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Analysis of Molecular Masses and Oligomeric States of Protein Complexes by Blue Native Electrophoresis and Isolation of Membrane Protein Complexes by Two-Dimensional Native Electrophoresis

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Blue native Electrophoresis is a "charge shift" method developed for isolation of native membrane protein complexes from biological membranes that also separates both acidic and basic water-soluble proteins at a fixed pH of 7.5. In combination with a second dimension sodium dodecylsulfate electrophoresis it provides an analytical method for the determination of molecular mass and oligomeric state of nondissociated complexes, of subunit composition, and of degree of purity and for the detection of subcomplexes. The method was applied to analysis of cytochrome bc/bf complexes. By combination of a novel colorless native polyacrylamide gel electrophoresis (CN-PAGE) with blue native BN-PAGE, a two-dimensional native technique was developed that is suitable for preparation of highly pure membrane protein complexes. © 1994 Academic Press, Inc.

Analysis of hydrodynamic properties of proteins, especially of membrane proteins, is difficult to perform by analytical ultracentrifugation methods (1). Therefore, less accurate sucrose density gradient centrifugation and gel filtration have commonly been used.

Resolution of mitochondrial multiprotein complexes by blue native PAGE (BN-PAGE)[1] was found to be much higher than that by density gradient centrifugation or gel filtration and required less protein, and a tentative assignment of the molecular masses of the complexes was possible (2).

We have extended these studies to establish BN-PAGE as a sensitive, high-resolution method for analysis of molecular mass, oligomeric state, and homogeneity of native proteins. A problem was the unavailability of suitable commercial membrane proteins with known molecular masses and isoelectric points. For that reason the previously neglected capability of BN-PAGE to also separate water-soluble proteins was studied and the possibility of using these as molecular mass standards was tested.

A second aim was to achieve an improved resolution of membrane proteins for preparative applications compared to BN-PAGE. Our effort concentrated on the development of an alternative, nondissociating technique that finally should be combined with blue native PAGE to yield a high-resolution, nondissociating, two-dimensional technique. Significant improvement of resolution, however, could only be expected with different separation principles in the first and second dimensions.

Since blue native electrophoresis is a charge shift method, where the electrophoretic mobility of the proteins is mainly determined by the negative charges of bound Coomassie dye, a novel method should make use of the intrinsic charge of the protein. Native isoelectric focusing (without urea) was not useful for resolution of the proteins studied (complexes I–V of the mitochondrial respiratory chain) because of severe protein aggregation; therefore, a novel method working at a fixed pH (colorless native PAGE) was developed.

[1] Abbreviations used: BN-PAGE, blue native polyacrylamide gel electrophoresis; CN, colorless native; SDS, sodium dodecyl sulfate; APS, ammonium persulfate; TEMED, tetramethylethylenediamine; Mops, 4-morpholinepropanesulfonic acid; APS, ammonium persulfate solution; AB, acrylamide and bisacrylamide; ISP, "Rieske" iron sulfur protein.

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TABLE 1

Buffers for Blue Native and Colorless Native PAGE

Cathode buffer (CN-PAGE):	50 mM Tricine, 15 mM Bistris/HCl, pH 7.0 (4°C)
(BN-PAGE):	+ Coomassie blue G-250[a] (0.02 or 0.002%)
Gel buffer (triple concentrated) (BN-PAGE and CN-PAGE):	150 mM Bistris/HCl, 1.5 M aminocaproic acid, pH 7.0 (4°C)
(CN-PAGE with membrane proteins)	+ 0.03% dodecyl maltoside
Anode buffer (BN-PAGE and CN-PAGE):	50 mM Bistris/HCl, pH 7.0 (4°C)

[a] We used exclusively Serva blue G, which is highly pure Coomassie blue G-250. Cathode buffer with 0.02% Coomassie was stored at room temperature. All other buffers were stored at 4°C.

MATERIALS AND METHODS

Chemicals

Dodecyl maltoside (*n*-dodecyl β-D-maltoside) was obtained from Boehringer, Mannheim, Germany; 6-aminocaproic acid was from Fluka, Buchs, Switzerland; acrylamide and bisacrylamide (the commercial 2× crystallized products) and Serva blue G (Coomassie blue G-250) were from Serva, Heidelberg, Germany. All other chemicals were from Sigma, Munich, Germany.

First Dimension: Blue Native PAGE or Colorless Native PAGE (CN-PAGE)

BN-PAGE. BN-PAGE was used as described in (2). Technical details necessary for performing the electrophoresis are described below. Buffers are listed in Table 1.

CN-PAGE. First described in the present paper, CN-PAGE is identical to BN-PAGE, but Coomassie dye is omitted from sample and cathode buffer (Table 1).

Gel types. Linear polyacrylamide gradient gels that were usually overlaid by a 4% sample gel were used for BN-PAGE as well as CN-PAGE (Table 2). Uniform acrylamide gels will not work satisfactorily. Five to thirteen percent acrylamide gradient gels cover the M_r range from 10^5 to 10^6 Da in BN-PAGE; for resolution of smaller proteins 5–18% acrylamide gradient gels are preferred. For CN-PAGE, lower initial acrylamide concentrations are better because the electrophoretic mobility of proteins based on the intrinsic charge usually is lower than that in BN-PAGE. Steep acrylamide gradient gels, e.g., from 4 to 20%, are recommended for first trials. If CN-PAGE is used for separation of membrane proteins, detergent is added to the gel buffer (Table 1).

Sample preparation and choice of cathode buffer. Samples applied to BN-PAGE and CN-PAGE can be total membrane protein extracts, partially purified membrane proteins, or water-soluble proteins. These three groups needed different handling in BN-PAGE as described below and schematically depicted in Fig. 1. The differences concern the addition (or omission) of Coomassie dye to the sample and the concentration of Coomassie dye in the cathode buffer. The handling in CN-PAGE, however, was identical for all three groups. Ponceau S (0.001%) was added to all samples just to color them faintly and a colorless cathode buffer was used. Ponceau S only served as a marker for the end of electrophoresis. This dye does not bind to proteins under the experimental conditions and proteins migrate according to their isoelectric points in contrast to BN-PAGE, where the binding of Coomassie dye induces a charge shift.

