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REVIEW

Two-dimensional electrophoresis of membrane proteins

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Abstract One third of all genes of various organisms encode membrane proteins, emphasizing their crucial cellular role. However, due to their high hydrophobicity, membrane proteins demonstrate low solubility and a high tendency for aggregation. Indeed, conventional two-dimensional gel electrophoresis (2-DE), a powerful electrophoretic method for the separation of complex protein samples that applies isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, has a strong bias against membrane proteins. This review describes two-dimensional electrophoretic techniques that can be used to separate membrane proteins. Alternative methods for performing conventional 2-DE are highlighted; these involve replacing the IEF with electrophoresis using cationic detergents, namely 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) and cetyl trimethyl ammonium bromide (CTAB), or the anionic detergent SDS. Finally, the separation of native membrane protein complexes through the application of blue and clear native gel electrophoresis (BN/CN-PAGE) is reviewed, as well as the free-flow electrophoresis (FFE) of membranes.

Keywords Membrane proteins · SDS/SDS-PAGE · 16-BAC/SDS-PAGE · BN-PAGE · Free-flow electrophoresis

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Introduction

Cells from all organisms are entirely enclosed by a phospholipid bilayer, the plasma membrane, through which cells communicate with their environment [1]. Channels, pumps and transporters formed from membrane proteins allow the controlled influx and efflux of metabolites and ions. Membrane receptors are used to relay extracellular signals, such as growth factors, to intracellular signals that trigger cellular responses. Eukaryotic cells are compartmentalized in organelles enclosed by further membranes. In mitochondria, for instance, the respiratory chain is embedded in the inner mitochondrial membrane, ensuring an energy supply for the cell. The importance of membrane proteins is highlighted by the fact that about one-third of all of the genes in various organisms code for this class of proteins [2, 3]. Over two-thirds of all medications exert their effects through membrane proteins [4, 5]. Therefore, the analysis of membrane proteins is highly relevant to our understanding of life and diseases.

Membrane proteins are proteins that are associated with cellular membranes [1, 6] (Table 1). Integral membrane proteins comprise one or several membrane-spanning regions. In most integral membrane proteins, these regions are formed from hydrophobic stretches of nonpolar amino acids that transverse the phospholipid bilayer as α-helices [1]. For instance, the visual pigment rhodopsin comprises seven transmembrane domains (Table 1). Hence, the hydrophobicity of membrane proteins usually increases with the number of membrane-spanning α-helices. Other transmembrane proteins, such as outer membrane proteins of bacteria (e.g., the outer membrane protein F from *E. coli*) and mitochondria (e.g., the outer mitochondrial membrane protein porin-1), form β-barrels that transverse the membranes as pores [1]. Here, the membrane-spanning domain

consists of alternate polar and nonpolar amino acids that face either the aqueous channel or the hydrophobic lipid bilayer. Therefore, the hydrophobicity of these proteins is markedly lower than that of proteins with several transmembrane α-helices (Table 1). In contrast to integral membrane proteins, peripheral membrane proteins do not contain membrane-spanning domains, and may be localized to the membrane via either transient interactions or via covalently attached lipid anchors [1] (Table 1). Thus, peripheral membrane proteins are markedly more hydrophilic than integral membrane proteins.

The biochemical analysis of (integral) membrane proteins is hampered due to their high hydrophobicity [6, 7]. Two main tasks must be performed: (i) the extraction of membrane proteins from their hydrophobic lipid environment and their solubilization in the aqueous environment; (ii) the membrane proteins must be kept solubilized and prevented from aggregating throughout the whole separation procedure. These problems become worse as the hydrophobicity of the analyzed proteins increases. Thus, the analysis and separation of integral membrane proteins, especially those containing several α-helical transmembrane domains, is very complex.

Membrane proteins can be extracted from their lipid environments and kept in solution by incorporating them into detergent micelles [7, 8]. Ionic detergents such as the anionic detergent sodium dodecyl sulfate (SDS) and the cationic detergent 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) have strong solubilizing powers [7, 9, 10]. However, their application results in the denaturing of the analyzed proteins. Nonionic detergents such as Triton X-100, Nonidet P40, digitonin, and dodecyl maltoside are milder and may allow the solubilization of native membrane proteins and membrane protein complexes [7, 8, 11, 12].

Solubilized membrane proteins may be separated by electrophoretic and nonelectrophoretic techniques. Density gradient centrifugation and chromatography (e.g., high-performance liquid chromatography, HPLC) have been successfully used for the nonelectrophoretic separation of membrane proteins and membrane protein complexes [8, 13–15]. Many of these nonelectrophoretic techniques have been coupled to electrophoretic techniques in the second dimension, such as SDS-PAGE. This review focuses on the two-dimensional (2-D) separation of membrane proteins in which electrophoretic methods are used in both dimensions. Both gel-based denaturing and native electrophoretic separations of membrane proteins are described, as well as gel-free isolation and purification of membranes by free-flow electrophoresis.

2-D separation of membrane proteins through the application of conventional 2-DE (IEF/SDS)

Conventional 2-D gel electrophoresis (2-DE) permits the separation of protein samples according to their isoelectric points (p*I*) and their molecular masses. It involves the application of isoelectric focusing (IEF) in the first dimension, and the application of SDS-PAGE in the second dimension [16]. This method allows the routine separation of more than 2000 proteins, and displays complex protein samples as 2-D protein patterns [17]. The high resolving power of conventional 2-DE and its

Table 1 Examples of membrane proteins

Protein name	SwissProt identifier[a]	GRAVY index[b]	Trans-membrane domain	Species
Capsule polysaccharide export inner-membrane protein kpsE	KPSE1_ECOLI	−0.16	2	*E. coli*
Inner membrane protein creD	CRED_ECOLI	+0.22	5	
UPF0126 inner membrane protein yadS	YADS_ECOLI	+0.95	7	
Outer membrane protein F	OMPF_ECOLI	−0.42	Porin	
ADP,ATP carrier protein 2	ADT2_YEAST	+0.10	5	*S.*
Pheromone α factor receptor	STE2_YEAST	+0.15	7	*cerevisiae*
Cytochrome *c* oxidase subunit 1	COX1_YEAST	+0.75	12	
Outer mitochondrial membrane protein porin 1	VDAC1_YEAST	−0.10	Porin	
Glycophorin A	GLPA_HUMAN	+0.01	1	*H. sapiens*
Rhodopsin	OPSD_HUMAN	+0.54	7	
Ras-related C3 botulinum toxin substrate 1 (Rac1)	RAC1_HUMAN	−0.15	Lipid anchor	
Triosephosphate isomerase	TPIS_HUMAN	−0.10	Soluble	

