

The use of amphipathic polymers for cryo electron microscopy of NADH:ubiquinone oxidoreductase (complex I)

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Summary

In the three-dimensional (3D) structure determination of macromolecules, cryo electron microscopy (cryo-EM) is an important method for obtaining micrographs of unstained specimens for the single-particle reconstruction approach. For cryo-EM, proteins are fixed in a frozen hydrated state by quick-freezing in a thin water layer on a holey carbon film. Cryo-EM of detergent-solubilized membrane proteins is hindered by the fact that detergents reduce the surface tension of water, so that it is difficult to control the ice thickness and the distribution of protein. Amphipols are a new class of amphipathic polymers designed to handle membrane proteins in aqueous solutions under particularly mild conditions. Amphipol A8-35 stabilizes NADH:ubiquinone oxidoreductase (complex I) from *Neurospora crassa* and keeps it water-soluble in the absence of free detergent. Electron microscope images of quick-frozen complex I/A8-35 samples were used for computer-based single-particle averaging and 3D reconstruction, and the reconstruction of unstained frozen-hydrated particles compared with previous detergent-based reconstructions. The potential of amphipols for cryo-EM is discussed.

Introduction

Integral membrane proteins, when removed from their normal lipid environment, are usually stabilized in aqueous solutions by detergents to prevent them from precipitating. Above the so-called critical micellar concentration (CMC), detergent molecules form regular aggregates (micelles), in which the hydrophobic parts of the molecules are sequestered in the core and the hydrophilic parts face the surrounding water. Above their CMC, detergents adsorb on to the transmembrane, hydrophobic surface of membrane proteins via their hydrophobic moiety, forming an amphipathic belt that substitutes for the natural lipid bilayer while keeping the protein water-soluble. An important characteristic of micelles and protein-adsorbed detergent belts is that they are not stable entities, but are in rapid equilibrium with free detergent monomers. If this equilibrium is disturbed by removal of monomers from solution, for instance by dialysis, more and more micelle detergent molecules return into solution until all micelles have disappeared. At that stage, most detergent molecules desorb from membrane proteins, which become water insoluble and aggregate.

For structural studies of proteins by cryo electron microscopy (cryo-EM), it is essential that they be evenly dispersed as single particles within a thin layer of amorphous ice on a holey grid. The presence of detergent reduces the surface tension of water so that, before freezing, the water film is too thin and large

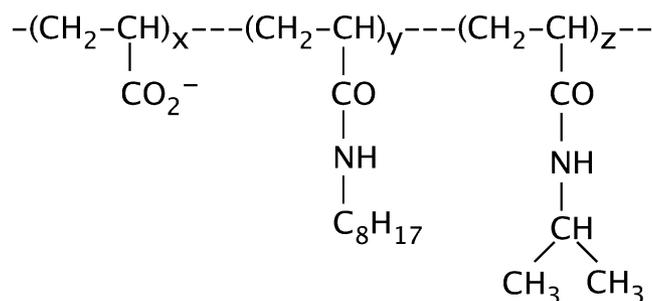


Fig. 1. Chemical structure of amphipol A8-35 (from Tribet *et al.*, 1996, and Gohon *et al.*, 2006). $\langle x \rangle \approx 35\%$, $\langle y \rangle \approx 25\%$, $\langle z \rangle \approx 40\%$, $\langle \text{MW} \rangle \approx 9$ kDa.

membrane protein complexes are forced away from the centre of the hole and tend to collect in a narrow band around its periphery. In the present work, we have examined whether this problem can be avoided by replacing the detergent with amphiphatic polymers ('amphipols').