Total membrane protein extracts. In solubilization of biological membranes the presence of salts, especially of potassium and divalent cations, was strictly avoided and substituted by aminocaproic acid. Salts may lead to precipitation of added Coomassie dye and of stained proteins. As an example, the sample preparation starting from bovine heart mitochondria is presented. Bovine heart mitochondria, prepared as described in (3), were sedimented by centrifugation (10 min, 10,000*g*). Sediments (200 μg of total protein) were solubilized by addition of 40 μl of 750 mM 6-aminocaproic acid, 50 mM Bistris, pH 7.0, and 5 μl of dodecyl maltoside (10%), and centrifuged (15 min, 100,000*g*). Shortly before starting BN-PAGE, Coomassie was added from a 5% stock solution in 500 mM aminocaproic acid to adjust to a detergent/Coomassie ratio of 4/1 (g/g). Addition of Coomassie to the sample and use of a cathode buffer with high

TABLE 2

Gel Composition for Blue Native and Colorless Native PAGE

	Sample gel	Gradient separation gel	
	4% T	5% T	18% T
AB (49.5% T, 3% C)	0.5 ml	1.8 ml	5.5 ml
Gel buffer (3×)	2 ml	6 ml	5 ml
Glycerol	—	—	3 g
APS (10%)	50 μl	100 μl	50 μl
TEMED	5 μl	10 μl	5 μl
Total volume	6 ml	18 ml	15 ml

Note. Linear gradient gels (1.6 mm) were cast at 4°C and maintained at room temperature for polymerization. AB (49.5% T, 3% C), 48 g acrylamide and 1.5 g bisacrylamide/100 ml; %T, total concentration of both monomers; %C, percentage of crosslinker to total monomer; Gel buffer (3×), see Table 1; APS (10%), ammonium persulfate solution (10%), freshly prepared; TEMED, tetramethylethylenediamine.

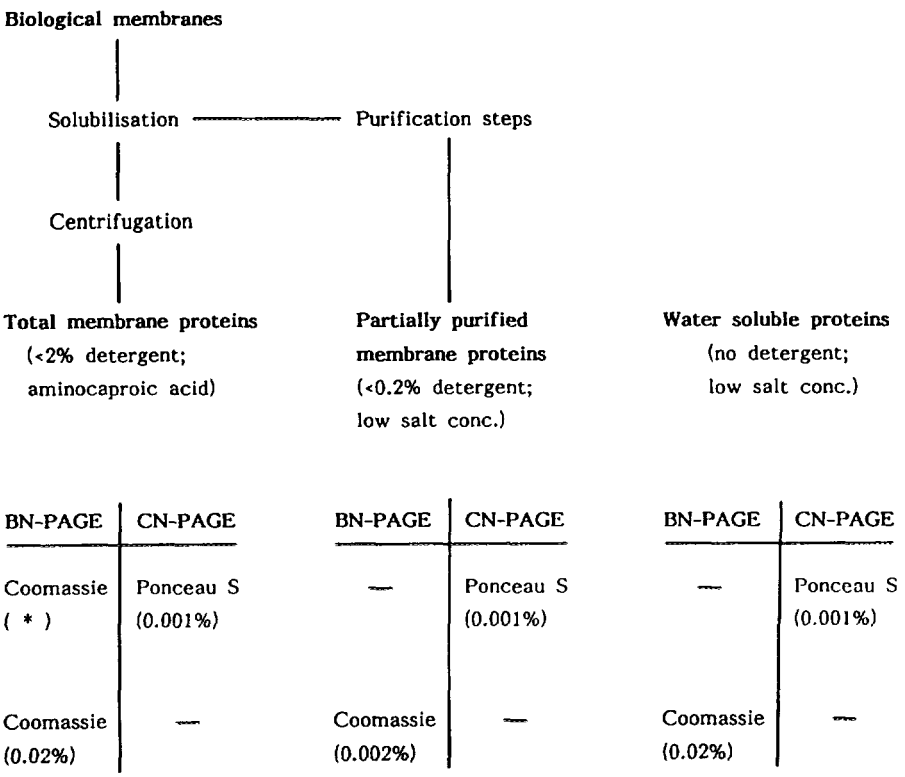

FIG. 1. Processing of different groups of proteins in blue native and colorless native PAGE. (*) Addition of Coomassie to the sample depended on the detergent concentration used. A detergent/Coomassie ratio of 4/1 (g/g) was adjusted. Reasons for using high or low Coomassie concentration in the cathode buffers are described in the text.

Coomassie concentration (0.02%) served for efficient removal of excess detergent and traction to the running front. For a better visualization of protein bands, the cathode buffer may be replaced after one-third of the run by a cathode buffer containing 0.002% Coomassie.

Partially purified membrane proteins. Partially purified membrane proteins dissolved in less than 0.2% detergent may contain low salt concentrations (up to 200 mM NaCl). Samples with low detergent concentrations needed no addition of Coomassie and precipitation was avoided. The Coomassie dye necessary for inducing the charge shift on the proteins in BN-PAGE was provided during electrophoresis by the cathode buffer containing 0.002% Coomassie.

Water-soluble proteins. Water-soluble proteins were dissolved in 15% glycerol, 50 mM Bistris/HCl, pH 7.0. Samples were applied without Coomassie. A cathode buffer with high Coomassie concentration (0.02%) was necessary for basic proteins.

Electrophoresis conditions. Native electrophoresis was performed at 4–7°C using a vertical apparatus according to Studier (4). Electrophoresis was started at 100 V until the sample was within the stacking gel and continued with voltage and current limited to 500 V and 15 mA. Running times were 3–4 h.

Second Dimension: Tricine–SDS–PAGE

The basic procedure for Tricine–SDS–PAGE was described in (5). Second-dimension Tricine–SDS–PAGE after BN-PAGE was described in (2). The following improved procedure gives better results with pieces as well as with whole lanes cut out from first-dimension gels.

Pieces or 0.5-cm-broad lanes of gel were placed on a glass plate at the usual position for stacking gels and soaked with 1% SDS, 1% mercaptoethanol for 2 h. Spacers were positioned (0.7 mm when pieces from 1.6-mm native gels were used) and the second glass plate was put on top. Mercaptoethanol solution was then removed as completely as possible because it inhibits polymerization of acrylamide. A separating gel mixture, usually for 16% acrylamide Tricine–SDS–PAGE (5), was poured between the glass plates leaving a 2-cm gap to the first-dimension gel. After polymerization, a 10% acrylamide Tricine–SDS–gel (1.5 cm) was overlaid. Finally the pieces of gel were embedded in a 10% acrylamide gel using the gel buffer of native gels (Table 1), but additionally 10% glycerol and 0.2% SDS were added. High ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) concentrations (70 μl of APS (10%) and 7 μl of TEMED per 10 ml) were necessary to allow complete polymerization within 30 min despite the presence of traces of mercaptoethanol.