[a] SwissProt database: http://www.expasy.org/; [b] GRAVY (grand average hydrophobicity) index was calculated with ProtScale software (http://www.expasy.org/tools/protscale.html). GRAVY specifies the hydrophobicity of proteins. High positive values suggest high hydrophobicity, whereas negative values indicate high solubility

ability to separate proteins with distinct post-translational modifications makes it an invaluable method for proteomic analyses [16, 17].

Conventional 2-DE has a strong bias against hydrophobic membrane proteins [6]. The higher the hydrophobicity of the protein, the lower the efficiency of separation by conventional 2-DE. This is largely due to the requirements of the IEF procedure. Proteins are polyampholytes—molecules that contain both positively and negatively charged residues. In IEF, the polyampholytic proteins migrate in a strong electric field along a pH gradient towards the pH at which they have no net charge [18]. These pH values are specific to the polyampholytes and are defined as their isoelectric points (p*I*). In conventional 2-DE, IEF is carried out in polyacrylamide matrices containing an immobilized pH gradient (IPG) [16, 17, 19]. Efficient separation in IPGs during IEF requires low concentrations of ions, because they migrate in the electric field and interfere with the focusing of the polyampholytic proteins in the pH gradient. This excludes the use of potent ionic detergents such as SDS. 2-DE lysis buffer instead contains the uncharged chaotropes urea and thiourea as denaturing components and uncharged or zwitterionic detergents [16, 17]. This composition results in three main problems in relation to membrane proteins: (i) the 2-DE lysis buffer has a lower capacity to extract membrane proteins from the lipid environment and keep them solubilized in the aqueous environment than buffers comprising ionic detergents; (ii) proteins are least soluble at their p*I*, so hydrophobic membrane proteins in particular tend to aggregate during IEF; (iii) the hydrophobicity of the membrane proteins hinders their transfer from the (hydrophobic) gel matrices of the IPG strips to the SDS gels of the second dimension. Thus, membrane proteins are markedly depleted during conventional 2-DE. Several approaches have been developed to reduce this effect:

- The use of detergents such as Triton X-100, dodecyl maltoside, and ASB14 in 2-DE lysis buffer have been shown to facilitate the separation of individual membrane proteins [20–23].
- Delipidation was shown to be beneficial for the conventional 2-DE of some membranous samples [17, 24, 25].
- Analysis of protein samples, in which integral membrane proteins were enriched from complex protein samples by, for example, (i) stripping membranes from soluble proteins by treatment with sodium carbonate [24–26], (ii) separating cell lysates in Tris-soluble and membranous Tris-insoluble fractions [27], or (iii) extracting membrane proteins via Triton X-114 phase partitioning [26], counteracted the loss of these proteins upon IEF.
- The application of multicompartment analyzers with isoelectric membranes as a prefractionation technique for conventional 2-DE enabled enhanced detection of membrane proteins [28].
- The use of soft IPG strips (<4%, i.e., large polyacrylamide pores) reduced the aggregation of membrane proteins to the hydrophobic polyacrylamide matrix [24, 29].
- Gel-free IEF in a free-flow device (FF-IEF) [30–32] or capillary electrophoresis (capillary IEF) [33] was applied, which did not use hydrophobic polyacrylamide matrices.

Despite all of these efforts, it should be noted that none of these approaches proved capable of solving the inherent issues associated with conventional 2-DE in respect to very hydrophobic membrane proteins. To give one very recent example, Mattow and colleagues analyzed the plasma membrane proteome of mycobacteria by conventional 2-DE and one dimensional (1-D) SDS-PAGE (Table 2, [26]). The authors purified the plasma membranes and enriched the membrane proteins by washing the crude membranes with sodium carbonate followed by the extraction of membrane proteins by Triton X-114 phase partitioning. Conventional 2-DE, in contrast to SDS-PAGE, failed to separate highly hydrophobic membrane proteins with more than three transmembrane domains [26]. However, membrane proteins with one or two membrane-spanning regions, i.e., membrane proteins with minor hydrophobicity, could be efficiently identified by conventional 2-DE. Similar effects were demonstrated in many other studies (e.g., [25, 34]), demonstrating the increased loss of highly hydrophobic membrane proteins during conventional 2-DE, but also the usefulness of this method for the separation of membrane-associated proteins and proteins with minor hydrophobicity. In fact, conventional 2-DE is frequently used for the analysis of outer membrane proteomes of bacteria (Table 2) comprising many porin-like outer membrane proteins with low hydrophobicity (see Table 1). Due to the limitations of conventional 2-DE for the separation of highly hydrophobic membrane proteins, alternative gel-based methods, such as SDS-, SDS/SDS-, 16-BAC/SDS- and CTAB/SDS-PAGE, are growing in popularity.