Amphipols were originally developed to overcome the dissociating effects of detergents, which generally entail the more or less rapid inactivation of detergent-solubilized membrane proteins (Gohon and Popot, 2003). Amphipols are short hydrophilic polymers carrying numerous hydrophobic side chains (Tribet *et al.*, 1996), thanks to which they bind noncovalently but quasi-irreversibly (Tribet *et al.*, 1997; Zoonens, 2004; Zoonens *et al.*, 2007) to the transmembrane surface (Zoonens *et al.*, 2005) of membrane proteins (reviewed by Popot *et al.*, 2003). The polymer used here, amphipol A8-35 (Tribet *et al.*, 1996; Gohon *et al.*, 2006), is an overall negatively charged molecule with an average molecular mass of 9–10 kDa (Fig. 1). A8-35 has been shown to stabilize membrane proteins against denaturation (Tribet *et al.*, 1996; Champeil *et al.*, 2000; Popot *et al.*, 2003; Picard *et al.*, 2006; Gohon *et al.*, unpublished data), to favour their renaturation (Pocanschi *et al.*, 2006) and to keep them water-soluble in the absence of excess detergent or amphipol (Tribet *et al.*, 1996; Gohon *et al.*, unpublished data; Zoonens *et al.*, 2007).

The mitochondrial membrane protein complex NADH:ubiquinone oxidoreductase (complex I) from the fungus *Neurospora crassa* was used for the present study. Complex I is the first enzyme of the mitochondrial respiratory chain. It catalyses the transfer of two electrons from NADH to ubiquinone, coupled with the translocation of four or five protons across the inner mitochondrial membrane (Weiss *et al.*, 1991). Complex I is one of the largest known membrane proteins, with up to 42 subunits in eukaryotes and a molecular weight of more than 1 MDa. The molecule is L-shaped, with a cytoplasmic (matrix) arm and a transmembrane arm approximately at right angles. Both arms have a length of about 20 nm as determined by EM and single-particle three-dimensional (3D) reconstruction of negatively stained samples (Hofhaus *et al.*, 1991; Guénebaut *et al.*, 1997; Radermacher *et al.*, 2006). A single-particle 3D reconstruction of unstained

detergent-solubilized complex I imaged in ice (Grigorieff, 1998) also gave an L-shaped structure, but the features of the two arms differed from those found in the negatively stained preparations. We have now carried out a 3D reconstruction of amphipol-stabilized complex I in ice which, although at a lower resolution, agrees substantially with the model obtained in negative stain.

Materials and methods

Reagents

Unless otherwise stated reagents were obtained from sigma-aldrich (Germany).

Complex I isolation

NADH-ubiquinone oxidoreductase (complex I) was isolated from *Neurospora crassa* mitochondria as described by Hofhaus *et al.* (1991) using anion-exchange chromatography, hydroxyapatite chromatography and sucrose gradient centrifugation in the presence of the detergent Triton X-100. For the experiments, a monodisperse solution of complex I at 2 g.L⁻¹ protein in 50-mM Tris-acetate, pH 7.2, 50 mM NaCl, 250 mM sucrose and 0.1% Triton X-100 (Serva, Germany) was used. Membrane arm preparations were made as described by Hofhaus *et al.* (1991). For negative staining, these were diluted in 0.1% dodecylmaltoside (DDM) in 50 mM Tris-HCl, pH 7.2, 700 mM NaCl.

A8-35 synthesis

Amphipol A8-35 was synthesized as described by Tribet *et al.* (1996) and Gohon *et al.* (2006). A stock solution of 10 g.L⁻¹ A8-35 in bi-distilled water was prepared by constant stirring at 4°C for 24 h.

Preparation of BioBeads

BioBeads were obtained from BioRad (BioRad, Germany). Before usage the beads were washed twice with methanol followed by five washes with triple-distilled water for 10 min each.

