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Surfactosomes: a novel approach to the reconstitution and 2-D crystallisation of membrane proteins

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Abstract The formation of vesicle-like structures (termed surfactosomes) and lamellar sheets from solutions containing ammonium perfluoroocanoate (APFO) is illustrated using conventional and cryo-transmission electron microscopy. It is shown how this detergent can be used for the solubilisation, reconstitution, and 2-D crystallisation of membrane proteins as demonstrated for the major protein of the membrane sector of the V-type H^+-ATPase (16-kDa protein). Electron microscopical analysis of 2-D crystals of the 16-kDa protein ($a = b = 13.0 \pm 0.2$ nm with $\gamma = 90°$ and p4 projection symmetry) revealed a unit cell comprising four dimeric complexes of the 16-kDa protein the significance of which is discussed.

Key words: Membrane protein; Structure; Reconstitution; 2-D crystallisation; Vacuolar H^+-ATPase; Electron microscopy

1. Introduction

The potential use of ammonium pentadecafluorooctanoate (ammonium perfluorooctanoate (APFO) as a 'biological detergent' in membrane biochemistry has been reported previously [1]. In this earlier study it was also noted that along with its many advantageous properties (e.g. very high solubilising capacity, stability, low cost), APFO shows an unusual phase behaviour characterised by a micellar-vesicular transition. This transition can be induced by changes in temperature and salt concentration suggesting its possible use for a unique reconstitution approach. In contrast to conventional reconstitution experiments (see e.g. [2]), APFO would serve as both the detergent and lipid component. The practicability of such an approach would also obviate the need for detergent exchange, which otherwise may constitute a further suitable option, if one wishes to use APFO for solubilisation only [1,3–5].

The phase behaviour of perfluorinated surfactants – other examples of which include perfluorinated carboxylic acids 4–8 carbon atoms in length and their Li^+, Cs^+, ammonium and quaternary ammonium salts – differs significantly from that of hydrocarbon surfactants [6–8]. The exchange of hydrogen atoms for bulky, highly electronegative fluorine atoms within the hydrophobic part of the molecule results in a rigid, rod-shaped molecule with an increased surface activity exhibiting a preferred formation of bilayers (lamellar phase) over columnar aggregates and low critical micellication concentration (cmc) values [6]. Furthermore, while both hydrocarbon as well as perfluorinated surfactants (i) exist as monomers in solution below their cmc and aggregate into isotropic micelles above their cmc and (ii) are able to exist in a lamellar phase [7,9–13], only the lamellar phase of perfluorinated detergents is stable over a wide range of conditions including variations in concentration, temperature and ionic strength [7,12–14] making them potential candidates for a 'detergent only' reconstitution approach.

Here we report how APFO can be used not only for the solubilisation and reconstitution, but also the 2-D crystallisation of the major protein of the membrane sector of the V-type H^+-ATPase (16-kDa protein) isolated from the hepatopancreas of *Nephrops norvegicus*. The 16-kDa protein belongs to a family of proteins with quite diverse functions ranging from proton pumping activity and cellular communication to the release of acetylcholine in response to elevated Ca^{2+} levels and cellular transformation processes [15–18]. Electron microscopical data of the first 2-D crystals grown from solubilised 16-kDa protein are presented, and it is suggested that this novel approach may be a valuable tool for the 2-D crystallisation and structural elucidation of a wide range of membrane proteins.

2. Materials and methods

2.1. Materials

Membrane preparations of *Nephrops norvegicus* hepatopancreas containing the 16-kDa protein as well as rabbit antibodies against the N-terminal domain of the 16-kDa protein were kindly provided by Dr. M.E. Finbow (Beatson Institute for Cancer Research, Glasgow).

APFO was purchased from Fluorochem Ltd. (Old Glossop, Derbyshire, UK), goat-anti-rabbit 10-nm gold-conjugated antibodies from Sigma Chemical Company Ltd. (London, UK), 400-mesh copper grids from Agar Scientific Ltd. (Essex, UK), and 1000-mesh copper grids from Gilder (Grantham, Lincolnshire, UK). All other chemicals were from Sigma Chemical Company Ltd. (London, UK) or BDH Chemicals Ltd. (Poole, Dorset, UK).