2-D separation of membrane proteins by applying PAGE with ionic detergents in the first dimension

Due to the unreachable solubilizing power of the anionic detergent SDS, SDS-PAGE is still the most commonly used method for the separation of protein extracts enriched with membrane proteins [5, 35–39]. 1.4 grams of SDS bind to each gram of protein, resulting in a near-constant charge-to-

Table 2 Recent proteomic studies of membranous samples that applied denaturing 2-D gel electrophoresis

Method	Sample	Reference
Conventional 2-DE (IEF/SDS)	Mouse liver plasma membrane	[25]
	Membrane fraction of *Sulfolobus solfataricus*	[27]
	Membrane fraction of *Streptococcus thermophilus*	[24]
	Plasma membrane of mycobacteria	[26]
	Cell envelope of *Brucella abortus*	[121]
	Outer membrane proteome of *Escherichia coli*	[122]
	Outer membrane proteome of *Pasteurella multocida*	[123]
	Meningococcal outer membrane vesicles	[124, 125]
SDS/SDS-PAGE	Membrane of *Clostridium thermocellum*	[45, 54]
	Rat synaptic vesicle proteome	[48]
	Outer membrane proteome of *Neisseria*	[47]
	Bovine respirasomes/mitochondria	[46]
	Mitochondrial subunit I of *Leishmania tarentolae*	[53]
	Membrane proteins of *E. coli*	[52]
16-BAC/SDS-PAGE	Membrane proteome of *Corynebacterium glutamicum*	[67]
	Rat synaptic vesicle proteome	[48, 56, 57, 60, 64]
	Membrane fraction of rat brain tissue	[66]
	Membrane fraction of mouse brain tissue	[55]
	Bovine heart mitochondria	[61]
	Rat liver peroxisomes	[62]
	Human platelet membrane proteome	[63]
	Outer membrane proteome mitochondria from *S. cerevisiae*	[69]
	Membrane proteome of mitochondria from *S. cerevisiae*	[68]
	Mammalian cells and tissues	[59]
	Mammalian nuclear envelope	[58, 65]
	Membrane proteome from *Halobacterium salinarum*	[126]
CTAB/SDS-PAGE	Membrane proteome of Jurkat T cells	[70]
	Plasma membrane proteins from *S. cerevisiae*	[71]

mass ratio for the SDS–protein complexes [40, 41]. In discontinuous SDS-PAGE, a polyacrylamide gel with large pores (stacking gel, e.g., 4%) is polymerized on top of a polyacrylamide gel with small pores (separating gel, e.g., 8–20%) [10, 42]. In the most common Tris–glycine system [10], glycine is almost in its uncharged zwitterionic form in the stacking gel (pH 6.8). The charged SDS–protein complexes are focused according to their electrophoretic mobilities into very sharp stacks behind the leading chloride ions but ahead of the trailing glycine ions (isotachophoretic stacking effect). This enables the concentration of proteins by a factor of up to 10000 [43]. In alkaline separating gel (pH 8.8), the electrophoretic mobility of glycine is drastically increased, and the SDS–protein complexes are separated according to their molecular masses in the small pores of the separating gel (sieving effect) [10, 44]. When applying 1D-SDS-PAGE, protein samples can only be resolved into about 50 individual protein bands [45]. Therefore, this method does not allow the separation of protein samples with high complexity, but it is widely used as a powerful and membrane-protein-compatible prefractionation method for subsequent analysis [5, 35–39].

SDS/SDS-PAGE

The resolving power of SDS-PAGE can be increased applying a 2-D approach (SDS/SDS-PAGE) [45–48] (Fig. 1). Proteins of a complex sample are separated by SDS-PAGE according to their molecular masses in both dimensions. The lane of the first dimension is cut out and laid on top of a second SDS polyacrylamide gel (Fig. 1a). Since the separation principle is the same for the first and the second dimensions, proteins are aligned as protein spots in a diagonal (Fig. 1a). Considerable dispersion of the protein spots around the diagonal and therefore increased resolution compared to 1D-SDS-PAGE can be achieved by manipulating the running buffers and the polyacrylamide gels, respectively (Fig. 1b–c):

– The use of different acrylamide concentrations in the first and second dimensions results in better resolution due to the anomalous migration of some SDS–protein complexes in gels with different acrylamide concentrations [46, 49, 50]. Highly basic proteins, glycoproteins and, most interestingly, highly hydrophobic membrane proteins demonstrate such a behavior [46,