Exchange of surfactants

For dilution and surfactant substitution, the following buffer was used: 50 mM Tris-acetate, pH 7.2, 50 mM NaCl, 0.1% Triton X-100, 0.1% NaN₃. For the substitution process, 25 µL protein were mixed with A8-35 in the ratio 1:2 (w/w) and diluted with the above buffer to a final protein concentration of 1 g.L⁻¹. The solution was incubated for at least 4 h. After the initial incubation, 6 mg of wet BioBeads were added, and the removal of detergent was carried out on a rotary shaker for either 2 h (samples for negative staining) or 10 h (samples for cryo-EM). To remove any remaining detergent, the protein solution was then subjected to a buffer exchange on a Micro-BioSpin P30 column (BioRad), with an

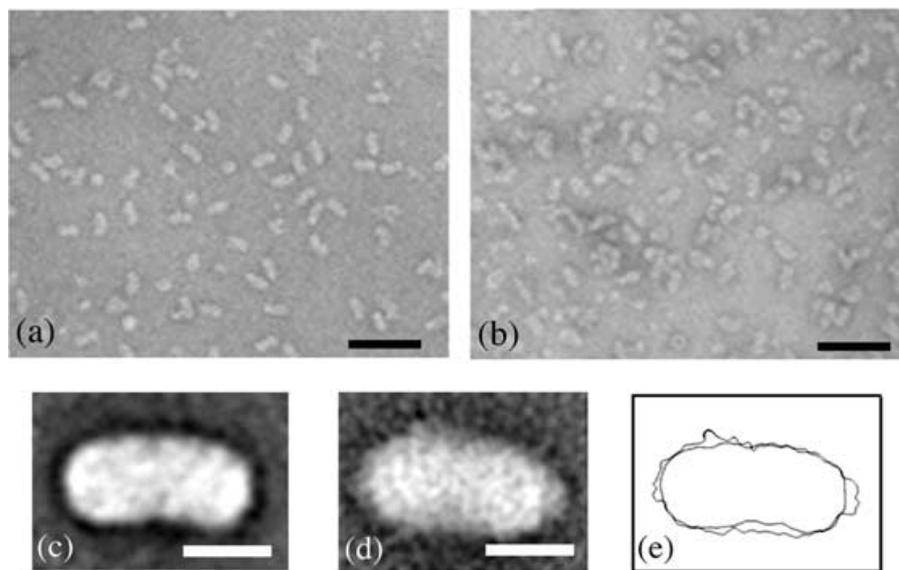


Fig. 2. EM pictures of negatively stained membrane arms of complex I. Particles were stained with 1% uranyl acetate on formvar/carbon-coated copper grids. (a) Proteins stabilized with 0.1% DDM in 50-mM Tris-HCl, pH 7.2, 700-mM NaCl. (b) Proteins stabilized with A8-35 in an amphipol/protein ratio of 2:1 (w/w), after 2-h incubation with BioBeads. (c, d) Averages of single particles after shift and rotational alignment in the SPIDER software package: (c) 104 particles in DDM; (d) 91 particles in A8-35 (a and b, scale bar 100 nm; c and d, scale bar 20 nm). (e) Overlay of the edge contours for the two averages.

exclusion limit of 40 kDa, against substitution buffer without Triton. For cryo-EM the protein was diluted 1:2 with the above buffer.

Estimation of detergent removal

A simple method was used to estimate the completeness of detergent removal based on the surface tension of the remaining solution. Small drops of solution (usually 50 μL) were pipetted on to a hydrophobic surface (Parafilm). The amount of detergent remaining was estimated from the diameter of the drop relative to standard drops containing known percentages of detergent. Greater amounts of detergent will give larger diameter drops by this method, which is equivalent to more accurate methods that measure the sessile drop contact angle. The surfactant exchange treatment described above was carried out until there was no further reduction in the drop diameter.

Negative staining

For negative staining, 400 mesh formvar/carbon-coated EM grids were glow-discharged in air prior to use. A drop of solution ($\sim 3 \mu\text{L}$ at $1\text{--}2 \text{ g.L}^{-1}$ protein) was put on the grid and incubated for 30 s. At the end of the incubation time the excess of protein solution was blotted away with filter paper (Whatman No.1) and the wet grid surface washed three times for 1 min with 1% aqueous uranyl acetate. After the last wash any excess of stain was blotted away and the grid dried.

