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High-Resolution Structure of a Membrane Protein Transferred from Amphipol to a Lipidic Mesophase

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Abstract Amphipols (APols) have become important tools for the stabilization, folding, and in vitro structural and functional studies of membrane proteins (MPs). Direct crystallization of MPs solubilized in APols would be of high importance for structural biology. However, despite considerable efforts, it is still not clear whether MP/APol complexes can form well-ordered crystals suitable for X-ray crystallography. In the present work, we show that an APol-trapped MP can be crystallized in meso. Bacteriorhodopsin (BR) trapped by APol A8-35 was mixed with a lipidic mesophase, and crystallization was induced by adding a precipitant. The crystals diffract beyond 2 Å. The structure of BR was solved to 2 Å and found to be indistinguishable from previous structures obtained after transfer from detergent solutions. We suggest the proposed protocol of in meso crystallization to be generally applicable to APol-trapped MPs.

Keywords Amphipol · Membrane protein crystallization · Bacteriorhodopsin · Monoolein · In meso crystallization

Introduction

Membrane proteins (MPs) are among major targets of structural biology. Serving for energy and sensory transduction, ion and solute transport, etc., they carry out the main functions of biological membranes. MPs represent roughly one-third of the proteins encoded in the human genome (Krogh et al. 2001), and about 60 % of all modern drugs have a MP as their target (Overington et al. 2006).

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Despite the high importance of MPs, studying their structure and function in vitro is challenging, because most of them quickly lose their functionality following their extraction from biological membranes. Indeed, detergents, which are traditionally used to render MPs water soluble by adsorbing onto their hydrophobic transmembrane surface, tend to inactivate them. Amphipols (APols) have been developed as milder substitutes to detergents (Tribet et al. 1996). APols are amphipathic polymers consisting of a relatively short main chain carrying interspersed hydrophobic and hydrophilic groups (reviewed by Popot et al. 2011; Zoonens and Popot 2014). Most MPs become significantly more stable, often dramatically so, following the substitution of APols to detergents (Popot 2010; Popot et al. 2003, 2011). This is thought to be due to a combination of several factors, among which i) the low critical association concentration (CAC) of APols ($\sim$0.002 g L^{-1} for APol A8-35; Giusti et al. 2012), which makes it possible to work at very low concentration of free surfactant, reducing the hydrophobic sink effect (dilution by the surfactant of hydrophobic and amphipathic cofactors and lipids); ii) their poorly dissociating character (responsible for their low detergency), which limits competition with stabilizing protein/protein and protein/lipid interactions (see, e.g., Popot et al. 2003; Dahmane et al. 2013; Marie et al. 2014); and iii) a probable damping effect on conformational excursions of MP transmembrane domains (Popot et al. 2003; Picard et al. 2006; Perlmutter et al. 2011, 2014; for general discussions, see Popot 2010; Popot et al. 2011; Kleinschmidt and Popot 2014). Whereas APols remain strongly bound to MPs in the absence of competing surfactants (Zoonens et al. 2007; Tribet et al. 2009), they are easily displaced by an excess of detergent, other APols, or lipids (Tribet et al. 1997, 2009; Nagy et al. 2001; Pocanschi et al. 2006; Zoonens et al. 2007; Damian et al. 2012). Over the years, APols have developed into precious tools for MP studies, including folding, cell-free synthesis, immobilization and ligand screening, NMR spectroscopy and electron microscopy, proteomics, and many other techniques (Popot et al. 2011; Zoonens and Popot 2014). Crystallization of MP/APol complexes, however, has remained an elusive goal (Popot et al. 2011; Charvolin et al. 2014).