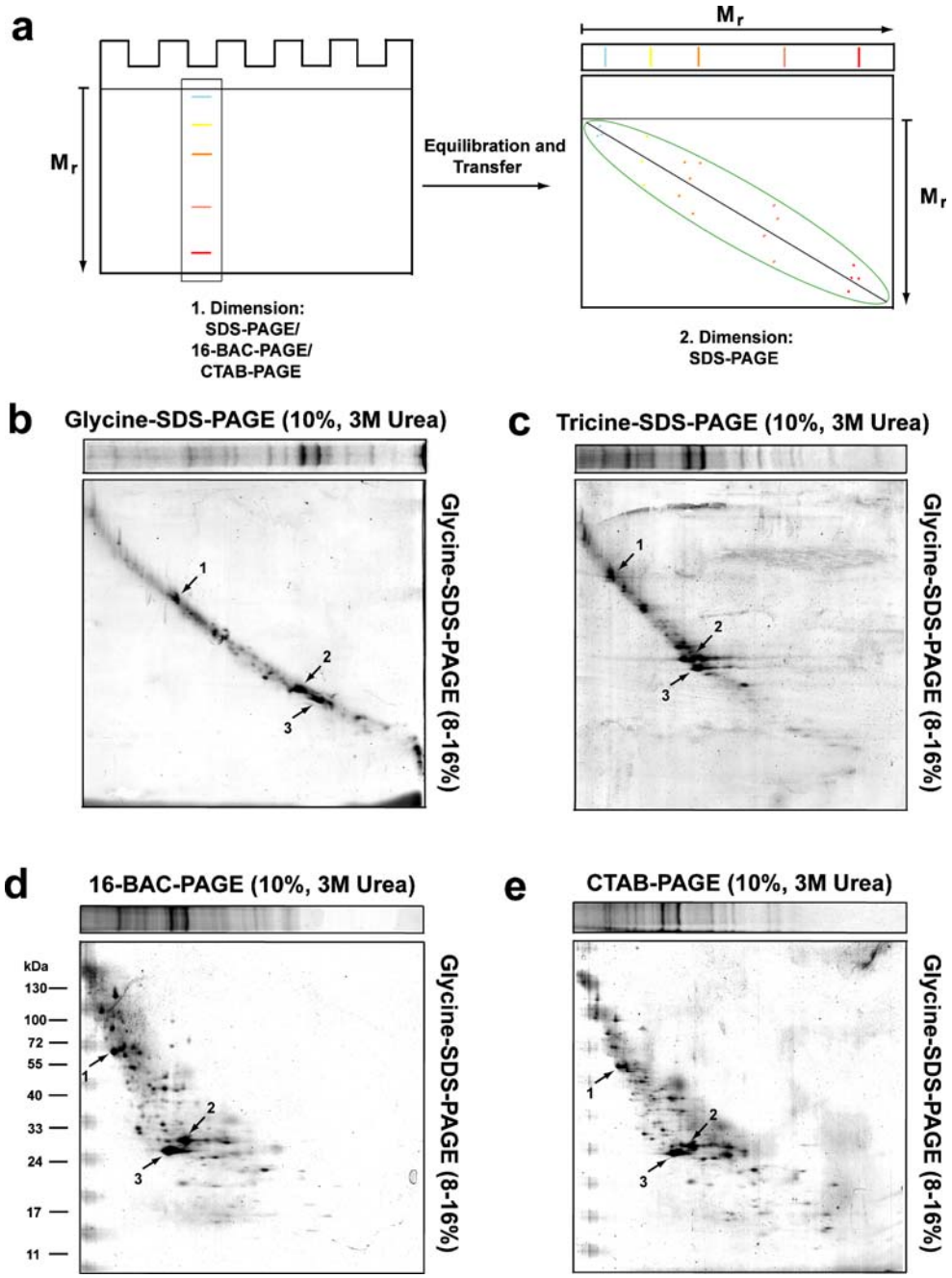

Fig. 1 **a–e** 2-D electrophoresis of membrane proteins with ionic detergents. **a** Method: proteins that are denatured and solublized with strong ionic detergents, such as SDS, 16-BAC and CTAB, are separated during polyacrylamide gel electrophoresis according to their molecular mass. Gel lanes are cut out, equilibrated, and transferred to a SDS polyacrylamide gel, and SDS-PAGE is performed. Proteins are separated and dispersed along a diagonal. **b–e** Application: mitochondria were isolated by differential centrifugation from baker's yeast [118]. Integral membrane proteins were enriched from 100 μg mitochondria by carbonate extraction [119], and subjected to the first dimension, i.e., glycine-SDS-PAGE (**b**, [10]), tricine-SDS-PAGE (**c**, [42, 51]), 16-BAC-PAGE (**d**, [74]), or CTAB-PAGE (**e**, [74]) with gels containing 10% acrylamide and 3 M urea. Lanes were cut from the gels, equilibrated and laid on top of a glycine-SDS gradient gel (8–16%) for the second dimension [60, 75]. SDS-PAGE was performed according to Laemmli [10]. Gels were stained with colloidal Coomassie [120]. Protein spots were analyzed with MALDI-TOF mass spectrometry. Examples of identified membrane proteins are marked by *arrows*: rotenone-insensitive NADH-ubiquinone oxidoreductase (spot 1, inner membrane, NDI1_YEAST), ADP/ATP translocase 2 (spot 2, inner membrane, ADT2_YEAST), and voltage-dependent anion-selective channel protein 1 (spot 3, outer membrane, VDAC1_YEAST). SwissProt entry names

49, 50]. The latter demonstrate increased SDS binding compared to soluble proteins, leading to an increased charge-to-mass ratio and to a higher electrophoretic mobility, especially in gels with large pores (≤10%) [46]. This effect is less pronounced in narrow-pore gels (≥15%) due to the predominant sieving effect [46]. When polyacrylamide gels with different percentages are used in both dimensions, this characteristic of highly hydrophobic membrane proteins can be used to obtain their specific dispersion from the diagonal in SDS/SDS-PAGE [46, 48]. Rais and colleagues separated the subunits of the bovine respirasome by SDS/SDS-PAGE, using a low-percentage gel (10%) in the first and a high percentage gel (16%) in the second dimension [46]. They located highly hydrophobic membrane proteins (GRAVY indices > +0.3) specifically above the diagonal [46].

- Urea influences the electrophoretic mobilities of individual proteins, possibly by influencing the binding of SDS to these proteins [46]. Rais and colleagues demonstrated that the use of urea in one dimension increases the effect of the dispersion of highly hydrophobic membrane proteins from the diagonal in SDS/SDS-PAGE [46].
- Increased concentrations of the crosslinker bisacrylamide and the addition of glycerol to the gel buffer in one of the two dimensions results in an enhanced sieving effect in the respective dimension [45]. Different sieving effects intensify the abnormal electrophoretic mobility of individual (membrane) proteins due to their anomalous SDS-binding behavior. As shown recently by Williams and colleagues, this leads to an increased resolution of SDS/SDS-PAGE [45].
- The relative electrophoretic mobilities of proteins in discontinuous SDS-PAGE can be further modified by using different trailing ions with distinct electrophoretic mobilities, e.g., glycine [10], tricine [42, 51], and bicine [45]. Glycine has a comparatively small mobility in the stacking gel. Under these conditions, small proteins (<20 kDa) cannot be separated from the SDS micelles during stacking and they comigrate with SDS in the separating gel, resulting in poor resolution [42]. In contrast, tricine has a markedly higher mobility in the stacking gel than glycine, enabling the resolution of small proteins from the SDS micelles during stacking, and the separation of these proteins in the separating gel [42]. Bicine possesses an intermediate mobility and has recently been proposed by Williams and colleagues as being especially suitable for the separation of membrane proteins [45]. Thus, the use of different trailing ions in the first and second dimensions of SDS/SDS-PAGE results in a higher resolution of membrane proteins [45, 46].

Taking into account these parameters that are used to improve the resolution of SDS/SDS-PAGE, approximately two- to five-fold more proteins can be resolved when compared to 1D-SDS-PAGE [45]. Highly hydrophobic membrane proteins appear to be particularly accessible using this method [45, 46]. Indeed, SDS/SDS-PAGE has been successfully used for recent biochemical and proteomic analyses of protein samples enriched with membrane proteins [45–48, 52–54] (Table 2).