Electrophoresis of 2D gels. Electrophoresis of 2D gels (0.7 mm; total length of 14 cm) was performed at room temperature (250 V; about 6 h) with the current limited to 40 mA.

Two-Dimensional Native PAGE

CN-PAGE as described above was always used in the first dimension of two-dimensional native PAGE. BN-PAGE then followed in the second dimension. The transition from first to second dimension was performed as follows: One "lane" (1–3 cm) of CN-PAGE was cut out and placed between two glass plates at the usual position for stacking gels. Then, a detergent-free 6–16% acrylamide gradient gel, preparing for the second-dimension BN-PAGE, was cast below the first-dimension gel. Finally a 4% stacking gel was cast below the gel strip. Electrophoresis with voltage and current limited to 500 V and 15 mA, respectively, was then started using a stacking buffer (100 mM glycine, 20 mM Bistris, 0.002% Serva blue G, pH 8.1, at 4°C). The protein bands from the first-dimension CN-PAGE were thereby moved out of the gel and concentrated. The glycine buffer was removed by suction when the blue protein bands were completely within the 4% gel and regular BN-PAGE followed (cathode buffer: 0.002% Coomassie, 50 mM Tricine, 15 mM Bistris, pH 7.0, at 4°C).

Third Dimension: Tricine–SDS–PAGE

The procedure of Tricine–SDS–PAGE, using protein spots after the two-dimensional native PAGE, was identical to the second-dimension Tricine–SDS–PAGE described above.

Extraction of Proteins from Gels

Extraction of proteins was performed either by native electroelution as described in (2) or in denatured form in a more convenient way as follows: Pieces of gel were incubated overnight in a 10-fold volume of 0.05% SDS. The protein solution was freeze-dried and redissolved in a minimal volume of 0.5% mercaptoethanol. The sample density was high enough for direct application to SDS-gels due to the presence of aminocaproic acid from the gel pieces.

RESULTS AND DISCUSSION

Preparative Applications of Native and Denaturing Techniques

Alternative Native First Dimensions: Comparison of Resolution of BN-PAGE and CN-PAGE

Solubilized bovine heart mitochondria (cf. Materials and Methods) were used to compare the resolving power of BN-PAGE and CN-PAGE in gels optimally resolving the membrane protein complexes I–V (Fig. 2). The identity of the bands was revealed by the characteristic polypeptide patterns of the complexes in second dimension Tricine–SDS–PAGE (Fig. 3).

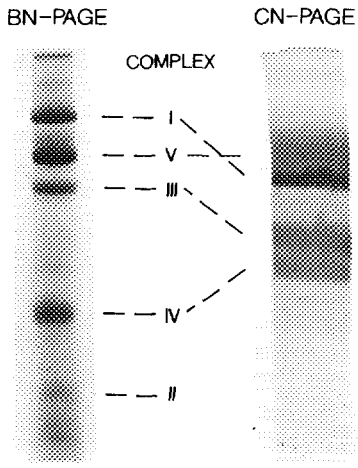

FIG. 2. Comparison of resolution of first dimension blue native PAGE (BN-PAGE) and colorless native PAGE (CN-PAGE) using solubilized bovine heart mitochondria. One hundred micrograms total protein was applied to an area of 16 mm^2. A 5–13% acrylamide gradient gel was used for BN-PAGE, a 4–16% gel with an extended 3% sample gel (4 cm) for CN-PAGE. Gels were fixed and stained by Coomassie as described in (5). The most prominent bands were the membrane protein complexes I–V of the system of oxidative phosphorylation. They were identified by their characteristic polypeptide patterns in second-dimension SDS–PAGE (Fig. 3).

CN-PAGE did not reach the quality of BN-PAGE with the special membrane protein sample used and complex II was not detected; however, an inversion of migration distances of complex I (M_r 880 kDa) and complex V (M_r 600 kDa) compared to BN-PAGE was observed, suggesting the idea of using CN-PAGE as a prepurification step before BN-PAGE. The inversion of migration distances was observed only with gradient gels starting at low acrylamide concentration (3%), where the molecular sieving effect did not dominate the charge dependent electrophoretic mobility. The 3% gel did not carry proteins of interest and was discarded after electrophoresis.

Resolution of the complexes by CN-PAGE was also dependent on the running time. The right side of Fig. 2 shows the best compromise for all complexes obtained when electrophoresis was stopped with Ponceau S about 2 cm before the gel front. Continuation of electrophoresis improved the resolution of complexes III and IV, but caused deterioration of the resolution of complexes I and V (not shown) and made the transition to the second-dimension BN-PAGE (next section) more difficult. It is therefore advisable to shorten running times in CN-PAGE in problematic cases with some loss of optimal separation. Proteins need not reach their individual end points of migration in order to achieve a sufficient electrophoretic mobility.

