necessary for a quantitative protein extraction not only from 10% T, but also from 16.5% T, 3% C gels.

Cathode buffer: 300 mM 6-aminocaproic acid, 30 mM Tris (pH 8.6–8.7).

Anode buffer: 300 mM Tris, 100 mM Tricine (pH 8.7–8.8).

A 3-mm stack of papers was soaked with anode buffer and placed on the lower electrode. A sheet of Immobilon, wetted with methanol and then transferred to the anode buffer, was next, followed by a 1.6-mm acrylamide gel not incubated in buffer. A 3-mm stack of papers soaked with cathode buffer was on top. An expansion of the gel during the protein transfer was avoided by placing a 5-kg load on the cathode.

The protein transfer of 16.5% T, 3% C gels (1.6 mm) was optimal at 5 V (overnight), but could also be performed for 5 h, at 10 V. Transfer times for 10% gels (1.6 mm) or for 16.5% T gels (0.7 mm) were 2 h at 10 V.

Staining of the sheets of Immobilon before protein sequencing was performed by soaking the wet membrane (directly after the blotting) in 0.1% Amido Black 10 B in 10% acetic acid for about 1 min. Methanol was omitted, because only then were very small hydrophilic polypeptides like Bacitracin (1.45 kDa) and Bradykinin (1.06 kDa) stained (not shown). Destaining was done with water.

RESULTS

Solubility of Membrane Proteins Supported by 6-Aminocaproic Acid

For efficient solubilization of mitochondrial membrane proteins (but not bacterial membrane proteins) by neutral detergents, salts like NaCl have very often been used because the solubilization of mitochondrial membrane proteins by Triton X-100 and laurylmaltoside is poor at low ionic strength (cf. Fig. 1). This dependence on ionic strength may be related to the mitochondrial phospholipid composition. However, since Cl^- has a much higher effective mobility in an electrical field than do proteins, the presence of high salt concentrations in the sample applied to the native gel would cause a separation of salt and protein with concomitant protein concentration and aggregation.

To overcome this problem we used 6-aminocaproic acid instead of NaCl. The pK values for the amino group (pK = 10.7) and for the carboxylic group (pK = 4.4) are so far apart that at a pH of 7–7.5 no migration of the compound in an electrical field occurs. The concentration dependence on protein solubilization by Triton X-100 is demonstrated in Fig. 1. The effect of 750 mM 6-aminocaproic acid on protein solubilization is comparable to that of 500 mM NaCl. Therefore, 750 mM 6-aminocaproic acid was used.

Within the gel the presence of 6-aminocaproic acid is of minor importance, probably because the binding of the negatively charged dye to the proteins increases their mutual electrical repulsion, thus lowering the need for a dielectric. Nevertheless, we introduced 500 mM aminocaproic acid also into the gel because the protein bands were somewhat sharper than without any aminocaproic acid.