16-BAC/SDS- and CTAB/SDS-PAGE

A significant increase in resolution compared to SDS/SDS-PAGE can be achieved by applying different ionic detergents in the two dimensions (Fig. 1). The use of the cationic detergents 16-BAC or CTAB in the first dimension and the anionic detergent SDS in the second dimension allows the separation of protein samples in up to several hundred protein spots for 16-BAC/SDS- [48, 55–69] and CTAB/SDS-PAGE [70, 71], respectively (Fig. 1d–e). Since 16-BAC and CTAB are potent ionic detergents that are suitable for solubilizing hydrophobic membrane proteins, these 2-D approaches are highly valuable for separating membrane proteins [60, 70].

When provided above their critical micellar concentrations, 16-BAC and CTAB bind in a constant ratio to proteins, analogous to SDS [9, 72, 73]. This confers a positive charge density to the proteins, masking their intrinsic charges, and leading to a near-constant charge-to-mass ratio. Similarly to SDS-PAGE, 16-BAC–protein or CTAB–protein complexes can then be separated in a sieving polyacrylamide gel according to their molecular masses [9, 72, 73]. Due to the positive charges of the 16-BAC–protein complexes, the electrophoresis is done—in contrast to SDS-PAGE—towards the cathode. Discontinuous 16-BAC-PAGE—which has been applied in most of the studies so far—uses acidic potassium phosphate buffers in the stacking (pH 4) and separating gels (pH 2) and glycine as the trailing ion [9]. However, this approach considerably prolongs, the electrophoresis and protein stacking is reduced compared to SDS-PAGE [9]. Very recently, Kramer described a new, superior, multiphasic buffer system for 16-BAC-PAGE. The author replaced the phosphate buffer system with methoxyacetic acid and acetic acid as buffer constituents in the separating (pH 3) and stacking gels (pH 4), respectively [74]. The introduction of this new buffer system, the optimization of the 16-BAC concentration, and the use of a new tracking dye yield highly efficient stacking and fast separation [74]. Thus, 16-BAC-PAGE is now as easy to handle as SDS-PAGE and highly comparable in terms of protein separation quality.

In 16-BAC/SDS-PAGE, the cationic detergent 16-BAC is removed from the separated proteins after the first

dimension and replaced by the anionic detergent SDS prior to performing the second dimension [60, 75]. Although both 16-BAC-PAGE and SDS-PAGE separate proteins according to their molecular masses, a significant dispersion of the protein spots around the diagonal can be observed [75] (Fig. 1d–e). This effect may be explained by differences in the binding of the two detergents to individual proteins, resulting in altered electrophoretic mobilities in the first and second dimensions. More than ten years ago, Hartinger and colleagues proved for the first time that 16-BAC/SDS-PAGE is highly suitable for the analysis of protein samples enriched with integral membrane proteins [60]. However, this method remained largely unnoticed for years. A dramatic increase in interest in this 2-D electrophoretic approach has taken place over the last four years. 16-BAC/SDS-PAGE is now successfully used as an alternative to conventional 2-DE for the separation of (integral) membrane proteins from various biological sources [48, 55–69] (Table 2). Zahedi and colleagues, for instance, performed a proteomic analysis of purified yeast mitochondria by applying conventional 2-DE and 16-BAC/SDS-PAGE [68]. Forty-two integral membrane proteins were identified exclusively by the 16-BAC/SDS-PAGE approach, whereas only five membrane proteins were solely identified by conventional 2-DE (Table 3, panel 4). Coughenour and colleagues analyzed the rat synaptic vesicle proteome by conventional 2-DE and 16-BAC/SDS-PAGE [57]. Consistent with the results of Zahedi and colleagues, only five membrane proteins were identified on conventional 2-D gels, in contrast to 12 on 16-BAC/SDS gels (Table 3, panel 3). Moreover, membrane proteins identified solely with 16-BAC/SDS-PAGE demonstrated significantly higher hydrophobicity, as gauged by increased GRAVY indices and an increased number of transmembrane domains, compared to proteins identified with conventional 2-DE [57]. These examples underline the usefulness of 16-BAC/SDS-PAGE for the separation of membrane proteins with increased hydrophobicity.

CTAB-PAGE, which uses the cationic detergent CTAB, has been introduced as an alternative to SDS-PAGE for the separation of protein samples [72, 73], and can be performed in the 16-BAC gel systems of Macfarlane [9] and Kramer [74], respectively. Combining CTAB-PAGE and SDS-PAGE in 2D-CTAB/SDS-PAGE results in very similar separations to 16-BAC/SDS-PAGE (Fig. 1d–e). CTAB/SDS-PAGE has also been proven to be suitable for the analysis of integral membrane proteins [70, 71]. For instance, Helling and colleagues identified 28 membrane proteins by applying CTAB/SDS-PAGE to the separation of the membrane fraction of Jurkat T cells [70] (Table 2). Notably, these authors demonstrated that this method is even useful for the analysis of scarce amounts (3 μg) of proteins [70]. In the future, CTAB/SDS- and 16-BAC/SDS-PAGE may thus enable efficient 2-D analyses of valuable protein samples that can only be obtained in small amounts.

A combinatorial approach is suitable for maximum coverage of a membrane proteome

Conventional 2-DE is a gel-based 2-D approach with a very high resolving power. It is applicable to the analysis of membrane proteins with minor hydrophobicity, but has a strong bias against the efficient separation of highly hydrophobic membrane proteins. In contrast, SDS/SDS-, 16-BAC/SDS-, and CTAB/SDS-PAGE are 2-D gel electrophoretic methods that are very efficient at separating integral membrane proteins (Table 3) [46, 68, 70]. Therefore, the percentage of proteins identified with these alternative 2-D methods that can be classified as membrane proteins is markedly higher than for conventional 2-DE (Table 3, panels 1, 3, and 4). Although 16-BAC/SDS- and CTAB/SDS-PAGE provide a higher resolving power than SDS/SDS-PAGE (Fig. 1b–e), SDS/SDS-PAGE may be slightly better for very highly hydrophobic proteins [48, 61], possibly due to the strongest solubilizing power of

Table 3 Comparison of gel-based electrophoretic methods for the separation of membrane proteins