2.2. Protein solubilisation, reconstitution and crystallisation

Prior to solubilisation, which was essentially carried out according to [1], protein concentrations were determined by the Lowry protein assay [19] using bovine serum albumin as a standard. Membranes preparations were solubilised by adding an aqueous solution of $NaHCO_3$ (23 mM, pH 8.3) containing 7% (w/v) APFO at a ratio of 69 mg protein:2.8 mg APFO, incubating for 60 min at 21°C and centrifuging in a Beckman Airfuge for 90 min at 178,000 × *g* and 21°C. The solubilised protein was reconstituted by slowly adding a solution of 0.8 M NH_4Cl (in 23 mM $NaHCO_3$) on ice up to a final concentration of 0.4 M NH_4Cl, 2% (w/v) APFO and 0.5 mg protein/ml, incubating at 21°C for 2 h, subsequent cooling below the micellar-lamellar phase transition temperature of 13.6°C (as determined in the absence of protein), and mixing by inversion. For crystallisation, reconstitution conditions were employed followed by temperature cycles

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between 4°C and 18°C. 2-D crystals were observed in samples taken at 6°C.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli [20] using 12% (w/v) acrylamide slab gels. Samples were mixed 1 : 1 with 0.13 M Tris-HCl (pH 6.7), 6% (w/v) SDS, 40% (v/v) glycerol, 10 mM dithiothreitol, and 20 mg/ml bromophenol blue and then incubated for 2 h at room temperature. Gels were silver-stained according to Morrissey [21].

2.4. Cryo-transmission electron microscopy (cryo-TEM)

Samples were prepared to be observed in their frozen-hydrated state as detailed by Lepault et al. [22]. 5 μl of the sample suspension was deposited on a bare 1000-mesh copper grid or a grid with a support film consisting of a perforated Formvar film coated with carbon and evaporated gold (for increased stability and decreased charging effects, respectively), blotted with filter paper and quickly plunged into liquid nitrogen-cooled ethane just above its freezing point. Grids were subsequently transferred into liquid nitrogen and then, via a cryo-transfer workstation, to a cryo-holder (Oxford Instruments, Oxford, UK). During the entire transfer process grids were kept in a liquid nitrogen atmosphere. Specimens were observed in a Philips CM10 electron miscroscope operated at an acceleration voltage of 100 kV at temperatures below −160°C. Images were recorded at calibrated magnifications in the range of 19,000–39,000× with a nominal defocus of 0.5 and 3 μm on Agfa Scientia 23 D 56 electron image sheet film or directly in digital format using a TVIPS (Tietz Video and Image Processing Systems, Gauting, Germany) on-line image acquisition and analysis system based on a Photometrics AT 200 Peltier-cooled sclow-scan 512×512 CCD camera controlled by Photometrics PMIS software (Photometrics, Tucson, AZ, USA).

2.5. Conventional TEM and image processing

Conventional TEM was carried out on negatively stained specimens which have been prepared as follows: 3-μl samples containing 0.4–0.6 mg protein/ml were allowed to adsorb onto carbon/collodium-coated copper grids (400 mesh) for 30 s, washed for 30 s on a droplet of double-distilled water, stained for 5 s on a droplet of an aqueous solution of uranyl acetate (2% w/v, pH 4.25), and blotted dry. All manipulations were performed under temperature-controlled conditions whereby solutions were allowed to equilibrate as required. Specimens were examined in a Philips CM 10 transmission electron microscope operated at an accelerating voltage of 100 kV. Electron micrographs were recorded at a calibrated magnification (37,100×) on Agfa Scientia 23 D 56 electron image sheet film. Electron micrographs of negatively stained 2-D crystals were assessed by optical diffraction and selected micrographs were digitised at 25 μm increments using a Joyce-Loebl rotating drum microdensitometer (Joyce-Loebl, Gateshead, Newcastle, UK). Suitable areas were boxed off and corresponding Fourier transforms indexed, lattices refined and Fourier projection maps calculated using the PC-based crystallographic image processing software package CRISP [23].