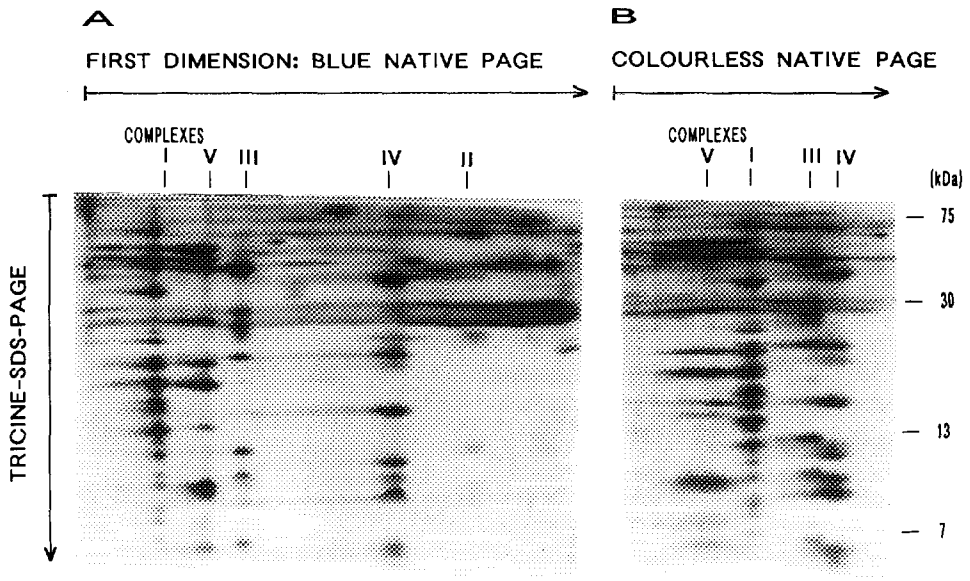

FIG. 3. Two-dimensional denaturing resolution of bovine heart mitochondria using blue native or colorless native PAGE in the first dimension (cf. Fig. 2) and denaturing Tricine–SDS–PAGE (16% acrylamide gels) in the second dimension: (A) BN-PAGE/Tricine–SDS–PAGE, (B) CN-PAGE/Tricine–SDS–PAGE. In (A), the five membrane protein complexes were separated and identified by their characteristic polypeptide patterns. In (B), more overlap of complexes was observed and complex II was not detected.

Two-Dimensional Native Electrophoresis: CN-PAGE Combined with BN-PAGE

After first-dimension CN-PAGE for preseparation of complexes I–V, one lane was processed by second-dimension BN-PAGE as described under Materials and Methods. Five prominent blue spots were detected during electrophoresis (Fig. 4) and were assigned to complexes I–V after identification by Tricine–SDS–PAGE in the third dimension.

Third Dimension: Tricine–SDS–PAGE

Blue spots from the two-dimensional native gel (Fig. 4), corresponding to complexes I and V, were cut out and processed further by third-dimension Tricine–SDS–PAGE (Fig. 5, lanes 2N). Similarly, blue bands of complexes I and V from first dimension BN-PAGE (Fig. 2) were used as references (Fig. 5, lanes 1N). The increase in purity using two preceding native separations (lanes 2N) instead of only one (lanes 1N) is seen best with complex I. Complex I comprises 41 subunits (6), but only about 30 subunits are usually resolved by SDS–PAGE. Major contamination by complex V (7) was removed by using two native separations. In addition to this technique using "implanted" pieces of gel, there is the potential to extract proteins first in the native or denatured state (cf. Materials and Methods), to concentrate the sample by freeze-drying, and to apply the proteins in free solution to SDS gels (not shown). Both the implantation and the extraction techniques offer advantages and disadvantages.

With the implantation technique, the proximity of individual complexes could be analyzed and impurities identified by band shapes differing from those of true subunits (Fig. 5). This information was lost with the extraction methods.

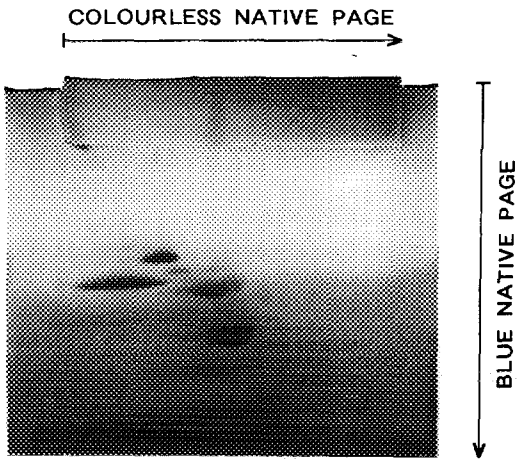

FIG. 4. Two-dimensional native resolution. Bovine heart mitochondria were resolved by CN-PAGE in first native dimension (cf. Fig. 2). One complete lane was then resolved by second dimension Blue Native PAGE. Individual complexes were detected as blue spots during electrophoresis. Assignment of complexes was possible after identification by Tricine–SDS–PAGE in the third dimension (cf. Fig. 5). Complex II, detected neither after CN-PAGE (Fig. 2) nor by two-dimensional denaturing resolution using CN-PAGE/Tricine–SDS–PAGE (Fig. 3B), was resolved from complexes III and IV by the second-dimension BN-PAGE.

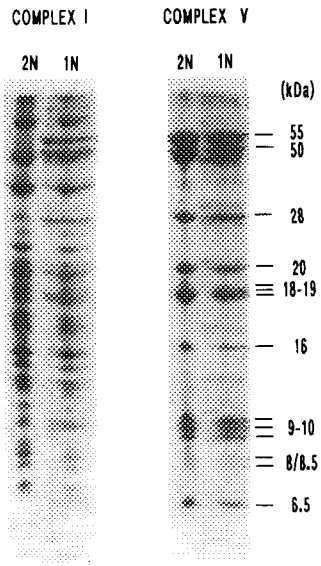

FIG. 5. Third-dimension Tricine–SDS–PAGE of complexes I and V. 2N: Pieces of gel after two-dimensional native PAGE (cf. Fig. 4) were used. 1N: Bands from BN-PAGE (cf. Fig. 2), using only one native purification step, were used as a reference. Tricine–SDS–PAGE using a 16% acrylamide gel, fixation and staining by Coomassie, was performed as described in (5).

An advantage of the extraction techniques was the free choice of protein load for optimal resolution and for protein sequencing after electroblotting. One spot from the two-dimensional native gel contained about 20–60 μg of individual complexes. In cases with demand for more protein, e.g., for immunization, spots from several gels were collected, eventually kept at −80°C, and extracted together.

Quarternary structure of membrane protein complexes I–V unexpectedly was retained after freezing and thawing gel pieces. This was tested by BN-PAGE (migration distances were unchanged) and by second-dimension Tricine–SDS–PAGE showing unchanged subunit compositions (not shown). The presence of 500 mM aminocaproic acid in the frozen gel pieces seems to protect complexes against dissociation similar to the commonly used protective effect of glycerol.

Analytical Applications of Native and Denaturing Techniques

Molecular Mass Calibration of BN-PAGE and CN-PAGE

Water-soluble standard proteins and membrane protein complexes I–V listed in Table 3 were used for molecular mass calibration in BN-PAGE and CN-PAGE. At a high protein load (15 μg), dimeric forms suitable as further molecular mass standards could be detected (Fig. 6).

