Novel method for evaluation of the oligomeric structure of membrane proteins

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Assessment of the quaternary structure of membrane proteins by PAGE has been problematic owing to their relatively poor solubility in non-dissociative detergents. Here we report that several membrane proteins can be readily solubilized in their native quaternary structure with the use of the detergent perfluoro-octanoic acid (PFO). Further, PFO can be used with

PAGE, thereby providing a novel, accessible tool with which to assess the molecular mass of homo-multimeric protein complexes.

Key words: ion channels, multimeric complexes, quaternary structure, SDS/PAGE.

INTRODUCTION

Analysis of the native multimeric structure of many membrane proteins has been limited by the requirement to extract the proteins from membranes with detergents. Detergents are required to extract integral membrane proteins from the lipid bilayer and to maintain these hydrophobic proteins in solution for subsequent separation by gel filtration or by electrophoretic methods. Unfortunately, depending on the nature and concentration of the detergents required for effective solubilization, multimeric complexes can dissociate. For example, although the resolution of molecular mass by SDS/PAGE is clearly superior to other methods for mass determination, it is incompatible with the analysis of multimeric complexes because SDS dissociates homo-multimeric protein interactions. Sucrose-density-gradient centrifugation and gel filtration have commonly been used to resolve the native quaternary structure of membrane complexes, solubilized with relatively mild detergents [1,2]. However, these methods typically require large amounts of recombinant protein and lack the resolving power of PAGE. In contrast, 'Blue native PAGE' [3,4] combines the resolving power of gel electrophoresis with the potential for preserving large membrane-protein complexes. Although this method is a powerful tool for the study of the structure–function relationships of membrane proteins it is technically demanding as it is incompatible with precast gel systems and requires the preparation of polyacrylamide gradient gels.

In this study we examined the efficacy of a novel detergent, perfluoro-octanoic acid (PFO), to protect interactions within protein oligomers and to permit the molecular mass determination of both cytoplasmic and transmembrane multimeric proteins when it replaces SDS in gel electrophoresis. The detergent is particularly effective in the solubilization of intractable membrane proteins such as the membrane-spanning V_0 subunit of the V-type ATPase [5] and the cystic fibrosis transmembrane conductance regulator ('CFTR') [6]. In contrast, PFO is capable of preserving certain high-affinity interactions. For example, unlike SDS, PFO is compatible with membrane protein purification by metal-affinity chromatography [6].

MATERIALS AND METHODS

Membrane preparation

Red-blood-cell ghosts were prepared and stripped of peripheral proteins by using extraction at high pH as described previously [1]. After centrifugation, the resulting membrane pellet was resuspended in 25 mM phosphate buffer, pH 7.2. Rat hippocampal membranes were prepared as described previously [7] except that we also included the protease inhibitors aprotinin (10 μ g/ml), leupeptin (10 μ g/ml), *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane ('E-64', 10 μ M) and benzamidin (1 mM) in the cell homogenization buffer. Furthermore, membranes were extracted with 1 M NaI at a protein concentration of 250 μ g/ml, for 5 min on ice. Then the extracted membranes were centrifuged at 100000 *g* and resuspended in PBS before storage at -80°C . *Xenopus* oocyte membranes were prepared as described in previous studies [8].

PFO/PAGE

The methods employed for PFO/PAGE were similar to those employed for SDS/PAGE. In the present studies, commercially available Tris/glycine precast gels without SDS were used. (As an alternative, freshly poured Tris/glycine gels without SDS could be used.) Resuspended membrane samples were solubilized at room temperature with sample buffer containing 25 mM dithiothreitol (diluted 1:1, v/v), vortex-mixed, centrifuged for 5 min at 10000 *g* and then applied to the gel. The doubly concentrated sample buffer contained 100 mM Tris base, 8% (w/v) NaPFO, 20% (v/v) glycerol, 0.005% Bromophenol Blue; the pH was adjusted to pH 8.0 with NaOH. The running buffer contained 25 mM Tris, 192 mM glycine and 0.5% (w/v) PFO; the pH was adjusted to pH 8.5 with NaOH. Electrophoresis was performed at 140 V with running buffer that had been precooled to 4°C . During the run, the electrophoresis box was kept in an ice–water bath. Protein bands were analysed either by Coomassie Blue staining or by Western blot analysis. A Bio-Rad transfer apparatus was used for transfer to nitrocellulose for immunoblotting. Transfer was usually complete within 1 h unless stated

Abbreviations used: GABA, γ -aminobutyric acid; PFO, perfluoro-octanoic acid.

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otherwise; the transfer buffer contained 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol. SDS/PAGE was performed by the method of Laemmli [9] and protein concentrations were determined with a modification of the Lowry method [10].