2.6. Immuno-TEM

Immunogold-labelling of surfactosomes after reconstitution was carried out essentially as described by Mayer and Rohde [24]. The protein was reconstituted as described above. Incubation of both the primary (2 μl of a 1 : 10 dilution per 35 μl reconstitution mixture) and the subsequently added gold-conjugated secondary antibody (dilution 1 : 100) was for 60 min at 7°C. Specimens were prepared and analysed by conventional or cryo-TEM (see above).

3. Results and discussion

In conventional reconstitution experiments, detergent-solubilised protein is mixed with lipid-detergent mixed micelles and the detergent subsequently removed causing the protein to be re-integrated into a membranous environment with the aim to reinstate the protein's biological activity (functional reconstitution) and/or to induce the formation of ordered 2-D protein–lipid arrays (2-D crystals) suitable for electron microscopical/crystallographic investigations [2]. In addition to the large number of parameters that critically influence the formation of 2-D crystals (e.g. the rate of detergent removal, temperature, initial detergent and protein concentrations, protein-to-lipid ratio) a detergent exchange step may be required since the detergents used for solubilisation often are undesirable for reconstitution [3,4]. To this end, the stable lamellar phase formed by APFO offers the tempting alternative to reconstitute APFO-solubilised protein directly into the bilayer structures formed by the detergent.

The use of APFO for the solubilisation of the 16-kDa protein crucially depended on the presence of $NaHCO_3$ which subseqently led to a change of the usually employed ternary system consisting of APFO, Cl^- (as counter ion, usually NH_4Cl) and H_2O (cf. [1]) to a quaternary system consisting of APFO/NH_4Cl/$NaHCO_3$/H_2O. In order to confirm the presence of a lamellar phase in the quaternary system – the introduction of $NaHCO_3$ is expected to alter the phase behaviour – phase transitions were monitored employing a cross-polariser making use of the fact that smectic and nematic phases exhibit characteristic birefringence patterns when observed in plane-polarised light [10,13,14]. Two results of this

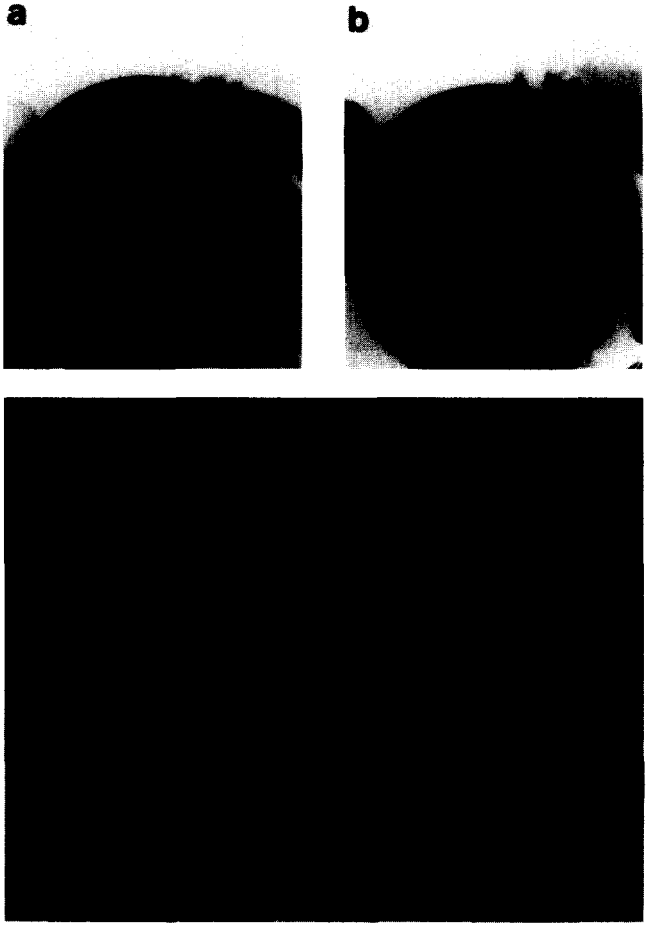

Fig. 1. The appearance of an aqueous solution of 5% (w/v) APFO, 0.5 M NH_4Cl and 23 mM $NaHCO_3$ observed with a cross-polariser above (a) and below (b) the micellar-to-lamellar phase transition temperature. While the solution in (a) at 25°C appears homogeneous, the solution in (b) at 11°C has undergone phase separation with a birefringent lower phase (arrowhead). In (c), a cryo-electron micrograph of the lamellar phase of APFO is shown. Frozen ethane appears as dark circular structures (arrowheads). The scale bar corresponds to 0.5 μm.