From migration distances in BN-PAGE and known molecular masses of the proteins, a calibration curve was deduced (Fig. 7A). A linear log M_r–distance relation resulted when 5–18% acrylamide gradient gels were used. All proteins that either had isoelectric points below pI = 5.4 or bound Coomassie fit the straight line except some extremely basic proteins. The basic proteins trypsinogen (No. 20, pI = 9.3) and cytochrome c (No. 21, pI = 10.7) did bind Coomassie and they migrated to the anode; however, the charge shift was not sufficient and broad bands were detected (Fig. 6A, lanes m and n). Both forms of lactate dehydrogenase (Nos. 7 and 10, pI = 8.6) fit the calibration line and the bands were much sharper (Fig. 6A, lane l). The third protein, which did not fit the line for BN-PAGE, was carbonic anhydrase II (No. 19, pI = 5.9). This protein did not bind Coomassie. There was no charge shift and the electrophoretic mobility due to the small difference between pI = 5.9 and the running pH 7.5 of the gel was too small (Fig. 6A, lane a).

In CN-PAGE, an almost identical calibration line was observed (Fig. 7B) when only proteins with isoelectric points below pI = 5.4 were considered. This means that migration distances of these acidic proteins were identical to BN-PAGE. Proteins with isoelectric points above 5.4 deviated from the line. The basic proteins (cytochrome c, No. 21; trypsinogen, No. 20; lactate dehydrogenase, No. 10; and the dimeric form, No. 7) migrated to the cathode and therefore were not detected (Fig. 6B, lanes l–n). Carbonic anhydrase II (No. 19; pI = 5.9) showed migration behavior in CN-PAGE similar to that in BN-PAGE because it did not bind Coomassie. Conalbumin (No. 14) with identical pI behaved similarly in CN-PAGE, i.e., it deviated strongly from the calibration line; however, it fit the line in BN-PAGE since it bound Coomassie. Complexes I, III, V, and IV (Nos. 2, 3, 4, and 9) deviated from the calibration line in CN-PAGE. Isoelectric points therefore seemed to be above pI = 5.4.

Conversely, it was deduced from the proteins fitting the calibration curve that CN-PAGE is only suitable for determination of molecular masses of acidic proteins with pIs at or below 5.4.

BN-PAGE has a much broader range of application. It can be applied to determination of molecular masses if one of the following conditions is fullfilled: (i) isoelectric points are at or below 5.4; (ii) analyzed proteins bind Coomassie blue G and pIs are below 8.6. All membrane proteins and most water-soluble proteins seem to bind Coomassie.

Binding of Coomassie certainly was helpful for increasing electrophoretic mobility; however, comparison with the molecular mass calibration of CN-PAGE showed that binding of Coomassie is not a necessary condition for fitting the calibration line, provided the isoelectric points of the proteins are low enough. Negative excess charges of the protein itself or of protein

TABLE 3

Native Membrane and Water-Soluble Proteins for Molecular Mass Calibration

No.	Protein	Source	p*I*	M_r (kDa)	Blue stain
1.	Ferritin (dimeric form of No. 6)	Horse spleen[a]	4.2–4.5	880	No
2.	Complex I (monomer)	Bovine heart[d]	<7	880	Yes
3.	Complex V (monomer)	Bovine heart[d]	<7	600	Yes
4.	Complex III (dimer)	Bovine heart[d]	<7	460	Yes
5.	Catalase (dimeric form of No. 8)	Bovine liver[a]	5.4	460	No
6.	Ferritin	Horse spleen[a]	4.2–4.5	440	No
7.	L-Lactate dehydrogenase (dimeric form of No. 10)	Rat muscle[b]	8.6	280	Yes
8.	Catalase	Bovine liver[a]	5.4	230	No
9.	Complex IV (monomer)	Bovine heart[d]	<7	200	Yes
10.	L-Lactate dehydrogenase	Rat muscle[b]	8.6	140	Yes
11.	Complex II (monomer)	Bovine heart[d]	<7	130	Yes
12.	Albumin (dimeric form of No. 15)	Bovine serum[c]	4.9	132	Yes
13.	Hexokinase	Yeast[b]	4.5–4.8	99	Weak
14.	Conalbumin	Chicken egg[b]	5.9	78	Yes
15.	Albumin (monomer)	Bovine serum[c]	4.9	66	Yes
16.	β-Lactoglobulin B	Bovine milk[b]	5.3	35	Weak
17.	β-Lactoglobulin A	Bovine milk[b]	5.1	35	Weak
18.	Trypsin inhibitor	Soy bean[b]	4.6	21	Weak
19.	Carbonic anhydr. II	Bovine ery.[b]	5.9	30	No
20.	Trypsinogen	Bovine pancreas[b]	9.3	24	Yes
21.	Cytochrome c	Horse heart[b]	10.7	12	Yes

Note. Isoelectric points (p*I*) and molecular masses (M_r) are from Righetti and Caravaggio (8) and from product information: Serva[a], Sigma[b], and Behring Werk[c]. Bovine heart mitochondria[d] were prepared and processed as described under Materials and Methods.

bound anions are responsible for anodic migration; however, the separating principle is the molecular sieving effect of the acrylamide gradient gel. In contrast to SDS–PAGE, protein migration ceases when areas of appropriate pore size within the polyacrylamide gradient gel are reached.

In order to avoid pitfalls in determination of molecular masses by BN-PAGE, determination of isoelectric

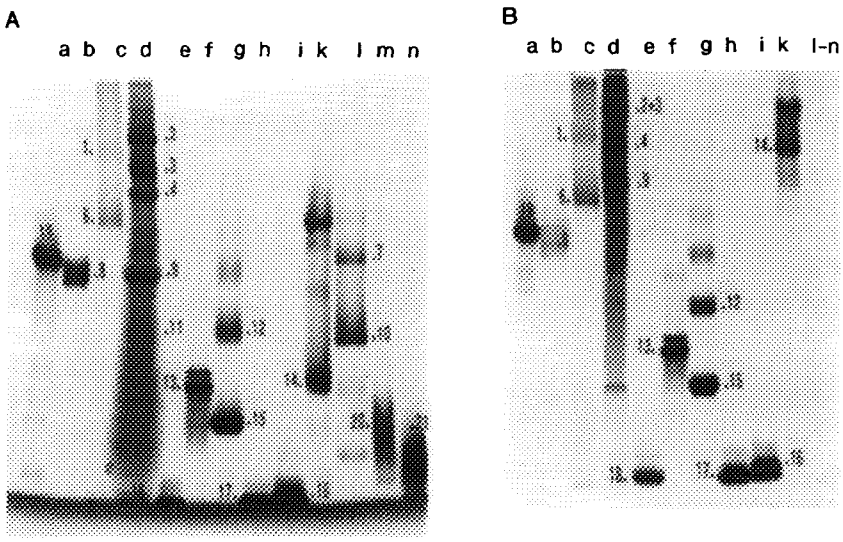

FIG. 6. Separation of water-soluble and membrane proteins by blue native PAGE (A) and colorless native PAGE (B). Numbering of protein bands followed Table 3. Fifteen micrograms of water-soluble proteins was applied to areas of 8 mm² in 5–18% acrylamide gradient gels. Lanes a–c, proteins that do not bind Coomassie; lane d, membrane protein complexes I–V from solubilized bovine heart mitochondria (cf. Materials and Methods); lanes e–n, proteins with isoelectric points increasing from e (p*I* = 4.6) to n (p*I* = 10.7). Basic proteins applied to lanes l–n in CN-PAGE (B) migrated to the cathode and were not detected. Fixation and staining by Coomassie were performed as described in (5).