One of the best investigated examples of MP trapping and stabilization by APols is that of bacteriorhodopsin (BR), the light-driven proton pump from *Halobacterium salinarum*. In brief, BR can be trapped in APols from its native state as a BR/lipid/detergent complex (Tribet et al. 1996; Gohon et al. 2008; Bazzacco et al. 2012; Dahmane et al. 2013), renatured by transfer from sodium dodecylsulfate to various APols either in the presence or absence of lipids (Pocanschi et al. 2006; Bazzacco et al. 2012; Dahmane et al. 2013), or synthesized in vitro in the presence of non-ionic APols (Bazzacco et al. 2012). In the course of these works, it was noted that archaebacterial lipids displaced by detergents rebind to BR upon its transfer to APols, with functional consequences on the photocycle (Gohon et al. 2008; Dahmane et al. 2013). APol-assisted folding has been extended to many other MPs, among which the pharmacologically important G protein-coupled receptors (Dahmane et al. 2009; Banères et al. 2011; Bazzacco et al. 2012; Damian et al. 2012; Kleinschmidt and Popot 2014; Mary et al. 2014). BR is well known as a pilot protein for MP studies and was instrumental in the development of new crystallization approaches involving different lipidic mesophases (Landau and Rosenbusch 1996; Takeda et al. 1998; Faham and Bowie 2002). In the present work, we used complexes between BR and an anionic APol, A8-35 (Tribet et al. 1996), to develop an approach for crystallizing APol-trapped MPs by direct transfer to a lipidic mesophase. The resulting crystals were of high quality and yielded a 2 Å-resolution structure.

Materials and Methods

Chemicals

n-Octyl-β-D-glucopyranoside (OG), potassium dihydrogen phosphate (KH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma Aldrich (France). Bio-Beads SM–2 adsorbent was obtained from Bio-Rad (France). Monoolein (1-monooleoyl-*rac*-glycerol, MO) was purchased from Nu-Chek Prep (USA). Amphipol A8-35 was synthesized by F. Giusti (UMR 7099) as described in refs. (Gohon et al. 2004, 2006).

Purple Membrane Purification and BR Solubilization

Purple membranes were extracted from *H. salinarum* S9 (Oesterhelt and Stoeckenius 1974), and purified and solubilized in OG as described in detail in refs. (Gordeliy et al. 2003; Borshchevskiy et al. 2011), except that 20 mM Na_2HPO_4/KH_2PO_4 (Na/K-P_i) buffer, pH 7.1, was used as a solubilization buffer instead of 20 mM Na/K-P_i buffer, pH 6.9.

The concentration of solubilized BR was estimated by UV–visible absorption spectroscopy using $\varepsilon_{554} = 47\ mM^{-1}\ cm^{-1}$ and $\varepsilon_{280} = 81\ mM^{-1}\ cm^{-1}$ (London and Khorana 1982; Gohon et al. 2008) and was typically equal to 2.5 g L^{-1}.

Preparation of BR/A8-35 Complexes for Crystallization

BR/A8-35 complexes were prepared as described in ref. (Gohon et al. 2008). A8-35 from a 10 % w/w stock solution

in water was added to BR solubilized in OG at 5:1 w/w APol/BR. After 15 min, detergent adsorption onto Bio-Beads SM-2 (10 g per g OG) was carried out for 2 h under gentle stirring, after which the Bio-Beads were removed. The concentration of BR was estimated by UV–visible absorption and typically was equal to 2–2.5 g L^{-1}. The solution was concentrated to 30 g L^{-1} by 15 min centrifugation at 3,000×*g* in a 100-kDa cut-off centrifugal filter (Vivaspin 500, Vivasciences, USA). Residual OG was estimated by Fourier transform infrared (FTIR) spectroscopy (*cf.* daCosta and Baenziger 2003) to be ~1 g L^{-1} (i.e., ~0.7 % of the mass of APol) with a Vertex 70 spectrophotometer (Bruker, Germany). The concentrated solution was used for crystallization attempts in the presence of an excess of APol.