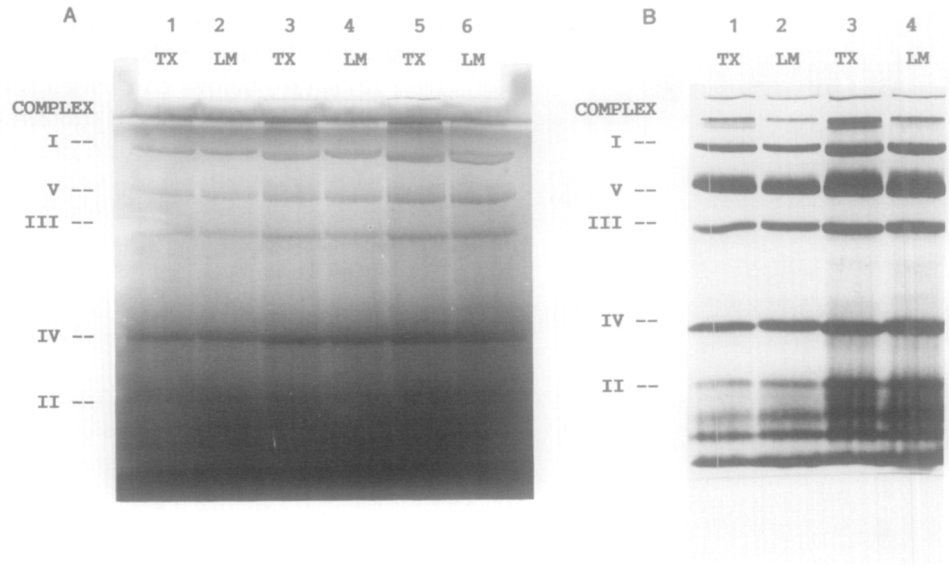

FIG. 2. First dimension: Blue Native PAGE for resolution of membrane protein complexes starting from whole mitochondria. (A) After Blue Native electrophoresis of bovine heart mitochondrial proteins the most prominent protein bands (complexes I–V) were detected on a light screen as dark blue bands over a blue background. (B) For a comparison of the intensity of the blue protein bands with the intensities observed after conventional staining, lanes 1–4 were fixed and stained as described in (14). Blue Native PAGE with cathode buffer A throughout the whole run was performed after solubilization of the proteins by laurylmaltoside (LM) or Triton X-100 (TX); 0.7-mm gels with 1% acrylamide steps from 6% T to 10% T were used. The protein load was 75 μg in lanes 1 and 2, 150 μg in lanes 3 and 4, and 225 μg in lanes 5 and 6. Electrophoresis was started at 100 V (30 min) and continued at 500 V (2 h) at 4°C.

First Dimension—Blue Native PAGE for Membrane Protein Complexes

Figure 2A shows how the Blue Native Gels (without removal of excess dye) appear prior to further processing. For a comparison of the intensity of the blue protein bands when inspected on a light screen with the intensities observed after conventional staining (Fig. 2B), lanes 1–4 were fixed and stained as described in (14). Complex III dimer with a molecular mass of 500 kDa, shown previously not to monomerize (16), was found in the 7–8% acrylamide range. Complex II with a molecular mass of about 130 kDa, described as a monomer, was found in the 10% acrylamide range. If one assumes that the complexes are separated according to their molecular masses, complexes I and V resolved in the 6–7% acrylamide range should be in the monomeric state with molecular masses of about 750 and 600 kDa, respectively. Complex IV, existing in both monomeric and dimeric forms, was found in the 9% acrylamide range, suggesting a molecular mass of about 200 kDa and the presence of the monomeric form. Occasionally a second band for complex IV with the same subunit composition was detected that might represent the dimeric form.

Second Dimension—Tricine–SDS–PAGE

The protein complexes separated first by Blue Native PAGE were further resolved into their individual subunits by denaturing Tricine–SDS–PAGE (Figs. 3 and 4).

Figure 3 shows the results from 5 mg (wet weight) of bovine heart muscle tissue which were comparable in quantity and quality to those from 50 μg of isolated mitochondria (not shown).

The two-dimensional gels originating from 50 μg of mitochondrial proteins could be used for a rough estimation of the percentage recovery of the complexes. They showed that possible protein losses due to dissociation or aggregation were low. Assuming that the five multiprotein complexes constitute 50% of the total mitochondrial protein, on average only 0.3 μg of each of the 76 protein subunits constituting complexes I–V were present. This is the lower limit of detection for the protein subunits of the complexes using Coomassie. As most of the protein subunits could in fact be detected in these 2D gels, we assume that the protein recovery of the complexes was nearly quantitative. The percentage recovery of the catalytic activity of complex III was also near 100% because the electroeluted complex III showed maximal catalytic activity.

The five well-characterized multiprotein complexes of the oxidative phosphorylation system of bovine heart mitochondria were identified by their characteristic polypeptide patterns.

Complex I comprises 32 protein subunits resolved by combining denaturing isoelectric focusing and SDS–PAGE (17). Most of them (30 protein bands) could be detected in our 2D gels (e.g., in Fig. 3), although we used only the second-dimension Tricine–SDS–PAGE for resolving the subunits.