Cryo electron microscopy

Samples were pipetted on to holey grids (Quantifoil, Germany) in a humid chamber, blotted with filter paper (Whatman No.1) for 15 s, and quick-frozen by plunging them in liquid ethane cooled with liquid N_2 . Cryo-EM was carried out on a Tecnai CM120 Biotwin with a Gatan Cryo holder. Images were taken at 100 kV at a magnification of $37\,000\times$ in minimal dose mode with an electron dose of $<100 \text{ e.nm}^{-2}$. Images were slightly underfocused such that the first zero of the contrast transfer function was less than 2.5 nm.

Image processing and 3D reconstruction

Micrographs were digitized on a Zeiss SCAI-Scanner at $14 \mu\text{m}$ raster resulting in an overall pixel size of 3.78 \AA . Single-particle picking and further processing were carried with the SPIDER processing package (Frank *et al.*, 1996).

Results

Electron microscopy of negatively stained complex I membrane arm in amphipol A8-35

An initial study was carried out using just the isolated membrane arm fragment of the complex (Hofhaus *et al.*, 1991) in order to determine whether stabilization by amphipols rather than detergent would significantly alter the structure of the transmembrane region. Figure 2 shows fields of

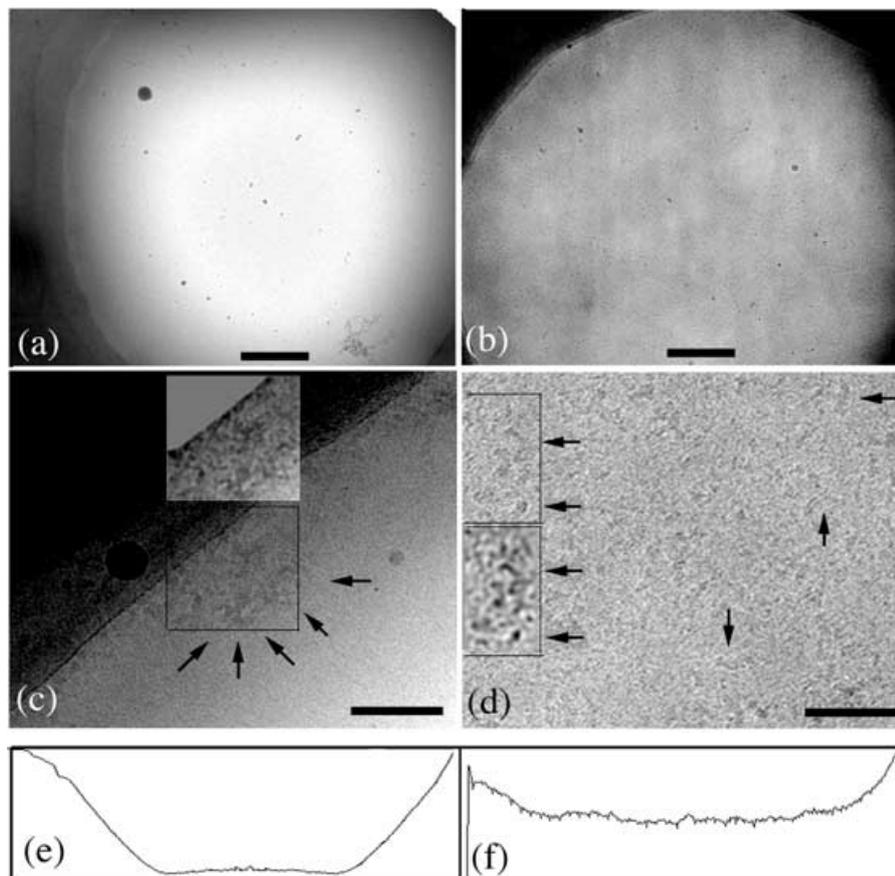


Fig. 3. Comparison of ice thickness and particle distribution. (a) Low magnification image of an ice film covering a fenestrated grid hole in the presence of Triton X-100; scale bar: 0.7 μm . (b) A similar image of the ice film for a sample stabilized by A8-35; scale bar: 0.7 μm . (c) Higher magnification image of a detergent sample showing clumping of complex I particles around the edge of the hole, where the ice is thicker (arrows); scale bar: 100 nm. (d) Image of complex I frozen in A8-35, showing a more even distribution of particles across the hole (arrows mark some of the particles); scale bar: 100 nm. The insets in (c) and (d) are low-pass filtered images of the adjacent boxed regions, in which the complex I particles can be seen more clearly. (e, f) Density scans for equal areas across the centre of the images shown in (a) and (b). In the case of the detergent solution (e), there is a marked thinning of the ice towards the centre of the hole, which is not the case for the amphipol preparation (f). The