According to ref. (Gohon et al. 2008), the weight ratio of A8-35/BR in the complexes is ca. 2:1, whereas to prepare them A8-35 is added to OG-solubilized BR in a weight ratio of 5:1. The concentration of A8-35 can be determined by FTIR spectroscopy in the wavelength range 400–3,000 cm^{-1}. To remove the excess of free unbound A8-35, the following procedure was carried out (*cf.* ref. (Gohon et al. 2008)). The FTIR absorption spectrum of the 30 g L^{-1} BR/A8-35 solution was measured, after which the solution was diluted 5× with 20 mM Na/K-P_i buffer, pH 7.1, and again concentrated to 30 g L^{-1} by 10 min of centrifugation at 3,000×*g* in the 100 kDa cut-off centrifugal filter. This dilution-concentration step was repeated ~4× until there were no detectable changes in the FTIR spectrum of the concentrated solution, indicating that unbound APol molecules had been eliminated from the solution. The final solution at 30 g L^{-1} BR concentration was used for crystallization with no excess of free APol.

Size-Exclusion Chromatography

The 0.5-mL BR/A8-35 samples, taken before and after the removal of unbound APol as described above, were injected into a Superdex 200 Increase 10/300 GL column (GE Healthcare Life Sciences, USA) driven by an Äkta Pure chromatographic system (GE Healthcare Life Sciences, USA). The column was developed in 20 mM Na/K-P_i buffer, pH 7.1 at a flow rate 0.75 mL min^{-1} at 8 °C, and BR was detected at 550 nm.

Crystallization

MO was melted at ~40 °C during 20 min and then mixed with a buffer solution using two gas–tight syringes (Hamilton, USA) (Cheng et al. 1998). The resulting phase was then mixed with the APol-trapped BR concentrated at 30 g L^{-1} in different volume ratios by two gas-tight syringes used for crystallization trials. Dispensing the resulting phases by 100 nL drops on the 96-well LCP Sandwich Set glass plates (Marienfeld, Germany) and adding 800 nL of the precipitant solution to each drop of the lipidic mesophase were performed by an NT8 Formulatrix (Waltham, MA) crystallization robot. Na/K-P_i, pH 5.6 solutions at concentrations from 0.8 to 2 M were used as precipitant. Hexagonal plate-like BR crystals of $P6_3$ symmetry were observed within 7–21 days after the precipitant was added. The lipidic mesophase that yielded the best-diffracting crystals was prepared by mixing MO, buffer, and the 30 g L^{-1} BR solution in a 1:0.25:0.75 volume ratio.

Small-Angle X-ray Scattering Experiments

To study the effects of A8-35 on the lipidic cubic phase, small-angle X-ray scattering (SAXS) measurements were performed on a SAXS pinhole camera system S-MAX3000 (Rigaku, Japan) equipped with a microfocus rotating Cu-anode X-ray generator MicroMax-007HF (Rigaku, Japan), as described in ref. (Joseph et al. 2011). The APol concentration in the lipidic cubic phase was varied from 0 to 7 % w/w with respect to MO (which corresponds to APol contents in crystallization trials), with other parameters held close to crystallization ones (the phase composed of MO, and a buffer was covered with the 1.5 M Na/K–P_i pH 5.6 precipitant).

X-Ray Data Acquisition and Treatment

X-ray diffraction data were collected at beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France, using a PILATUS 6 M detector, at the wavelength of 0.972 Å. The dataset consisted of 1,250 images collected at 74 % beam transmission and 0.04-s exposure time, with oscillation of 0.1°. Diffraction patterns were processed using the MOSFLM (Leslie and Powell 2007) and SCALA softwares from the CCP4 program suite (Winn et al. 2011). The best-diffracting crystal was perfectly twinned (twin fraction 50 %). Diffraction data were collected to the resolution of 1.945 Å and integrated to 2.0 Å. The diffraction signal was slightly weaker along unit cell vector *c* ($F/\sigma F$ of 4 at ~2.36 Å) as compared to the diffraction along unit cell vectors *a* and *b* ($F/\sigma F$ of 4 at ~1.96 Å). The diffraction anisotropy was assessed using the truncate program from the CCP4 program suite (Winn et al. 2011).