Sample	2-DE (IEF/SDS)	16-BAC/SDS	SDS/SDS	BN/SDS	Reference	Panel
Bovine heart mitochondria	16/123 (13%)	6/30 (20%)	8/41 (20%)	14/71 (20%)	[61]	1
Rat synaptic vesicle proteome	-	14/59 (24%)	28/96 (29%)	-	[48]	2
Rat synaptic vesicle proteome	5/22 (23%)	12/31 (39%)	-	-	[57]	3
Yeast mitochondria	46% 5	56% 42	-	-	[68]	4

Panel 1: number of proteins with positive GRAVY index vs. total number of proteins identified with this method. *Panel 2*: number of integral synaptic vesicle proteins vs. total number of proteins identified with this method. *Panel 3*: number of membrane proteins vs. total number of proteins identified with this method. *Panel 4*: percentage of membrane proteins of all proteins identified with this method; number of membrane proteins exclusively identified with this method

SDS. For instance, in a recent analysis of a synaptic vesicle proteome, 28 integral synaptic vesicle proteins were identified with SDS/SDS-PAGE compared to only 14 with 16-BAC/SDS-PAGE [48] (Table 3, panel 2). Thus, for maximum coverage of the proteome of a membranous fraction, a combinatorial approach (as described in [61]) that applies conventional 2-DE, SDS/SDS-, and 16-BAC/SDS- or CTAB/SDS-PAGE appears to be a reasonable one.

2-D separation of membrane proteins by applying native PAGE in the first dimension (blue native (BN)/ clear native (CN)-PAGE)

IEF, SDS-PAGE, 16-BAC-PAGE, and CTAB-PAGE separate proteins under denaturing conditions [9, 10, 17, 70]. Due to the use of strong ionic detergents, the latter three methods are especially suitable for solubilizing and separating membrane proteins. However, membrane proteins can also be solubilized using comparatively mild nonionic detergents, such as Triton X-100, dodecyl maltoside or digitonin [7, 8]. These detergents permit the solubilization of native membrane proteins and native membrane protein complexes [7, 8], which can then be separated by native electrophoretic methods. These techniques use the inherent negative charge of proteins (with pI<7) in clear-native (or colorless-native) electrophoresis (CN-PAGE) [76–78]. Alternatively, negative charges (charge shift) are provided by adding the negatively charged protein-binding dye Coomassie Brilliant Blue G-250 in blue-native electrophoresis (BN-PAGE) [11, 12, 79]. Segregation of the solubilized protein complexes occurs in native, nonreducing polyacrylamide gradient gels according to their molecular mass. Protein complexes migrate in these gradient gels with decreasing pore size until they reach their specific size-dependent pore-size limit.

To identify and characterize the composition of native membrane protein complexes, a 2-D approach with native BN-PAGE (or CN-PAGE) as the first and denaturing SDS-PAGE as the second dimension can be applied (BN/SDS-PAGE) [12, 79, 80] (Fig. 2). The first dimension separates membrane extracts in individual native membrane protein complexes according to their molecular mass (Fig. 2a). The lane of the first dimension or individual protein bands are treated with SDS under reducing conditions, resulting in the denaturation of the membrane protein complexes. The gel strip or protein bands are then laid on top of a SDS gel and SDS-PAGE is performed (Fig. 2a). All proteins released from one protein complex are separated along the electric field and positioned in a straight line, one below the other, according to their molecular masses (Fig. 2a–b). BN/SDS-PAGE is therefore an alternative method to immunoprecipitation for identifying physiological protein–protein interactions and determining the compositions of membrane protein complexes [12, 78–80]. It has the advantage that no antibodies are necessary, but the conditions are not as sensitive due to the failure to detect detergent-labile interaction partners [80].

CN-PAGE and BN-PAGE were developed by Schägger and colleagues for the separation of mitochondrial membrane protein complexes [76, 79]. Upon applying these methods, the authors determined the compositions of supercomplexes in the respiratory chain of mitochondria [76, 80–82]. CN-PAGE usually yields a lower resolution than BN-PAGE. However, it enables the separation of labile supramolecular assemblies of membrane protein complexes that would dissociate in BN-PAGE, presumably due to the presence of the charged Coomassie Brilliant Blue G-250

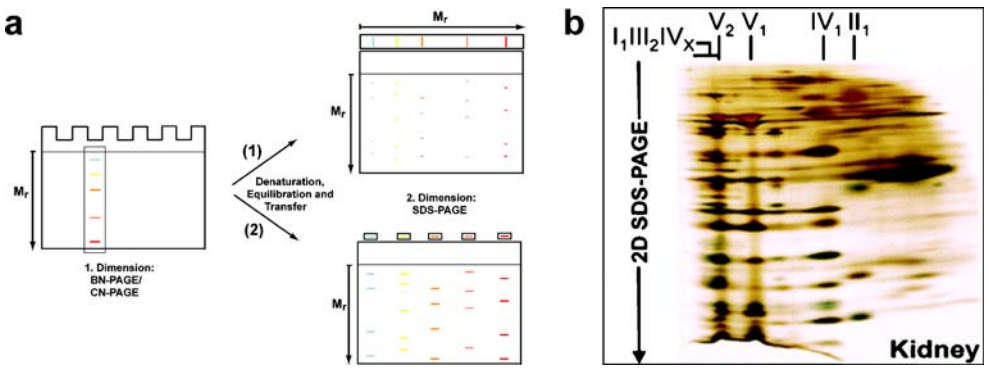

Fig. 2 a–b 2-D electrophoresis of native membrane protein complexes. **a** Method: membrane protein complexes are separated electrophoretically in a polyacrylamide gel under native conditions according to their molecular mass (BN- or CN-PAGE). Gel lanes (*1*) or individual BN/CN bands (*2*) are cut out, equilibrated under reducing and denaturing conditions, and transferred to a SDS polyacrylamide gel. SDS-PAGE is performed, resulting in the separation of potential protein complex subunits. **b** Application: crude mitochondria from rat kidney were purified by sucrose-density gradient centrifugation and separated by BN/SDS-PAGE. The mitochondrial supercomplexes $I_1III_2IV_X$ ($I_1III_2IV_{0-4}$), monomeric (V_1), and dimeric (V_2) ATP synthases, as well as monomeric complexes II and IV are indicated on top of the gel. This image is reprinted in part from [87] with the permission of the American Chemical Society

[77]. The selection of the type and the concentration of detergents is critical for the isolation of membrane protein complexes or supercomplexes, and therefore has to be determined empirically for every type of membrane sample [11, 12, 78]. Since BN-PAGE allows the separation of membrane protein complexes in a native and enzymatically active state, the BN gel can be used for "in-gel" activity assays, e.g., to measure the activities of mitochondrial enzyme complexes [83, 84]. Alternatively, native proteins can be electroeluted from the gel, reconstituted, and further analyzed with techniques such as 2-D crystallization and electron microscopy [85, 86].