Chemicals

PFO produced by Fluorochem (Old Glossop, Derbyshire, U.K.) was obtained through Oakwood Products (West Columbia, SC, U.S.A.). Precast Tris/glycine gels (4 % and 4–12 %) and the electrophoresis system were obtained from Novex (San Diego, CA, U.S.A.). The high-molecular-mass rainbow marker kit and the ECL[®] enhanced chemiluminescence kit were purchased from Amersham (Oakville, ON, Canada). Albumin for non-denaturing PAGE (A-8654) and the protein molecular mass marker kit (MW-GF-1000) were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Analysis of the oligomeric states of water-soluble proteins by PFO/PAGE

To assess the capacity of PFO/PAGE to determine the molecular mass of proteins accurately, we compared the resolution of a standard mixture of 'marker' proteins by PFO/PAGE and SDS/PAGE with a 4–12 % gradient gel. Each of the marker proteins, ranging from 30 to 220 kDa, was clearly resolved by PFO/PAGE (Figure 1, right panel). To determine whether this novel method could be used to assess the native multimeric structure of proteins, we first analysed several commercially available multimeric water-soluble proteins by PFO/PAGE. The proteins are listed in Table 1; their separation on 4–12 % gradient and 4 % polyacrylamide gels is shown in Figure 2(A). The native, multimeric structure and certain products of multimer dissociation were detected for each of the water-soluble proteins tested. PFO/PAGE analysis of cross-linked albumin (which contains a mixture of monomer and reversible oligomers; Figure 2A, lane 2) shows multiple bands corresponding to monomeric, dimeric, trimeric and possibly tetrameric albumin. The monomeric form of albumin exhibits a mobility corresponding to a molecular mass that is less than that of the modified albumin from Figure 2(A), lane 1. This discrepancy between monomeric

Figure 1 Separation of a mixture of protein standards (rainbow markers) on 4–12 % gradient gels with the modified Laemmli (SDS/PAGE) system (left panel) and the PFO/PAGE system (right panel)

Protein standards (10 μ l) in the appropriate solubilizing buffer were applied to each well. The positions of molecular mass markers are indicated (in kDa) at the left of each panel.

Table 1 Molecular masses of monomers and multimers of soluble proteins

Band no.	Protein	Molecular mass (kDa)
1	Myosin	220
2	Phosphorylase <i>b</i>	97.4
3	BSA	66
4	Ovalbumin	46
5	Carbonic anhydrase	30
6	Albumin, tetramer	264
7	Albumin, trimer	198
8	Albumin, dimer	132
9	Albumin, monomer	66
10	Urease, trimer	273
11	Urease, dimer	182
12	Urease, monomer	91
13	Contaminant	
14	Apo ferritin, 24-mer	475
15	Apo ferritin, 12-mer	272
16	β -Amylase, tetramer	224
17	β -Amylase, dimer	112
18	Thyroglobulin, monomer	304

forms of albumin is also apparent on SDS/PAGE (results not shown) and possibly relates to the nature of the chemical modification of the albumin in the marker lane to incorporate a chromophore. Urease, a trimeric protein, showed the presence of three bands corresponding to the monomer, dimer and trimer (Figure 2A, lane 3). A lower-molecular-mass contaminant was also present. Apoferritin, a protein with 24 subunits, each with a molecular mass of 19.4 kDa, was resolved to show two bands, an upper band corresponding to the 24-mer and a broader lower band with the average predicted molecular mass of a 12-subunit protein (Figure 2A, lane 4). The molecular mass of the native 24-mer protein was resolved better on a 4 % gel (right panel). Analysis of β -amylase (Figure 2A, lane 5) revealed two bands, one corresponding to that expected for the molecular mass of a dimer (twice the mass of the monomer, 56 kDa) and the other band corresponding to the native tetramer. Thyroglobulin, a dimeric protein, was analysed on a 4–12 % gradient gel and a 4 % gel (Figure 2A, lane 6). Only one band was apparent on the 4–12 % gel, corresponding to the monomer (304 kDa). On the 4 % gel (right panel), both the monomer (304 kDa) and the native dimer (608 kDa) were detected. A molecular mass calibration curve for a 4–12 % PFO/PAGE gel was deduced from migration distances and known molecular masses of the proteins studied in Figure 2(B), from which it is clear that most polypeptides fit a log-linear molecular-mass- R_F relation and therefore that most proteins are adequately separated on the basis of molecular mass.