Structure Determination and Refinement

To avoid bias, initial phases were obtained by a molecular replacement method using MOLREP (Vagin and Teplyakov 2010) and a poly-alanine model derived from PDB ID

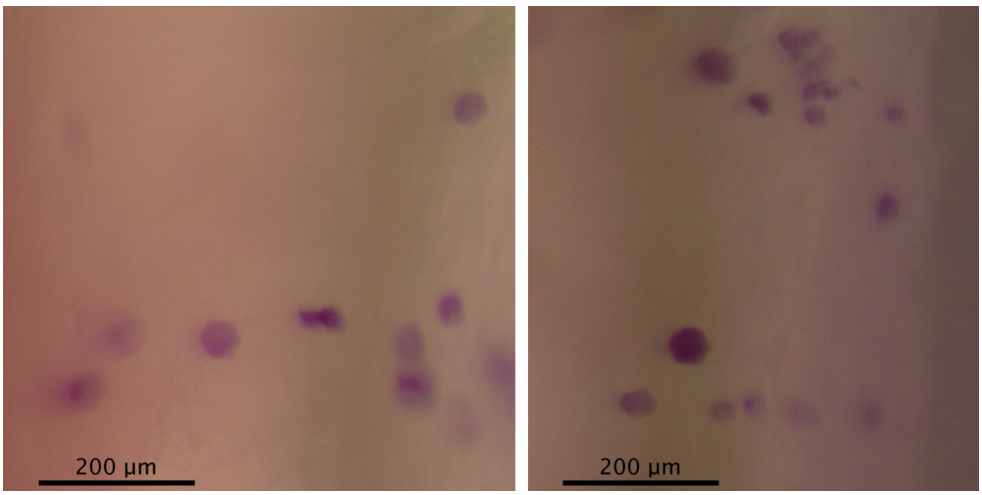

Fig. 1 Crystals of A8-35-trapped BR grown in meso following transfer of BR/A8-35 complexes in the absence of an excess of APol

Table 1 Data collection and refinement statistics

Data collection	
Space group	P63
Cell dimensions	
a, *b*, *c* (Å)	60.999, 60.999, 108.174
α, *β*, *γ* (°)	90, 90, 120
Resolution (Å)	52.83–2.00 (2.11–2.00)*
R_{merge} (%)	12.1 (81.4)
$I/\sigma I$	9.7 (2.2)
Completeness (%)	99.9 (100.0)
Redundancy	6.4 (6.5)
Refinement	
Resolution (Å)	52.83–2.00
No. reflections	15418
R_{work}/R_{free}	17.9/20.9 %
No. atoms	
Protein	1661
Retinal	20
Water	14
B-factors (Å^2)	
Protein	20.7
Ligand/ion	13.7
Water	19.1
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.5

* Highest resolution shell is shown in parenthesis

1C3W (Luecke et al. 1999). The resulting model was extended using the automated model building software ARP/wARP (Langer et al. 2008). After that the model was iteratively corrected in Coot (Emsley and Cowtan 2004) and refined using intensity-based twin refinement with the REFMAC5 (Murshudov et al. 2011) and PHENIX software suites (Adams et al. 2010). The final model includes the protein atoms, the covalently bound retinal, and ordered water molecules. The lipid tails were not added to the model, and the electron densities presented in Fig. 4c were calculated in their absence. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (Berman et al. 2000), PDB ID code 4OV0.

Results and Discussion

Crystallization of A8-35-trapped BR

Following solubilization of *H. salinarum* purple membrane with OG, BR was trapped in A8-35 using an $\sim 2.5\times$ excess of APol over the amount that actually binds to the protein (Gohon et al. 2008). Two different kinds of crystallization experiments were then carried out, depending on whether unbound APol was removed or not before mixing the sample with monoolein. Crystallization in the presence of an excess of APol did not provide high-quality crystals. On the contrary, after most of the free APol had been removed by several cycles of dilution/ultracentrifugation, well-shaped crystals of $P6_3$ symmetry were obtained (Fig. 1), which diffracted beyond 2.0 Å (Table 1). The crystals were of type I (Michel 1989), that is formed of stacked planar layers, each layer resembling the native purple membrane, where BR trimers are organized in a hexagonal 2D crystalline lattice (Henderson and Unwin 1975).