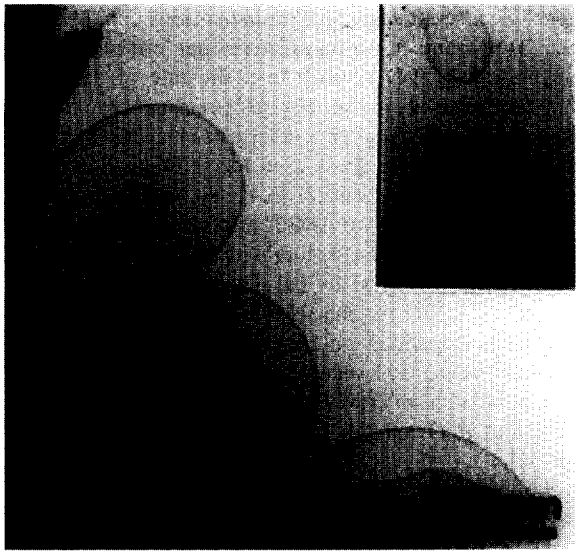

Fig. 2. Cryo-electron micrographs of surfactosomes formed from aqueous solutions of 5% (w/v) APFO, 0.5 M NH_4Cl and 23 mM $NaHCO_3$. The arrowheads mark the margin of the support film delineated by evaporated gold.

analysis are depicted in Figs. 1a and 1b. The presence of lamellar sheets was also confirmed by cryo-TEM (Fig. 1c), which is the method of choice since the object can be kept in its frozen-hydrated (native) state throughout the course of analysis. The formation of vesicular structures in aqueous solutions of APFO and NH_4Cl has been discussed [1], however, no rigorous proof of their existence has been presented to date. Fig. 2 depicts the typical appearance of vesicle-like structures of APFO (termed surfactosomes) in the presence of NH_4Cl and $NaHCO_3$ as revealed by cryo-TEM. The diameter of surfactosomes is in the range of 0.1 to >2 μm.

Assuming that the arrangement of APFO molecules in the lamellar phase is similar to that of lipids (lipid bilayers within biological membranes are ~5 nm thick with the length of the lipid chains being ~2 nm) and using a value of 1.2 nm for the chain length of APFO [25], the thickness of the bilayer it forms can be estimated to be around 3.5 nm. Measurements of the bilayer thickness carried out on cryo-electron micrographs arrive at a value of around 4 nm which is in excellent agreement with the theoretical considerations and confirm that surfactosomes are unilamellar. Having established the presence of unilamellar surfactosomes in the system under study, reconstitution experiments were attempted using the 16-kDa protein as the protein component and surfactosomes as the *quasi-lipid* component. Prior to the reconstitution experiments, a successful solubilisation of the 16-kDa protein was confirmed by SDS-PAGE (Fig. 3). In order to ascertain as to whether the 16-kDa protein can be reconstituted using APFO, surfactosomes with putatively integral 16-kDa protein were subjected to immunogold-labelling. Specimens were analysed by conventional as well as cryo-TEM (Fig. 4), and in both cases labelling was almost entirely restricted to surfactosomes. Control experiments in the absence of protein/presence of antibodies revealed a negative result. The presence of some gold particles in the background of the cryo-specimens can be ascribed to the bare grid technique used in preparing the specimens which does not allow for washing steps to be carried out. The appearance of gold particles right on the edge of the surfactosomes (Fig. 4) does furthermore suggest that the antibodies could not have been unspecifically trapped inside nascent surfactosomes (a possibility which cannot be rules out since the labelling was performed in solution) but were specifically bound to antigenic target sites residing within the APFO bilayer. Similar results were obtained with negatively stained specimens, although the overall appearance of the surfactosomes after negative staining (Fig. 4) suggests that the surfactosomes have suffered to some extent from dehydration. These findings are also supported by observations made with liposomes [26].