Analysis of the oligomeric states of membrane proteins by PFO/PAGE

To determine the fidelity of PFO/PAGE in reporting the oligomeric structure of membrane proteins, we assessed the mobility of membrane proteins for which direct structural studies have been published, i.e. Band 3 protein [11] and aquaporin 1 [12]. Band 3, the chloride-bicarbonate exchanger in red blood cells, migrates as a monomer with an estimated molecular mass of approx. 100 kDa on SDS/PAGE. However, electron microscopical studies of two-dimensional crystals provided direct evidence that Band 3 forms a dimeric complex [11]. Furthermore, in biological membranes, it has been suggested that Band 3 exists

Figure 2 Separation of multimeric water-soluble proteins by PFO/PAGE

(A) Left panel: the resolution of protein standards on a 4–12% gradient gel. Right panel: the resolution of subgroups of these proteins on a 4% gel. lane 1, rainbow markers [molecular masses shown (in kDa) at the left]; lane 2, albumin for PFO/PAGE; lane 3, urease; lane 4, apoferritin; lane 5, β -amylase; lane 6, thyroglobulin; 25 μ g of each protein standard (lanes 2–6) was applied to each lane. Gels were stained with Coomassie Blue. (B) The mobilities of the protein bands relative to the front (R_f) were plotted against the logarithm of the molecular masses (MW). The identities of the bands (numbered 1–18) are shown in Table 1.

Figure 3 Western blot analysis of Band 3 and aquaporin 1 after resolution by PFO/PAGE and SDS/PAGE

Left panel: Band 3 protein migrates as a monomer, a dimer and a tetramer on PFO/PAGE (6% gel) and only as a monomer on SDS/PAGE (6% gel); 250 ng of total membrane protein was loaded on each gel. Right panel: aquaporin 1 migrates as a tetramer on PFO/PAGE and as a monomer on SDS/PAGE. Stripped red-cell ghosts were solubilized in PFO or SDS sample buffers. Both buffer solutions contained 25 mM dithiothreitol. SDS (0.25%) was added to the aquaporin sample just before PFO/PAGE. Samples for SDS/PAGE were incubated for 3 min at 37 °C before electrophoresis; 25 μ g of total membrane protein was loaded on each gel. The positions of molecular mass markers are indicated (in kDa) at the left of each panel.

both as a dimer and as a tetramer, in which the dimeric form of Band 3 is the minimum functional unit [13] and the tetramer forms the site for ankyrin attachment to the membrane [14]. We found that Band 3 (from alkali-stripped red-cell ghosts) migrated as two bands on PFO/PAGE. The predominant band corresponds to the molecular mass of the dimeric form of the protein (Figure 3, left panel), and the second band corresponds to the approximate molecular mass of a tetrameric complex of Band 3 protein. These results are consistent with the suggestion that PFO can protect the native oligomeric structure of membrane proteins.

Aquaporin 1 is another abundant protein in red blood cells and it functions as a constitutively active water-conducting pore [12]. On SDS/PAGE, aquaporin 1 migrates as a band with an estimated molecular mass of 28 kDa (Figure 3, right panel). However, electron-diffraction studies of two-dimensional crystals has established that aquaporin exists as a homotetramer in

membranes with four independent water permeation pathways [12]. PFO/PAGE of red-cell ghosts (stripped of peripheral proteins) did not give a detectable signal for aquaporin in a Western blot unless a low concentration of SDS (0.5%) was added to the PFO-solubilized sample just before electrophoresis. Under these conditions, described in detail in the legend to Figure 3, aquaporin 1 migrated as a diffuse band with a mobility corresponding to a molecular mass of approx. 115 kDa, which is consistent with the native tetrameric structure. The necessity for the addition of a small concentration of SDS to the PFO-solubilized membranes probably relates to unmasking of the antibody epitope for aquaporin 1 rather than to increasing its solubility, because PFO was fully effective in solubilizing aquaporin (results not shown).

Next we assessed the ability of PFO/PAGE to protect the oligomeric structure of ion channels. Kir 2.1 is a member of a family of inwardly rectifying K^+ channels that has an important

Figure 4 Determination of oligomeric structure of the K⁺ channel Kir 2.1

Left panel: *Xenopus* oocyte membranes (40 μ g of total membrane protein) expressing Kir 2.1 were analysed by PFO/PAGE (4% gel). The molecular mass of the band observed on PFO/PAGE was determined as approx. 230 kDa with a graph (inset) of the linear relationship between the mobility of protein standards relative to the front (R_f) and the logarithm of their molecular masses (MW). The standards used were: 1, thyroglobulin dimer (608 kDa); 2, apoferritin (475 kDa); 3, thyroglobulin monomer (304 kDa); 4, myosin (220 kDa). The molecular mass of 230 kDa corresponds to the estimated mass of a tetrameric complex (4' mer) of Kir 2.1. Right panel: when run on SDS/PAGE (8% gel), Kir 2.1 protein migrated as two bands, one corresponding to the predicted molecular mass of the monomer (53 kDa) and the other an SDS-resistant complex of approx. 170–180 kDa. Kir 2.1 was detected with a rabbit polyclonal antibody (generously provided by P. Backx). The positions of molecular mass markers are indicated (in kDa) at the extreme left and right.