The crystallization conditions for APol-trapped BR are nearly the same as those used for crystallizing in meso BR solubilized in OG. This suggests that at least up to a certain concentration, the presence of A8-35 in the mesophase

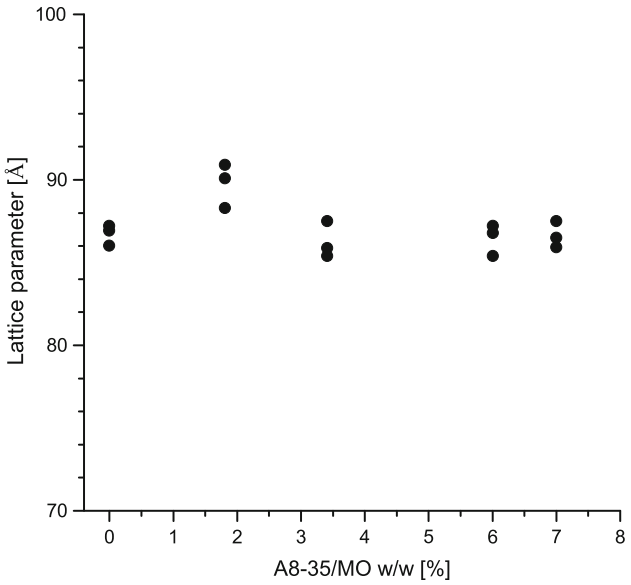

Fig. 2 Cubic–Pn3m phase lattice parameter variation with A8-35 concentration in MO/APol/buffer (1.5 M Na/K–P_i, pH 5.6) system. Three independent experiments were performed at each APol/MO ratio

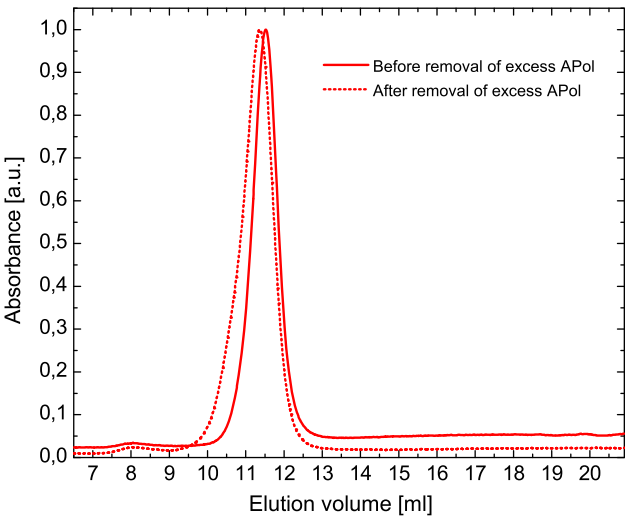

Fig. 3 Size-exclusion chromatography elution profiles at 550 nm of BR/APol A8-35 complexes before (*solid line*) and after removal of free APol A8-35 (*dotted line*). The two profiles have been normalized to the same maximum

matrix does not interfere with MP crystallization. This view is supported by the fact that, according to SAXS data, MO/APol/buffer (1.5 M Na/K-P_i, pH 5.6) systems form a cubic phase with Pn3 m symmetry (Lindblom and Rilfors 1989), and no significant lattice parameters changes are observed upon increasing the APol/MO ratio up to 7 % w/w with respect to MO (Fig. 2). Our best crystals were obtained at an APol/MO ratio of ~5%. Increasing the APol/MO ratio above 7 % induces a transition to a cubic phase with Ia3d symmetry and then the phase transforms to a lamellar phase at mass ratios higher than 20 % (Sintsov et al. preliminary results). This is likely related to the reason why no high-quality BR crystals were observed in the presence of an excess of APol. Indeed, under the conditions used, the APol/MO mass ratio was around 12 %.