It seems worth pointing out that a successful reconstitution using APFO requires a particularly careful tuning of all parameters. When using protocols different from the one reported herein and involving e.g. changes in the final protein concentration, mode of addition of NH_4Cl, temperature, omission of $NaHCO_3$ etc., the protein tended to precipitate. Perhaps more importantly, when cyclic temperature changes followed effective reconstitution conditions, the formation of 2-D crystals was observed. Fig. 5 shows 2-D crystals of the 16-kDa protein obtained within vesicular, lamellar and tubular structures. Unit cell dimensions are $a = b = 13 \pm 0.2$ nm with $\gamma = 90°$ and a *p*4 plane group could be assigned with a phase residual of 6°. There was a tendency of the 2-D crystals to stack on top of each other which might be explained by the strong longitudinal interactions known to exist between 16-kDa protein complexes arranged in gap-junction-like structures [15]. In contrast to previously published results which were based on the analysis of hexagonal 2-D protein-lipid arrays of the 16-kDa protein [15,27] and which showed a star-shaped hexameric complex around a central channel running perpendicular to the membrane plane, the protein was found to crystallise as a dimer after solubilisation and reconstitution in APFO. Fourier projection maps of the reconstituted 16-kDa protein calculated in *p*4 to a resolution of about 2 nm (Fig. 5c) reveal eight stain-excluding densities per unit cell which are organised in pairs. The diameter of each density is ~3 nm which is consistent with the diameter of a single four α-helical bun-

Fig. 3. SDS-PAGE of the solubilised 16-kDa protein. In the left lane, the band corresponding to the 16-kDa protein is marked. The right lane contains molecular mass standards of 14.1, 20.1 (both of which are labelled), 30.0, 43.0, 67.0, and 94.0 kDa.

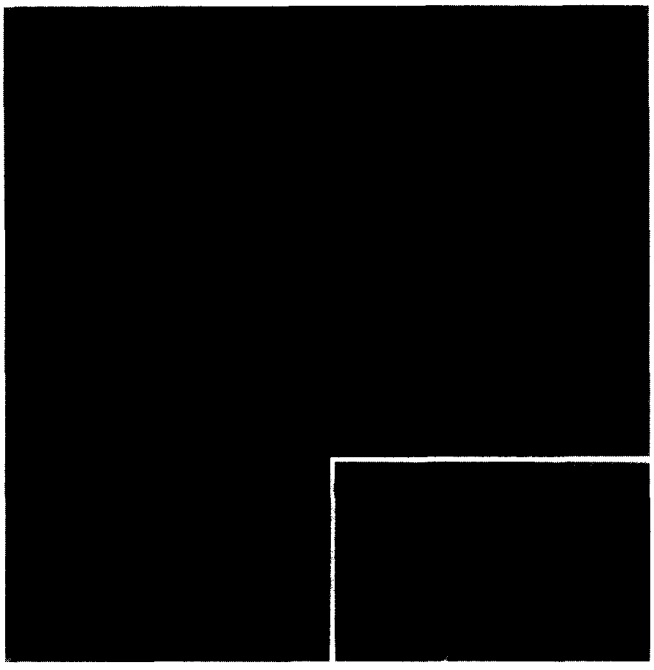

Fig. 4. Conventional (after negative staining) and cryo-electron micrograph (insert) of immunogold-labelled 16-kDa protein reconstituted into surfactosomes. The inverted contrast of the gold particles in the cryo-electron micrograph (gold appears white) is due to defocusing. Both scale bars represent 100 nm.

dle of the 16-kDa protein in a projection perpendicular to the membrane plane (cf. [15]). An SDS-stable dimer of the 16-kDa protein has been observed previously after solubilising gap-junction-like structures from *N. norvegicus* [27] and one may speculate that APFO causes effects similar to SDS when used over prolonged periods of time. The questions as to whether the 16-kDa protein indeed occurs as a dimer in the native membrane and whether this dimeric form bears any functional significance must await further studies. However, while the gap-junction-like structures formed by the 16-kDa protein from *N. norvegicus* and other sources typically suffer from short-range rotational disorder which has so far precluded the application of any high-resolution electron crystallographic studies [15,28] and which may be due to unfavourable interactions between the hexameric complexes forming the lattice, it appears that the 16-kDa protein can achieve a much better packing when in its dimeric state.