The significance of BN/SDS-PAGE for the separation of membrane protein complexes has noticeably increased over the last few years. Aside from the systematic analysis of mitochondrial membrane protein complexes (e.g., [87]), BN/SDS-PAGE has also been successfully applied to the analysis of protein complexes of various other membrane preparations. Examples are thylakoid membranes of chloroplasts [88], microsomal membranes [89], and rod photoreceptor outer segments [90]. Thus, BN/SDS-PAGE is a gel-based 2-D approach that is highly suited to the systematic proteomic analysis of membrane protein complexes. Furthermore, BN/SDS-PAGE permitted the identification of membrane proteins that were not found by other gel-based methods [61]. Therefore, the combination of this technique with denaturing conventional 2-DE, SDS/SDS- and/or 16-BAC/SDS-PAGE (as described in [61], Table 3, panel 1) allows the description of both the membrane protein inventory of a given sample and its interaction in functional complexes.

Isolation and purification of membranous compartments by free-flow electrophoresis for biochemical analysis

The efficient analysis of membrane proteins by gel-based electrophoretic methods requires samples enriched in or consisting of membranes. Gel-free zone electrophoresis in a free-flow device (ZE-FFE) is applied as a gentle, fast and efficient method for the isolation and purification of cells, organelles and membranes [91]. In ZE-FFE, the sample is injected into a carrier buffer of a constant composition, uniform pH and conductivity. Separation of the individual components of the sample is based on their charge-to-size ratios in an electric field oriented perpendicular to the buffer stream (Fig. 3a). One advantage of FFE compared to classical prefractionation and separation methods is the continuous sample application, enabling the relatively rapid separation of large amounts of sample.

ZE-FFE has been used to separate cells from different organisms and tissues [91, 92], and to isolate organelles such as lysosomes and endosomes [93–95], golgi apparatus subfractions [96, 97], zymogen granules of pancreatic cells [98], peroxisomes [99, 100], and mitochondria [23, 101]. Using FFE combined with conventional 2-DE and subsequent MS analysis, Zischka and coworkers [101] identified markedly more mitochondrial proteins in ZE-FFE-purified mitochondria than in mitochondrial extracts isolated by differential centrifugation, concomitant to a significant reduction in nonmitochondrial proteins. In a subsequent study, Zischka and coworkers successfully applied ZE-FFE as an analytical tool to analyze mitochondria from yeast cells grown under respiratory and fermentative conditions, respectively [23]. Mitochondria isolated from fermenting cells showed a more cathodal deflection than mitochondria from respiratory cells. This may be a result of the observed marked alterations in the proteome of their outer membranes as resolved by SDS-PAGE and MS. By applying ZE-FFE, we separated yeast mitochondria enriched with microsomal membranes (referred to as the side fraction, SF) from mitochondria with high purity (referred to as the main fraction, MF) [23]. Figure 3 illustrates the gel results from a 1D-SDS-PAGE analysis of the obtained ZE-FFE fractions and 16-BAC/SDS-PAGE of the mitochondrial fraction enriched with microsomes.

Beyond cells and organelles, FFE purification procedures have been reported for a variety of membranes [102–106], which include mitochondrial membranes [107, 108], and membrane subfractions from erythrocytes [109]. FFE improved the separation and subsequent analysis of plant membranes [110, 111]. From the same homogenate, tonoplast and plasma membranes could be separated with high purities, and due to the continuous flow mode used, large sample amounts could be processed for further biochemical analysis [111]. Furthermore, ZE-FFE was used to isolate plasma membrane and intracellular membrane fractions from a mixed membrane fraction of human platelets [112, 113] for proteomic analysis using SDS-PAGE and conventional 2-DE [114]. Recently, Senis and coworkers performed a study aimed at identifying novel membrane proteins in human platelets [115] using three different membrane enrichment methods, namely lectin and biotin/NeutraAvidin affinity chromatography and ZE-FFE, combined with SDS-PAGE and with LC–MS/MS. The FFE approach resulted in the identification of 74 and 116 integral membrane proteins when analyzing the plasma membrane and the intracellular membrane fractions, respectively. This was considerably more than the number identified using the other approaches applied. Therefore, FFE is a valuable technique for prefractionating and enriching membranes at the preparative scale in preparation for the further analysis of membrane proteins by gel-based denaturing (e.g., conventional 2-DE, 16-BAC/SDS-PAGE) and native (e.g., BN-PAGE) electrophoretic methods.