It is worth mentioning that upon removal of free APol, MP/APol complexes, including BR/A8-35 ones, have been observed to self-aggregate into small oligomers (Zoonens et al. 2007) or vermiculate arrangements (Gohon et al. 2008; Arunmanee et al. 2014). Indeed, after removal of free APol, the main peak observed in the size-exclusion chromatography elution profile of the BR/A8-35 complexes becomes slightly broader and elutes slightly earlier than before removal (Fig. 3). This behavior is similar to that observed with tOmpA/A8-35 complexes (Zoonens et al., 2007). It indicates that small oligomers have formed, as well as a very small amount of larger aggregates, which elute in the void volume. At the time of mixing with MO, BR/A8-35 complexes depleted of free APol therefore are not homogeneous, which does not prevent them from subsequently reorganizing into well-ordered 3D crystals once inserted into the lipidic mesophase. Similarly, in the presence of lipopolysaccharide, OmpF/A8-35 filaments can evolve into 2D crystals (Arunmanee et al. 2014).

Crystal Structure of BR Crystallized From the APol-Trapped State

The structure of BR crystallized from APol-trapped complexes was solved to 2.0 Å (Fig. 4 and Table 1). Besides the atoms of the protein, the structure reveals the covalently bound *all-trans*-retinal (Fig. 4a). The cluster of hydrogen-bonded water molecules (W401, W402, and W406) close to the retinal's Schiff base is easily discernible (Fig. 4b). These features are absolutely identical to those observed in the crystals grown by the traditional in cubo technique, starting from BR in detergent solution (Luecke et al. 1999; Lanyi 2004). Apart from the electron densities corresponding to BR or ordered water molecules, some residual densities are seen in the lipid tail region. The position of these densities corresponds closely to that of the lipid molecules recognized at higher resolution (Luecke et al. 1999). Typical densities along the extracellular side of helices E, F, and G are shown in Fig. 4c. We assume that in our crystals these densities also correspond to the lipids and not to A8-35 molecules. This is consistent with the observation that purple membrane lipids initially solubilized along with BR are present in BR/A8-35 complexes (Gohon et al. 2008) and contribute to modulating the photocycle of BR (Dahmane et al. 2013). Their presence is probably essential to the formation of the crystals.