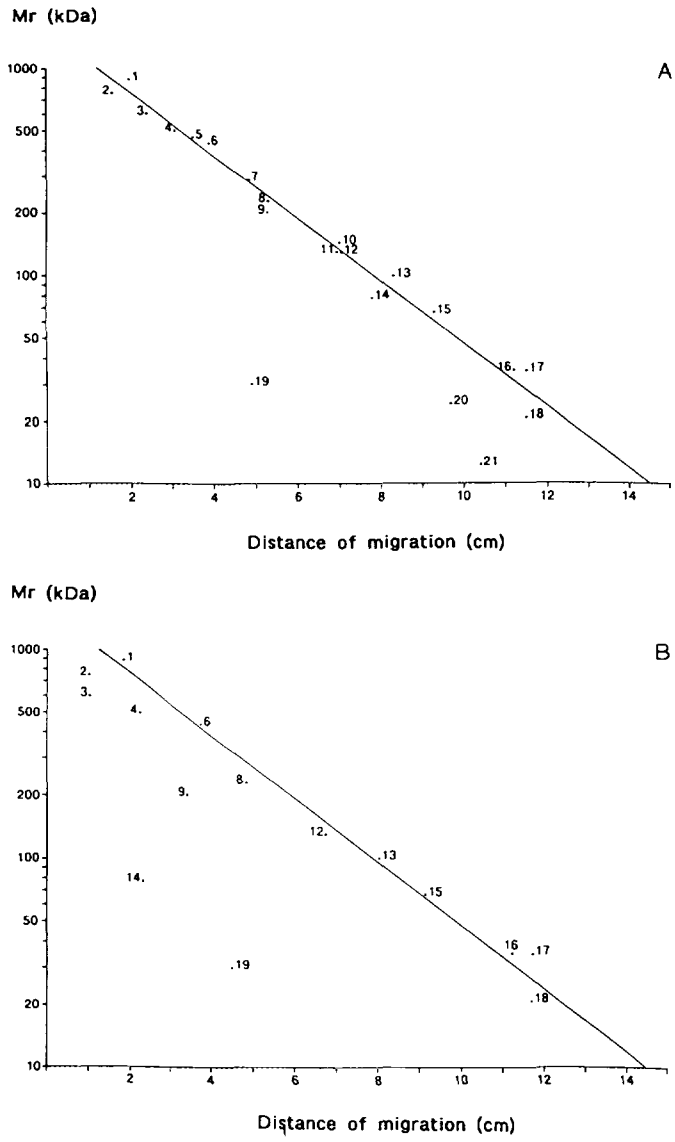

FIG. 7. Molecular mass calibration curves for blue native PAGE (A) and colorless native PAGE (B) using proteins and gels as in Fig. 6. Calibration line of CN-PAGE was almost identical to that of BN-PAGE when only proteins with isoelectric points at or below 5.4 were considered.

points by independent methods should be performed if proteins, capable of binding Coomassie, are not detected in CN-PAGE (basic proteins) and if proteins do not bind Coomassie.

Maximal deviations from known molecular masses were below 20% with proteins binding Coomassie (p*I*s below 8.6) and with proteins not binding Coomassie (p*I*s below 5.4). Apparent molecular masses of the membrane protein complexes were not those of the protein moieties but of the protein–lipid complexes. About 20 molecules of the negatively charged cardiolipin were bound to complex III dimer (9) and 10 molecules of cardiolipin to complex IV monomer (10) besides varying amounts of neutral phospholipids. Although neither ligands (phospholipid, detergent, Coomassie) nor the influence of globular or rod-like protein shape were considered in BN-PAGE, the apparent molecular masses, given in Table 3, were consistent (±20%) with data from the literature (1, 2, 6, 7, 11).

Quality Control and Analysis of Hydrodynamic Properties of Membrane Protein Preparations by Blue Native PAGE/Tricine–SDS–PAGE

Discrimination between structurally associated and separated proteins. "Core" proteins I and II of yeast complex III (12) with molecular masses of 48.3 and 38.7 kDa (13, 14), respectively, were dissociated from hydroxyapatite-bound complex III by 2 M urea, 0.5% Triton X-100, 400 mM NaCl, 50 mM Na-phosphate, pH 7.2. Both proteins were eluted in the same fraction. Our question was: Do subunits I and II remain associated under the eluting conditions or are single subunits coeluted from the column? Since the density of the sample was still high enough after dilution with the same volume of water, 50 μl of the diluted sample was directly applied to a 16-mm^2 area of a linear 5–16% acrylamide gel without addition of Coomassie to the sample. BN-PAGE was performed with low dye concentration (0.002%) in the cathode buffer from the beginning. Two blue bands near the front were observed during BN-PAGE (Fig. 8). Tricine–SDS–PAGE in the second dimension demonstrated that these two bands were core proteins I and II that migrated as dissociated single subunits in BN-PAGE. This shows that the application range of BN-PAGE also includes native proteins with a molecular mass below 10^5 kDa.