horizontal scale in (e) and (f) is the same as in (a) and (b). The vertical scale is the same in (e) and (f) and is in arbitrary ice density units. If the thickness of the carbon film at the edge of the hole is taken to be 30 nm (Karlsson, 2001), we can approximately estimate the thickness of the ice at the centre of the hole to be about 2 nm in detergent and 15 nm in amphipols. The apparently higher noise in (f) is density variation caused by a more random distribution of complex I particles in (b).

particles stained with uranyl acetate and dried on a carbon film. The distribution and appearance of those in detergent (Fig. 2a) compared with those in A8-35 (Fig. 2b) are very similar. Particles were picked randomly from each image, cross-correlated and averaged. The two-dimensional (2D) projections (Fig. 2c, d) are essentially the same, the A8-35 image (Fig. 2d) showing slightly higher granularity or noise. The edge contours made at the point of maximum descent are also very close (Fig. 2e). For another large membrane protein complex, cytochrome bc_1 (~ 500 kDa), size exclusion chromatography has also revealed no significant size difference between detergent-stabilized and amphipol-stabilized complexes (D. Charvolin and J.-L.P., unpublished observations).

Cryo electron microscopy of whole complex I in detergent and amphipol A8-35

Figure 3 (a, c, e) shows samples of complex I in Triton X-100, which were blotted on perforated carbon films and fast-frozen in liquid ethane. The resulting ice films show a pronounced thickness gradient across the holes (Fig. 3e) and the protein tends to clump and aggregate at the edges (Fig. 3c). If the detergent was replaced with amphipol (Fig. 3b, d, f), an ice film much more uniform in thickness was obtained (Fig. 3f) and individual complex I molecules could be identified (Fig. 3d) and picked for image processing. These are typical images and could be reproducibly obtained by using Quantifoil grids.

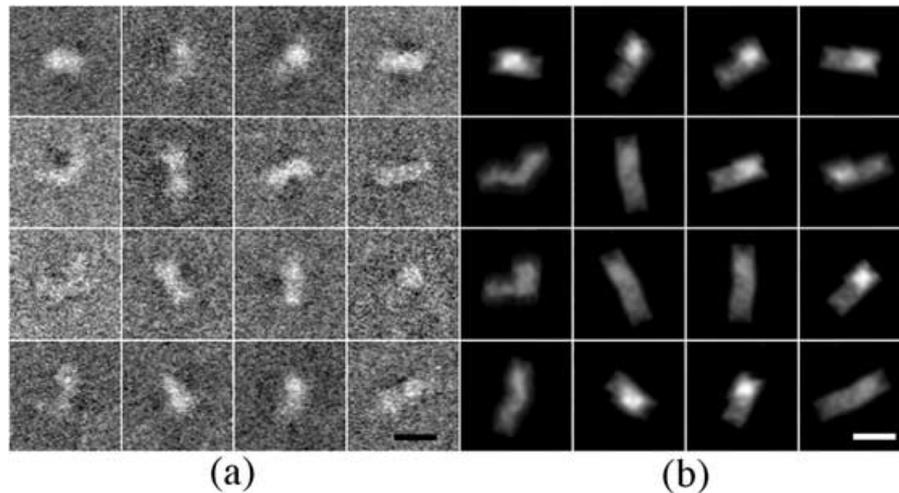


Fig. 4. Averaged images. (a) The top 16 class averages of cryo-EM images for the full set of complex I/A8-35 particles. (b) Corresponding 2D projections of the 3D model from negative staining (from Guénebaut *et al.*, 1998); scale bars 20 nm. The projections are sampled at a pixel size of ~ 0.4 nm.