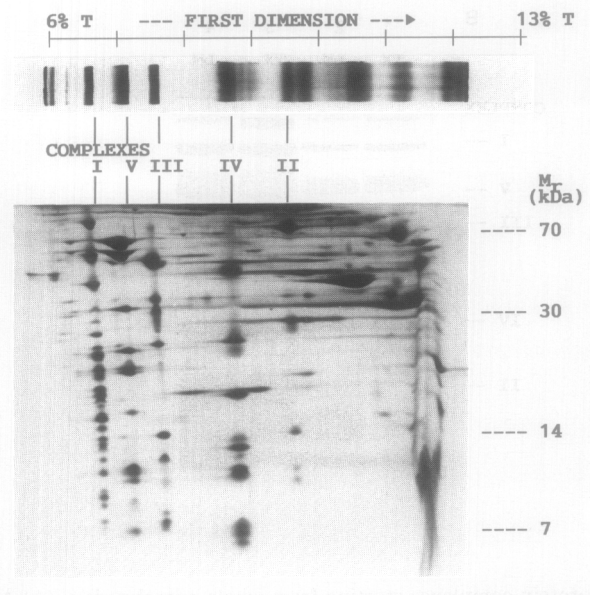

FIG. 3. Two-dimensional resolution of the respiratory chain complexes from bovine heart. First dimension: Blue Native PAGE of the proteins and complexes extracted from 5 mg (wet wt) bovine heart muscle tissue. A linear 6–13% T acrylamide gradient gel (1.6 mm) was used. Second dimension: Denaturing Tricine–SDS–PAGE (14) for resolution of the complexes into their individual protein subunits. A uniform 16.5% T, 3% C gel was used. The individual respiratory chain complexes were identified by their characteristic polypeptide patterns and, with the exception of complexes I and II, by their catalytic activities after extraction from preparative gels.

Complex II comprises six protein subunits, but only five protein bands could be detected because two subunits could not be separated by electrophoresis (18). These five protein bands can be seen in Fig. 3.

Complex III comprises 11 protein subunits (16,19). Two of these 11 subunits, the "Rieske" iron sulfur protein (22 kDa) and the smallest protein of the complex (the 6.4-kDa protein), are relatively loosely attached to the holo complex and are easily removed by detergents (20). The effects on catalytic activity of dissociating one or the other subunit are quite different. Dissociation of the 6.4-kDa subunit has no effect on electron and proton transfer activities of complex III (16). The removal of the Rieske iron sulfur protein, however, leads to complete loss of catalytic activity (21). During development of the Blue Native PAGE method the degree of retention of the iron sulfur protein served as an indicator for determining the mildest conditions (especially the lower limits of detergent concentrations). In the 2D gels (Blue Native PAGE/Tricine–SDS–PAGE) 9 protein bands corresponding to 10 subunits could be detected. The 2 largest proteins, core proteins I and II (22), were not resolved. The smallest subunit, the 6.4-kDa protein, was not detected because it was removed during the Blue Native PAGE. The iron sulfur protein, however, was retained (Fig. 3). The full electron transport activities measured with the complex electroeluted after Blue Native PAGE (see below) confirmed this result.

Complex IV comprises 13 subunits (23). In the SDS gels, used here for the second dimension, only 12 subunits were detectable; however, catalytic activity was retained (see below). This does not mean that only 12 subunits are necessary for functionality, but we know that for resolution of all 13 subunits special gels containing 6 M urea are necessary (24). This gel type, however, would not resolve the other complexes equally well.

Complex V probably comprises 14 subunits (25). In our 2D gels usually 12 (occasionally 13) subunits could be detected. However, measurements of catalytic activity showed (see below) that all proteins necessary for the catalytic activities were present (at least 20% of the oligomycin sensitivity conferring protein).

Figure 4 shows the analogous polypeptide patterns of fungal mitochondrial membranes, obtained after Blue Native PAGE (Fig. 4A) or after Native PAGE (Fig. 4B), followed by Tricine–SDS–PAGE. A band for complex I cannot be expected as *S. cerevisiae* does not possess complex I. Since the yeast cells were broken in the french press and only the sedimented membranes obtained after ultracentrifugation were processed further, cytoplasmic and matrix proteins were removed and therefore only membrane proteins or proteins associated with the membrane were present.

In order to see what polypeptide patterns of the membrane proteins of bacteria might be expected, *E. coli* membrane proteins were resolved by Native PAGE (Fig. 5) without identification of individual proteins. The resolution in the low molecular mass range is better than the resolution with the Blue Native PAGE; however, the largest and most prominent protein complex observed in the Blue Native PAGE was not detected (not shown).

Catalytic Activities of the Extracted Complexes

Bovine heart mitochondria were separated by Blue Native PAGE after solubilization with laurylmaltoside or Triton X-100 as described under Materials and Methods.

Extracted complex III (bc_1 complex) was tested for catalytic activity directly and after addition of phospholipid according to the method described in (16). The nonylubihydroquinone:cytochrome c reductase activity was low (30 s^{-1} at 25°C) when measured directly. However, the Antimycin-sensitive catalytic activity increased more than 10-fold within 15 min (400 s^{-1}), when egg yolk phospholipid dissolved in Triton was added to a molar ratio of 5000 phospholipids/complex dimer. After overnight incubation at that phospholipid/protein ratio, catalytic activity further increased to that measured