The novel 2-D crystallisation approach presented in this report offers for the first time ways to obtain better ordered 2-D crystals of the 16-kDa protein with the scope to finally resolve high-resolution details by electron microscopy/crystallography. It furthermore appears almost certain that the presented methodology will prove to be a valuable tool for structural/functional studies of many other ion channels and transmembrane proteins, and may possibly be adapted for drug delivery purposes as suggested for different surfactant structures [29].

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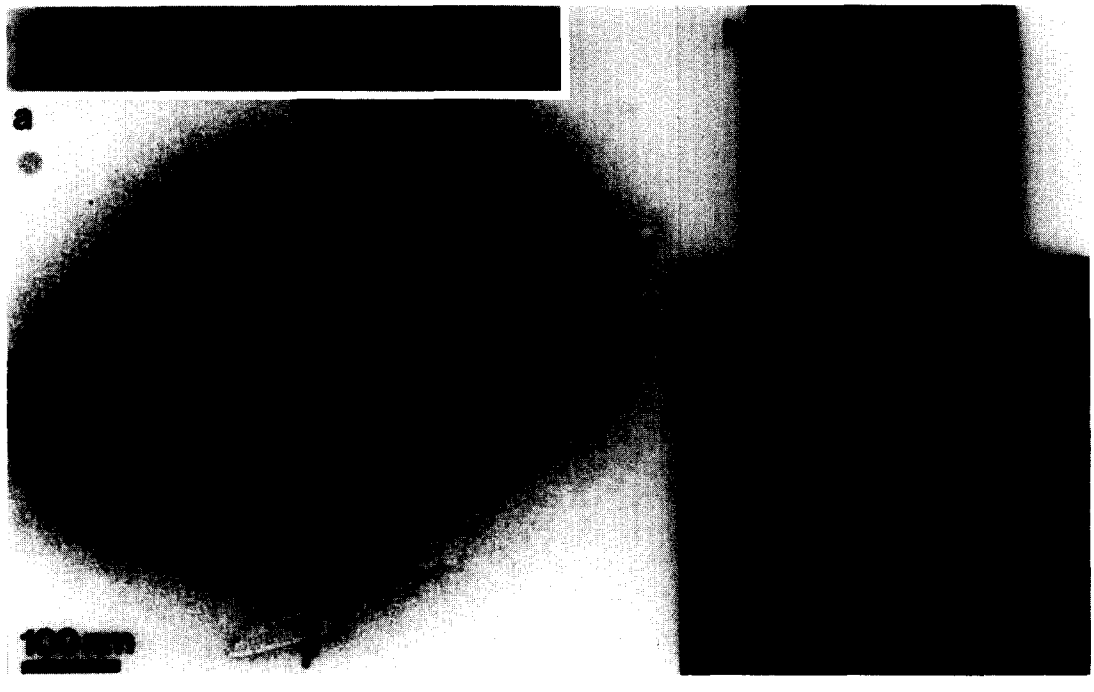

Fig. 5. Electron micrographs of negatively stained (protein appears white) 2-D crystals of the 16-kDa protein within vesicular (surfactosome), lamellar, and tubular (insert) structures of APFO (a). Note the folded over area (arrow). A typical diffraction pattern of a 2-D crystal of the 16-kDa protein is shown in (b), and in (c) a corresponding Fourier projection map calculated in $p4$ is depicted. The reflections (h,k) = (0,−4) and (h,k) = (−4,1) in (b) are marked (boxed and circled, respectively) and the scale bar represents 0.2 nm^{-1}. Unit cell dimensions are $a = b = 13.0\ nm \pm 0.2\ nm$ with $\gamma = 90°$.

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