The individual images of single particles were too noisy to identify any details, so an initial correspondence analysis and averaging were carried out in order to establish whether reproducible features were present. A total of 1200 particles were selected, normalized and subjected to multivariate analysis (van Heel and Frank, 1981). The top 16 class averages which, in total, included about 750 particles are shown in Fig. 4a. The class average projections were then cross-correlated with a set of angular projections of a 3D model taken from the reconstruction of negatively stained complex I (Guénebaut *et al.*, 1997). The matching projections of the model are shown in Fig. 4b. There is good agreement between the class average projections and the corresponding projections of the L-shaped model, which gave us the confidence to use the latter as a reference

for the angular reconstitution method (Serysheva *et al.*, 1995).

A 3D reconstruction by this method, using the full data set of 1200 particles, was then carried out. As a reference to determine the tilt angles for the individual images, we used 3248 projections of the 2.8-nm resolution 3D reconstruction made from negatively stained complex I (Guénebaut *et al.*, 1998; Fig. 5c). These were cross-correlated with the single-particle images to obtain a set of projections at known Euler angles, which were then combined by weighted back-projection to give a 3D model (Fig. 5a). The resolution estimated by Fourier shell correlation (Saxton and Baumeister, 1982) for reconstructions from two half data sets was 5.8 nm. We also made a low-pass filtered model of the negatively stained reconstruction

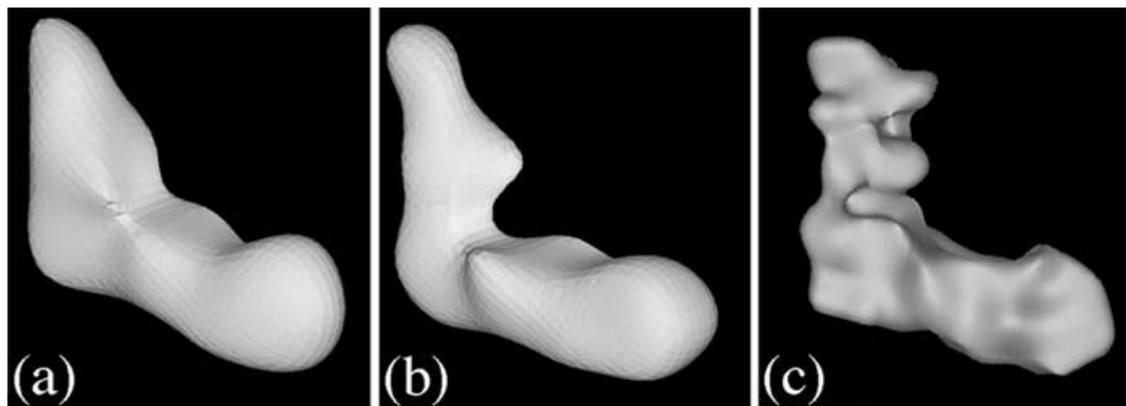


Fig. 5. Reconstructed images. (a) 3D reconstruction of unstained complex I stabilized by amphipol A8-35 and imaged in ice. (b) 3D reconstruction of negatively stained complex I in detergent (from Guénebaut *et al.*, 1998) with the corresponding resolution cutoff (6 nm). (c) 3D reconstruction shown in (b) at 2.8-nm resolution; scale bar 10 nm.

(Guénebaut *et al.*, 1998) at 6 nm for comparison (Fig. 5b).