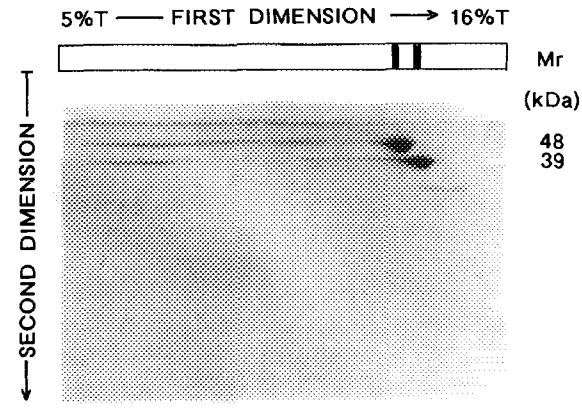

FIG. 8. Two-dimensional resolution of core proteins I and II from yeast complex III by blue native PAGE/Tricine–SDS–PAGE. A linear 5–16% acrylamide gradient gel was used in BN-PAGE. In Tricine–SDS–PAGE, a uniform 16% gel was used and proteins were stained by Coomassie (5).

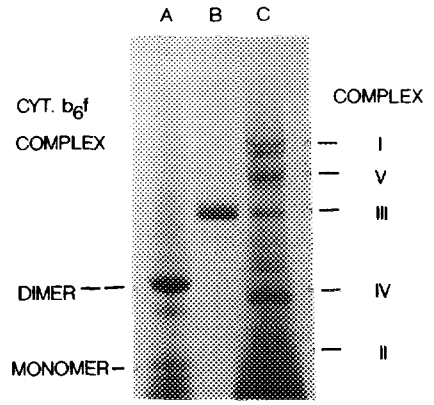

FIG. 9. First-dimension blue native PAGE of (A) cytochrome b_6f complex of spinach chloroplasts, (B) isolated complex III from bovine heart mitochondria, and (C) solubilized bovine heart mitochondria. Proteins were stained in native state during BN-PAGE and the photograph was taken when the gel was still between the glass plates. A linear 5–16% acrylamide gradient gel was used. Further resolution of subunits by second-dimension Tricine–SDS–PAGE is shown in Fig. 10.

Analysis of cytochrome b_6f complex. Cytochrome b_6f complex (6.6 mg/ml) from spinach chloroplasts (15) was stored at −80°C in 0.2% dodecyl maltoside, 30 mM Tris–succinate at pH 7.8. The density was increased by adding 4 μl of 2 M 6-aminocaproic acid to 8 μl of the sample before application to BN-PAGE. Our question was: Does the complex exist in monomeric or in dimeric form?

Several bands were observed in the first-dimension BN-PAGE (Fig. 9, lane A). One band was slightly larger than complex IV with a molecular mass of about 250 kDa. Another band was slightly smaller than complex II, i.e., the estimated molecular mass was somewhat below 140 kDa. The resolution of the essential lower part of lane A by second-dimension Tricine–SDS–PAGE (Fig. 10A) showed that these two bands corresponded to two different aggregation states. From the apparent molecular masses of the subunits (cytochrome b and the iron–sulfur protein with apparent molecular masses of 19 kDa were not resolved), a minimal molecular mass of 110 kDa was calculated.

Therefore, the predominant form of the b_6f complex is the dimer and a minor part is present in monomeric form.

Analysis of purified complex III. Complex III from bovine heart was prepared according to (16). The purified enzyme (2.5 mg protein/ml) in 0.05% Triton X-100, 10% glycerol, 100 mM NaCl, 20 mM Mops at pH 7.2 was stored at −80°C. Ten microliters of the sample was applied directly to an area of 16 mm^2.

Purified complex III seemed to be a rather pure, homogeneous and monodisperse preparation, as only one blue band at the position of dimeric complex III was detected in BN-PAGE (Fig. 9, lane B). Resolution of lane B by second dimension Tricine–SDS–PAGE (Fig. 10B) showed that almost no impurities were observed and all 11 subunits including the detergent labile 6.4-kDa subunit were present. The band shapes of individual subunits, however, were not exactly the same for all 11 subunits. Bands of the labile subunits, 6.4 kDa (17) and 21 kDa-ISP (18), were somewhat shorter as indicated in Fig. 10B. This means that a minor portion of a subcomplex missing the 2 subunits was running just ahead of the main band of intact complex III in the first-dimension gel. The 2 subunits were partially removed by BN-PAGE and migrated near the gel front; these subunits were detected as single spots in the right area of the 2D SDS gel. The dissociation of the 6.4-kDa and ISP subunits has been reported previously (19).

A comparable discrimination between a major part of intact dimeric complex III (M_r 460 kDa) and a minor part of a subcomplex (M_r 400 kDa) is not possible by density gradient centrifugation, gel filtration, or analytical ultracentrifugation methods. Therefore the resolution of the two-dimensional electrophoretic technique in this respect is superior to that of analytical ultracentrifugation.

CONCLUSIONS

Electrophoretic techniques in the presence of SDS are well established methods used for analytical and preparative purposes. Electrophoretic methods separating native proteins, however, are rarely used for water-soluble proteins and even less for membrane proteins (20). Some reasons are as follows: (i) Methods relying on the protein intrinsic charge are not generally applicable because the effective electrophoretic mobility of proteins differs strongly. (ii) The running pH not only influences the resolution and the p*I* range of proteins that can be separated but also the stability of proteins. The running pH in the commonly used Ornstein–Davis system (21,22), i.e., the Laemmli system (23) without SDS, for example, is at pH 9.5 and therefore covers a wide range of proteins. However, only that part of the proteins applied that is insensitive to pH 9.5 will be separated in native form. The membrane protein complexes studied here, for example, were found to dissociate irreversibly into their protein subunits. Therefore the running pH of the novel techniques CN-PAGE and BN-PAGE was fixed to pH 7.5. (iii) A further problem, especially concerning membrane proteins, is the tendency of membrane proteins to form aggregates even in the presence of detergents and/or to denature because of the detergents.