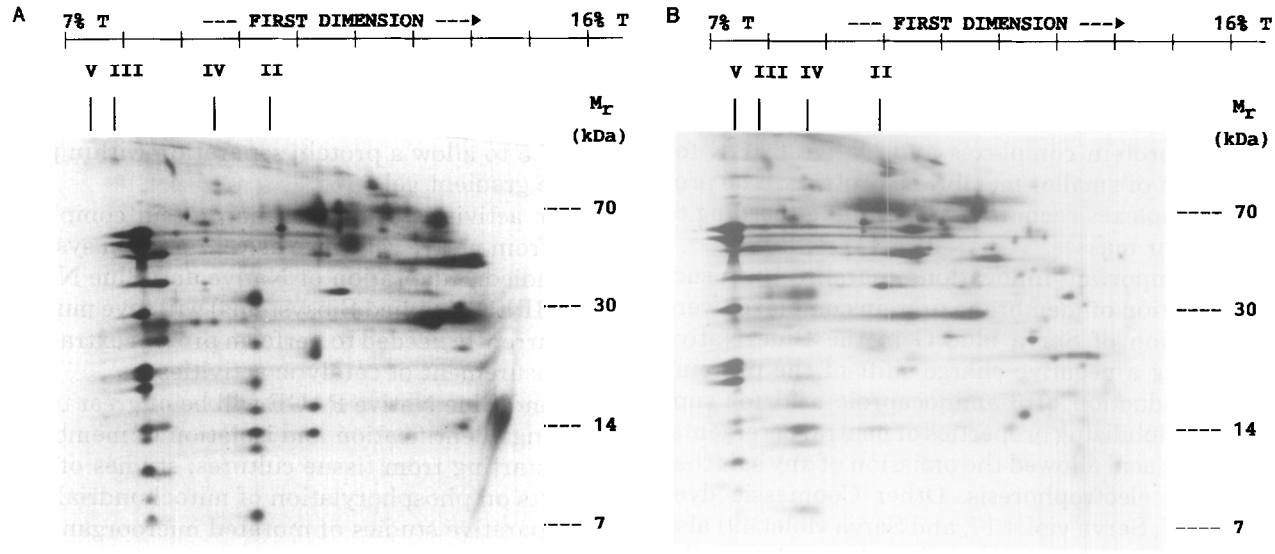

FIG. 4. Two-dimensional resolution of yeast membrane proteins. (A) Blue Native PAGE with 7–16.5% acrylamide gradient gels (1.6 mm), followed by Tricine–SDS–PAGE (10% gels, 1.6 mm). Multiprotein complexes were resolved without severe aggregation. Smaller proteins were crowded in the high acrylamide range. (B) Native PAGE followed by Tricine–SDS–PAGE (gel types as above). Multiprotein complexes, with the exception of complex V, were prone to aggregation and dissociation; however, smaller proteins were resolved better than in (A). Yeast cells were broken and processed further as described under Materials and Methods.

in chromatographically prepared complex III (1000 s^{-1}). Although the catalytic activities of the extracted complex III and those of a chromatographical preparation (16,19) were finally the same, differences in the optimal stoichiometry of phospholipid/protein and in the kinetics of activation by phospholipid were observed. Chromatographically delipidated complex III was immediately and maximally activated by addition of 200 molecules of phospholipid/complex III dimer (16). The extreme activation delay and the need for 25 times more phospholipid seem to be due to competition with bound Coomassie.

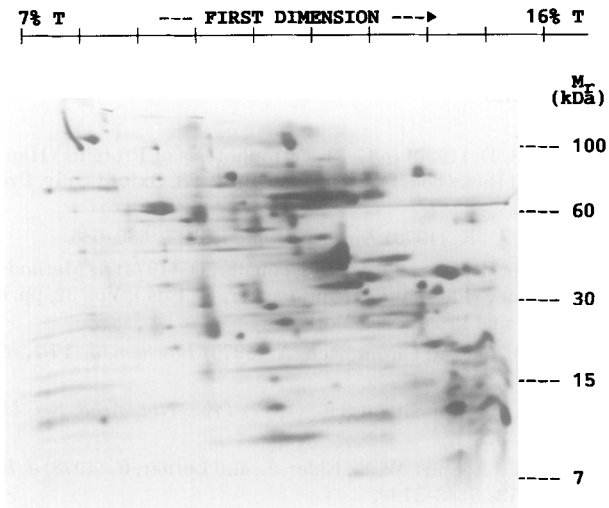

FIG. 5. Two-dimensional resolution of *E. coli* membrane proteins. *E. coli* membrane proteins were resolved by Native PAGE with 7–16.5% acrylamide gradient gels (1.6 mm), followed by Tricine–SDS–PAGE (10% gels), without trying to identify individual proteins or complexes. *E. coli* cells (XL-1 Blue) were broken and processed further as described under Materials and Methods. Native PAGE was useful for separation of proteins in the low molecular mass range; however, the largest and most prominent protein complex observed in Blue Native Gels (not shown) was not detected.