Discussion

Using mitochondrial complex I as a test specimen for exploring the use of amphipols in cryo-EM, it has been possible to obtain a model for the unstained enzyme which, although at lower resolution, preserves the essential features seen in stained reconstructions. At this resolution, there does not appear to be any structural change induced by the amphipol. How thick a layer amphipols form over the transmembrane surface of the membrane proteins they stabilize is not known accurately yet. However, small-angle neutron-scattering measurements (Gohon *et al.*, unpublished data) and molecular sieving (Zoonens, 2004; Zoonens *et al.*, 2007) suggest that it is unlikely to exceed 1.5–2 nm. By analogy with free amphipol particles (Gohon *et al.*, 2006), this layer is likely to be significantly hydrated, which would lower its electron density. Amphipols may thus contribute some extra density to the surface of the reconstruction, which could differ somewhat from that contributed by detergents, but this is likely to be weak and confined to a relatively thin surface layer. Larger aggregates of amphipols randomly bound to the protein surface, should there be any, would tend to be averaged out in a large set of particles.

The lower resolution of the 3D reconstruction obtained in this exploratory study, as compared with that obtained previously by negative staining, can be explained partly by the smaller number of particles sampled. A greater number of particles may have improved the resolution. However, a more important factor may have been the inherent flexibility of the structure of complex I. A hinge movement of the two arms relative to each other was recognized in the early negative staining work and has recently been studied in more detail by Radermacher *et al.* (2006). Because we did not take this into account when carrying out the reconstruction from the cryo-EM images in amphipols, it is likely that some 'blurring' of the structure may have taken place. The membrane arm of complex I having the greater contrast, both in negatively stained and cryo-EM images, tends to dominate the alignment. The result is that the transmembrane arm is very similar in shape and size to that obtained in negative stain, whereas the cytoplasmic arm is reduced.

Why the use of amphipols results in more uniform ice films and, therefore, a more even distribution of particles compared to the use of detergents deserves a closer examination. Above its critical aggregation concentration, and at equilibrium, A8-35 decreases the surface tension of water to about the same extent as a detergent like DDM (A. Kumpulainen, E. Blomberg and P. Claesson, personal communication). It seems unlikely that the procedure used in the present work to remove

free amphipol was efficient enough for its concentration to drop below the critical aggregation concentration, which is lower than 0.1 g.L^{-1} (unpublished data). A huge difference in equilibrium surface tension therefore is unlikely to account for the formation of thicker films. However, amphiphatic polymers self-organize at the air/water interface with a significantly slower rate than detergents or lipids. It is thus possible that the increased thickness result from a kinetic phenomenon. A close analogue of A8-35, amphipol A8-75 (see Tribet *et al.*, 1996), has been used to form centimetric Newton films (free-standing, air-exposed surfactant bilayers of thickness below 20 nm), which are macroscopic equivalents of those that form in the holes of an EM carbon film. At an ionic strength comparable to that of the buffer used in the present work, these films reach an equilibrium thickness of <10 nm (see Millet *et al.*, 2001, where A8-75 is denoted '5.8 25C8Na'). It is therefore far from certain that, despite the small diameter of the holes ($\sim 5 \mu\text{m}$), the equilibrium thickness of the EM films be reached within the short time ($\sim 2 \text{ s}$) that elapses between blotting the excess fluid from the grids and freezing them. Whatever the underlying mechanism(s), the end result is, however, the production of more uniform ice films, better suited to single-particle studies.

Membrane proteins extracted from their original environment and kept in detergent solutions are exposed to a dissociating environment. This generally results, more or less rapidly, in their losing subunits, lipids or cofactors, in their quaternary and tertiary structures being affected, and in their biological function being lost or altered (for discussions, see e.g. Breyton *et al.*, 1997; Bowie, 2001; Garavito and Ferguson-Miller, 2001; Gohon and Popot, 2003). This problem is particularly severe when the forces that hold together transmembrane structures are weak, as can be the case for large supramolecular structures made up of several membrane proteins. Amphipols were developed with the aim of providing membrane proteins with a less aggressive environment. They are likely to help stabilizing such fragile superstructures, which cryo-EM is particularly well suited to investigating. The present results suggest that, due to their dual characteristics of being less dissociating than detergents and of enabling easier cryo-EM data collection, amphipols will become useful tools in this important area of membrane structural biology.

Acknowledgments

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