In BN-PAGE, the problem of aggregation is not completely solved but largely reduced by the use of the negatively charged dye Coomassie blue G-250. This dye binds to hydrophobic domains on the surface of proteins

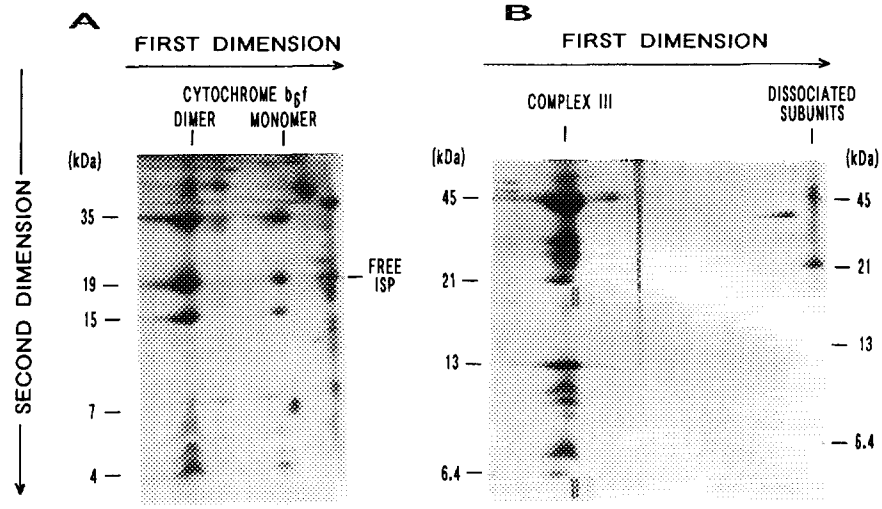

FIG. 10. Two-dimensional resolution of cytochrome b_6f complex from spinach leaves (A) and of isolated complex III from bovine heart (B) by blue native PAGE/Tricine–SDS–PAGE. Preceding first-dimension BN-PAGE is shown in Fig. 9. In (B), a subcomplex of complex III was detected just ahead of the main band. The missing subunits of the subcomplex are marked by II. A 16% acrylamide gel was used in Tricine–SDS–PAGE and proteins were stained by Coomassie (5).

(not all water-soluble proteins in native state bind the dye) and exerts four main effects: (i) The binding of the anionic dye induces a charge shift on the proteins; even basic proteins then migrate to the anode at pH 7.5 and the applicability range is extended. (ii) The induced negative surface charge reduces the problem of protein aggregation. (iii) The danger of denaturation is low because a transition to detergent-free areas of the gel follows. Coomassie is not a detergent, but it keeps membrane proteins solubilized in the absence of detergent and it binds tightly in the absence of detergent due do its hydrophobicity and poor solubility in water. (iv) A side effect is the staining of native proteins during electrophoresis, which facilitates protein detection.

BN-PAGE differs from SDS–PAGE in several respects: (i) In BN-PAGE, proteins are solubilized by a minimal amount of a neutral detergent, whereas in SDS–PAGE proteins are solubilized by an excess of the denaturing detergent SDS. (ii) Coomassie dye binds to the protein surface; SDS dissociates and unfolds proteins. (iii) The ratio of bound Coomassie/protein and the charge/mass ratio is variable in BN-PAGE, whereas the SDS/protein ratio usually is 1.4 (g/g) and a largely uniform charge/mass ratio results. (iv) Despite the differences concerning the charge/mass ratio, both BN-PAGE as well as SDS–PAGE allow analysis of molecular masses of proteins. In BN-PAGE, the pore-size distribution of the acrylamide gradient gel determines the individual *end points of migration of proteins.* The electrophoretic mobility of proteins gradually approaches zero when the restrictive acrylamide concentration, which varies with the size of the protein, is reached. In these circumstances, proteins could not pass through the gel and be eluted even at extended running times. In SDS–PAGE, however, acrylamide concentrations are used that would allow proteins to pass the whole gel at extended running times. The appropriate acrylamide concentrations used in SDS–PAGE thus have a modulating but not totally restrictive effect on the electrophoretic mobility. Molecular mass determination of *migrating proteins* is possible in this case because the charge/mass ratio is identical for "all" proteins. The modulation of the electrophoretic mobility by the gel pores thus becomes the only protein-size dependent factor.

Quantitation after BN-PAGE/Tricine–SDS–PAGE was possible with cytochrome-containing complexes III and IV from solubilized mitochondria. These could be quantitated spectrophotometrically before application to BN-PAGE. The bands of the complexes then were excised from BN-PAGE and resolved by a second dimension Tricine–SDS–PAGE. Defined amounts of chromatographically purified complexes were applied as standards to the same gel (not shown). The yield was 95% with complex III and 80% with complex IV.

How native are the proteins isolated by BN-PAGE and CN-PAGE? Is the term native justified or would the term nondissociating be more appropriate? The retainment of catalytic activity of three of the five complexes of the system of oxidative phosphorylation was already described in a previous publication concerning the preparative aspects of BN-PAGE (2). Catalytic activity of complexes III and IV has now been performed after two-dimensional native electrophoresis (first-dimension CN-PAGE, second-dimension BN-PAGE, followed by electroelution). The turnover numbers of electro-

eluted complexes III and IV were 600 and 130 s^{-1}, respectively; these are the turnover numbers of good chromatographic preparations. The term native electrophoresis seems to be justified for CN-PAGE as well as BN-PAGE, although not every membrane protein will necessarily be isolated in enzymatically active form.

Summarizing, the novel electrophoretic techniques offer the following advantages:

(i) *Blue native PAGE,* performed on an analytical and preparative scale, allows isolation of native membrane protein complexes directly from biological membranes or homogenized animal tissue and is a valuable means for final purification of milligram amounts of partially purified membrane proteins, e.g., for immunization and protein sequencing.

(ii) *Two-dimensional denaturing PAGE,* combining BN-PAGE in the first dimension with SDS–PAGE in the second dimension, is a high-resolution method for analysis of molecular masses and oligomeric states of microgram amounts of native proteins, especially of native membrane protein complexes. BN-PAGE thereby is the essential part because the desired protein as well as the molecular mass markers can be detected as blue bands during the first-dimension run. SDS–PAGE only serves for unambiguous identification of proteins by their characteristic polypeptide patterns. Alternatively, blue bands from BN-PAGE can be electroeluted and identified by enzymatic tests.

(iii) *CN-PAGE* has lower resolution than BN-PAGE and is restricted to acidic proteins. There is no dye that might interfere with measurement of catalytic activity.

(iv) *Two-dimensional native PAGE,* combining the two native techniques CN-PAGE and BN-PAGE to a two-dimensional system, yields significantly higher purity of proteins than BN-PAGE alone. Since CN-PAGE separates only proteins with p*I*s below pH 7.5, this technique is restricted to acidic proteins. After 2D native PAGE and electroelution, functionally active proteins can be recovered.

(v) *Three-dimensional denaturing PAGE,* combining the two-dimensional native PAGE with SDS–PAGE in the third dimension, allows identification of true subunits of a multiprotein complex and discrimination from impurities. Using mitochondria, for example, the subunits of the complexes are detectable by Coomassie staining. Thus, the amount of protein recovered is close to the amount required for direct protein sequencing, even when the separation is performed on the analytical scale.

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