Extracted complex IV (cytochrome oxidase) had a cyanide-sensitive ferrocytochrome c oxidase activity that was measured according to the procedure described in (24). It increased linearly up to 60 s^{-1}, when the concentration of reduced cytochrome c in the test was increased to 60 μM, the highest feasible concentration. Hence the K_m for ferrocytochrome c was shifted compared to a chromatographically prepared oxidase with a K_m of 20 μM cytochrome c (24). This K_m shift is probably due to the binding of the negatively charged dye to the basic cytochrome c. The turnover number of the chromatographic preparation was 350 s^{-1}.

Extracted complex V (F_0F_1-ATPase) had a DCCD-sensitive ATPase activity of 5 μmol ADP/mg protein $\times$ min. However, inhibition by oligomycin was only about 20%. The activity measurements were performed as described in (26).

Extracted complexes I and II had no NADH:nonylubiquinone or succinate:nonylubiquinone oxidoreductase activities. The enzymatic measurements were performed as described in (27,28), but coenzymes Q_1 and Q_2 were replaced by nonylubiquinone. Artificial electron acceptors instead of nonylubiquinone were not tested.

DISCUSSION AND PERSPECTIVES

Two variants of a new native electrophoresis method are presented, namely Blue Native PAGE for the separation of multiprotein complexes and Native PAGE for the separation of smaller membrane proteins. The proteins and complexes seem to be separated according to their molecular masses.

The most important innovations guaranteeing a successful resolution of membrane protein complexes were the introduction of Serva blue G in the Blue Native PAGE causing a negative charge shift of the proteins and the introduction of 6-aminocaproic acid for supporting the solubilizing properties of neutral detergents. Aminocaproic acid allowed the omission of any salt that would impair electrophoresis. Other Coomassie dyes (Serva blue W, Serva violet 17, and Serva violet 49) also functioned, but it was more difficult to detect the stained protein bands in the first-dimension gel. Using Serva blue R, detergent had to be added to the cathode buffer to disaggregate the dye. This caused a partial dissociation of special proteins from the complexes, e.g., of the iron sulfur protein of complex III. With Serva blue G, however, detergents were not required. This means that Serva blue G alone or in combination with some residual detergent was able to maintain the complexes in solution. In contrast to detergents that dissociate from membrane proteins upon dilution below the critical micelle concentration, Coomassie dyes like Serva blue G remained tightly bound to the protein. Membrane proteins were thus converted to water soluble proteins. Selected proteins will therefore be used for crystallization attempts.

The properties of a dye to bind tightly to the proteins and to be sparingly water soluble seem to play an important role because the electrophoretic system did not work at all with two other more water soluble dyes, Amido black 10 B (Serva) and Ponceau S (Sigma).

Taurodeoxycholate instead of Serva blue G in the cathode buffer was found to be less suitable for the separation of multiprotein complexes, because some complexes were prone to aggregation or dissociation; however, small membrane proteins were very well resolved. The Native PAGE described here is distinct from other methods using deoxycholic acids (5,6) in several respects: the introduction of 6-aminocaproic acid to improve protein solubilization, the running pH of 7.5 to avoid protein denaturation, and the use of Tricine as a trailing ion for optimal resolution in acrylamide gradient gels. We preferred to use taurodeoxycholate instead of deoxycholate because of its better solubility at pH 7.0. With cholate or taurocholate more protein aggregation was observed.

The first component that was fixed during development of the Blue Native PAGE was Bistris within the gel buffer because it is one of the rare commercially available bases with a pK in the slightly acidic range (pK 6.5–6.8). Thus it is able to stabilize pH 7.5 in the gel. Tricine as trailing ion was found empirically to have the optimum pK (8.15) and the appropriate relative mobility at pH 7.5 to allow a protein separation within polyacrylamide gradient gels.

Catalytic activities of membrane protein complexes extracted from native gels have as yet not been systematically studied. Adaptation of Native and Blue Native PAGE to HPEC (Applied Biosystems) will save much of the time currently needed to perform protein extraction before measurement of catalytic activities.

Native and Blue Native PAGE will be of great use in the following: identification and isolation of membrane receptors starting from tissue cultures; studies of hormone effects on phosphorylation of mitochondrial proteins; comparative studies of mutated microorganisms; studies on the organ specificity of mitochondrial respiratory chain complexes and analysis of protein defects in inborn mitochondrial myopathies; and identification and isolation of new multiprotein complexes, e.g., which proteins are associated with known components of the mitochondrial import machinery located in mitochondrial contact sites.

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