Figure 5 Determination of the oligomeric structure of the GABA-A receptor

Left panel: rat hippocampal membranes were solubilized in PFO sample buffer containing a final concentration of 0.5%, 2% or 4% (v/v) PFO (lanes 1, 2 and 3 respectively). In some cases (lane 4), membranes were first treated with 1 M NaI to extract extrinsic membrane proteins before solubilization in PFO. Membrane proteins were then analysed by PFO/PAGE with a 4–12% gel; 100 μ g of total membrane protein was loaded in lanes 1–3; in lane 4, 50 μ g of total membrane protein solubilized in 2% PFO was loaded. On PFO/PAGE, a primary band corresponding to the predicted molecular mass of a trimeric complex (3' mer) of GABA-A was detected and a fainter band, corresponding to a pentameric complex (5' mer), was removed by pretreatment of membranes with NaI. Right panel: the predominant band detected in the same membrane preparation by SDS/PAGE with an 8% gel corresponded to the molecular mass of a single subunit. GABA-A protein was detected with a polyclonal antibody generated against the α -subunit of GABA-A. The positions of molecular mass markers are indicated (in kDa) at the left of each panel.

role in determining the resting potential of the cell [8]. The three-dimensional crystal structure of the inwardly rectifying prokaryotic K⁺ channel was recently solved [15]. This direct structural information supports previous functional results suggesting that these ion channels exist as tetramers in membranes [16]. In our analysis of membranes isolated from *Xenopus* oocytes expressing Kir 2.1 channel by PFO/PAGE and immunoblotting, we detected a prominent band at approx. 230 kDa that corresponded to the molecular mass estimated for a tetrameric channel complex (Figure 4, left panel). Therefore PFO/PAGE seems to preserve the native quaternary structure of this K⁺ channel. Analysis of membranes expressing Kir 2.1 protein by SDS/PAGE revealed both a band corresponding to the molecular mass predicted for a single subunit (53 kDa) and another band corresponding to a Kir 2.1 complex of approx. 170–180 kDa (Figure 4, right panel). Therefore, unlike the other protein

complexes studied in this report, the Kir 2.1 protein complex was not completely dissociated by SDS/PAGE.

The γ -aminobutyric acid (GABA)-A receptor ion channel complex is thought to be a hetero-pentameric oligomer composed of at least one α , one β and one γ subunit from a 'repertoire of 13 known members' (six distinct α subunits, three β subunits, three γ subunits and one δ) [17]. The major GABA-A receptor subtype in the adult rat hippocampus has the following subunits composition: $\alpha 1\beta 2\gamma 2$ [17]. It has been suggested that a pentameric complex would consist of two $\alpha 1$ subunits, one $\beta 2$ and two $\gamma 2$ subunits with a predicted total molecular mass of approx. 260 kDa (if one assumes that the long isoforms of the $\beta 2$ and $\gamma 2$ polypeptides were expressed). PFO/PAGE of membranes from hippocampal membranes obtained from adult rats, followed by immunoblotting with an antibody against the $\alpha 1$ subunit, revealed two bands, a major band at approx. 150 kDa and a

minor band at 270 kDa (Figure 5, left panel). The predominant band in the PFO/PAGE gel corresponds to the predicted molecular mass of a trimeric complex comprising $\alpha 1/\beta 2\gamma 2$ and the minor band corresponds to the predicted mass of the pentameric complex described above [17]. The intensities of both bands peaked at a detergent-to-protein ratio of 10:1 in the solubilization buffer (Figure 5, left panel, lane 2). We suggest that the trimeric complex probably represents the more stable of the two complexes, because increasing the detergent-to-protein ratio as well as pretreatment of the hippocampal membranes with NaI resulted in the loss of the higher band. However, the molecular basis and the physiological significance of the trimeric form of the receptor complex have yet to be determined.

To summarize, we have described a novel method that can be used to study the quaternary structure of membrane proteins. This method can be considered to be complementary to Blue native PAGE as a high-resolution method for the analysis of molecular mass and oligomeric status of membrane protein complexes. PFO/PAGE has advantages over native PAGE with Coomassie Blue staining in that it is relatively simple and accessible to most laboratories.

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