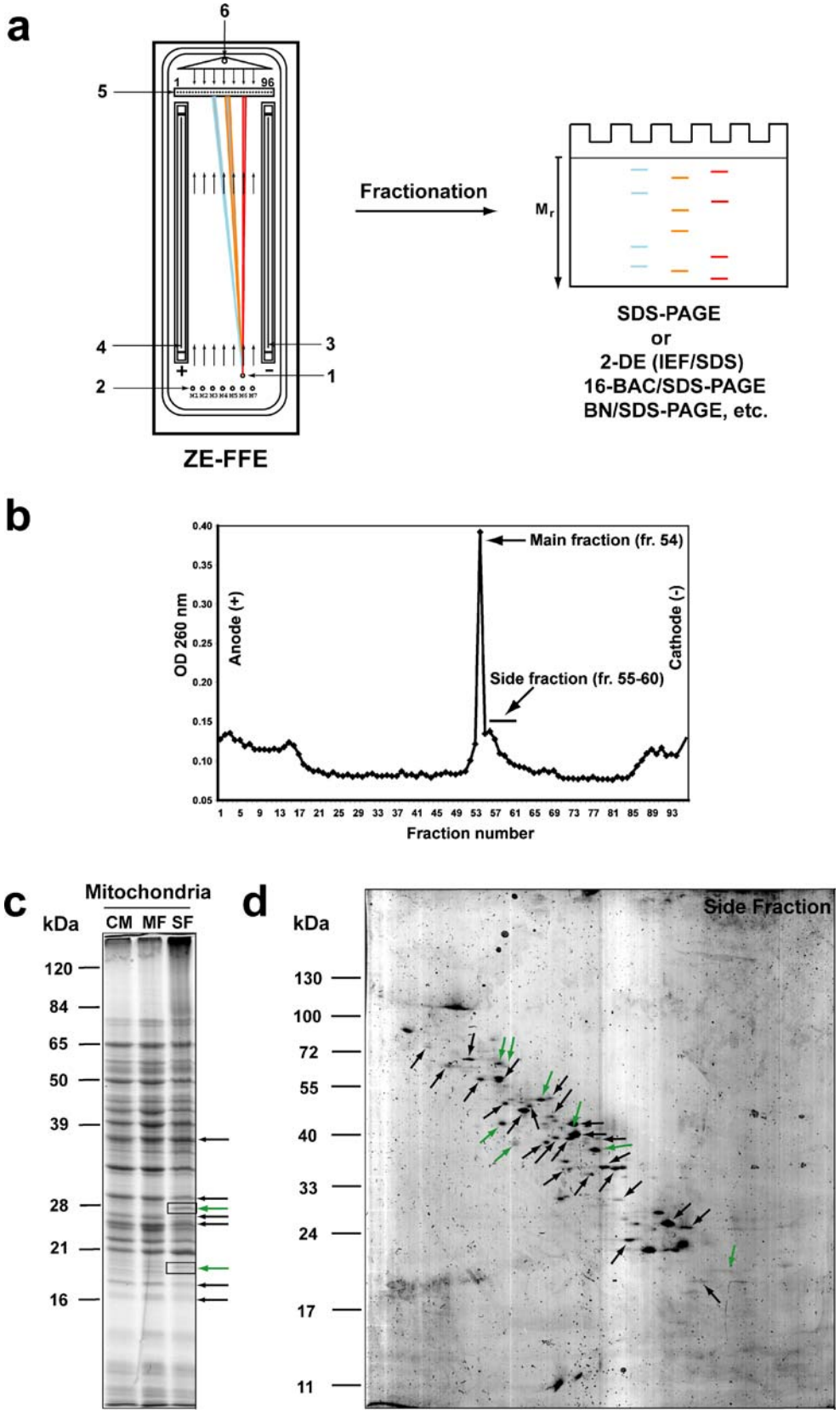

Fig. 3 a–d Use of free-flow electrophoresis for prefractionation of membranous samples. **a** Method: membranous compartments, such as organelles, and membranes can be separated by zone electrophoresis in a free-flow device (ZE-FFE). In the electrophoresis chamber, a laminar buffer flow is guaranteed by the continuous input of separation media via the media inlets (*2*). Samples are applied into the buffer flow at the cathodal side of the fractionation chamber (*3*) via the sample inlet (*1*), and are deflected in the electric field towards the anode (*4*). At the end of the electrophoresis chamber, the laminar buffer flow is blocked via the counter flow (*6*) and samples are fractionated (*5*). The protein contents of the different fractions can be analyzed by either SDS-PAGE or by 2-D electrophoretic methods, such as 2-DE (IEF/SDS), 16-BAC/SDS-PAGE and BN/SDS-PAGE. **b–d** Application: crude mitochondria (CM) were isolated by differential centrifugation from baker's yeast [118]. CM were applied to ZE-FFE and separated into a main fraction (MF) containing purified mitochondria and a side fraction (SF) comprising mitochondria enriched with microsomes (**b**), as described in [23]. MF and SF were subjected to SDS-PAGE (**c**), illustrating the mitochondrial protein band patterns of both fractions (e.g., *black arrows*). The *green arrows* mark proteins specifically found in the SF. The microsome-containing SF was analyzed by 16-BAC/SDS-PAGE (**d**) and MALDI-TOF mass spectrometry, resulting in the identification of mitochondrial (*black arrows*) and nonmitochondrial (*green arrows*) proteins. One-third of all identified proteins are predicted to have at least one transmembrane domain (TMPred, http://www.expasy.org)

Conclusions

2-D gel electrophoresis with IEF in the first dimension and SDS-PAGE in the second dimension (conventional 2-DE) is the electrophoretic method with the highest resolving power for complex protein samples. Conventional 2-DE can be used for the separation of membrane-associated proteins and membrane proteins with minor hydrophobicity, e.g., proteins with one and two transmembrane domains or porin-like proteins. However, due to the limitations of IEF, conventional 2-DE fails to efficiently separate highly hydrophobic membrane proteins. In contrast, SDS/SDS-, 16-BAC/SDS- and CTAB/SDS-PAGE are comparatively easy to handle and fast 2D electrophoretic methods that permit the efficient separation of integral membrane proteins with multiple transmembrane domains. In addition, these approaches, in contrast to conventional 2-DE, enable the analysis of very small amounts of proteins. BN/SDS- and CN/SDS-PAGE are 2-D electrophoretic methods that are suitable for the analysis of native membrane protein complexes. The description of (functional) protein complexes completes the information on the membrane protein inventory of a given sample obtained by the denaturing methods. Finally, ZE-FFE is a highly useful and versatile electrophoretic fractionation method that is used for the prefractionation of membranous compartments prior to denaturing and the native gel-based 2-D analysis of membrane proteins.

We apologize that we are, due to space limitations, unable to mention and discuss all of the approaches that have been developed in the field of the 2-D electrophoresis of membrane proteins. Applications of BN/CN-PAGE were discussed only briefly, because excellent reviews are available for this method [11, 12, 77, 78, 80]. The application of capillary electrophoresis to the analysis of membrane proteins is also not described. We refer the interested reader to appropriate papers and reviews [116, 117].

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