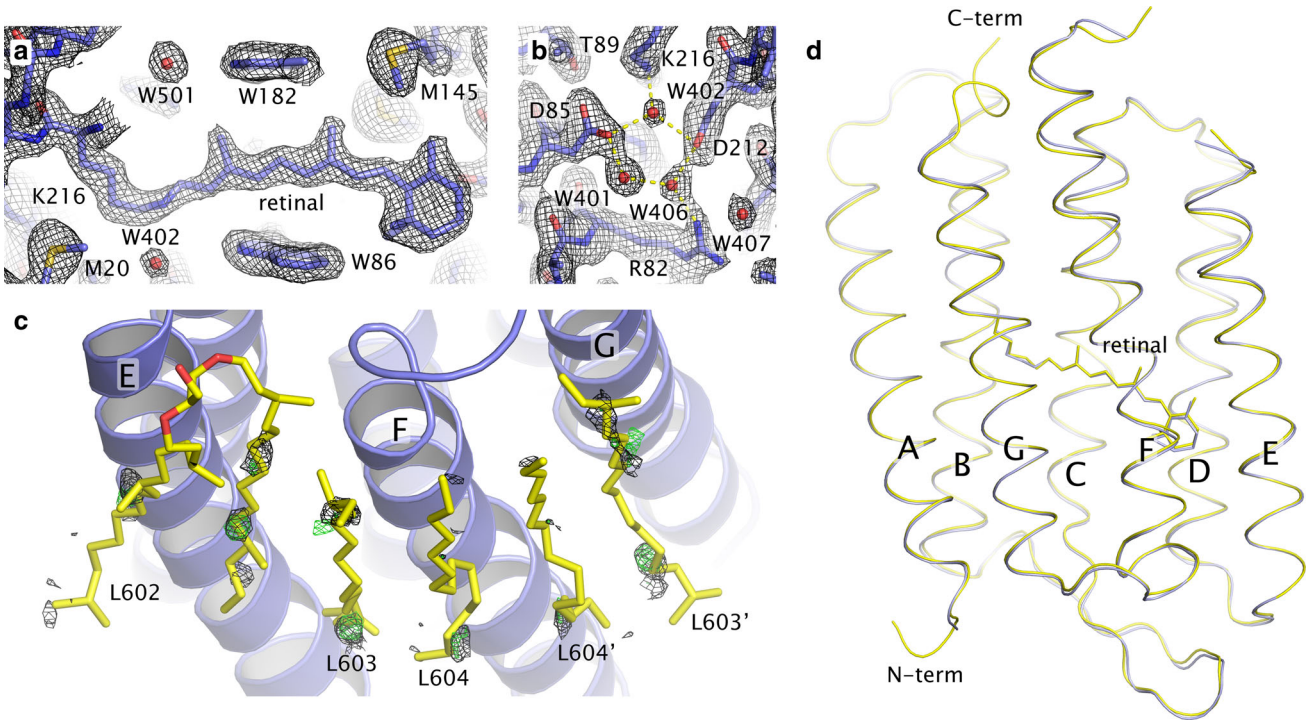

Fig. 4 Structure of BR in crystals grown from BR/A8-35 complexes. **a.** $2F_o$–F_c electron density map around the retinal drawn at the level of 1.5 σ. **b.** Position of the water molecules close to the retinal's Schiff base. The configuration is identical to that observed in the crystals grown by traditional *in meso* technique. **c.** Residual $2F_o$–F_c (*black*) and F_o–F_c (*green*) electron densities in the lipid tail region at the levels of 1 and 3 σ, respectively. The F_c coefficients and phases were calculated in the absence of any lipid molecules in the model. The densities are overlaid with the lipid molecule positions from a higher resolution reference structure (*yellow*, PDB ID 1C3W (Luecke et al. 1999)). **d.** Comparison of the overall BR crystal structure obtained from BR/A8-35 complexes (*blue*) with the structure observed in the crystals grown by traditional *in meso* technique (*yellow*, PDB ID 1C3W (Luecke et al. 1999)). The structures are essentially identical. The root mean squared deviation (RMSD) of atomic positions is 0.86 Å for all the atoms and 0.38 Å for the backbone atoms (Color figure online)

Apart from the aforementioned, no unaccounted densities are observed. Thus, it can be concluded that molecules of A8-35 are not present in the crystals grown from APol-trapped BR, or, if they are, that they are significantly disordered. Taking into account that the average molecular weight of A8-35 is ~4.3 kDa (Giusti et al. 2014) and that the packing of BR molecules in type I crystals is very tight (Pebay-Peyroula et al. 1997; Luecke et al. 1999), the presence of APol molecules seems highly unlikely. Indeed, the lattice constants are essentially the same for the crystals obtained from BR/APol complexes (61.0, 61.0, and 108.2 Å) and from detergent-solubilized BR (61.0, 61.0, and 110.0 Å; Borshchevskiy et al. 2011). It has been shown before that MPs can be transferred to lipid bilayers upon exposure of MP/APol complexes either to lipid vesicles (Nagy et al. 2001) or to black films (Pocanschi et al. 2006). It seems safe to assume that upon mixing BR/A8-35 complexes with monoolein, the APol dissociates from BR and dissolves in the mesophase, possibly adsorbing at the interface between water channels and lipid bilayer. Then, during the formation of the crystal, MP molecules and lipids likely diffuse in the lipid bilayer and assemble into crystals without interference by the APol. The high electrostatic charge density of A8-35 therefore cannot oppose the formation of protein crystal contacts, as is suspected to be the case during conventional vapor diffusion crystallization of MP/A8-35 complexes (Popot et al. 2011; Charvolin et al. 2014) .

Conclusions

Using the in meso crystallization approach, we have crystallized an APol-trapped MP. The crystals obtained are of a high X-ray diffracting quality, suitable for high-resolution protein structure determination. The lipid bilayer of the crystallization matrix likely displaces A8-35 molecules from the transmembrane surface of the protein and dissolves them. Thereby, they do not interfere with crystallization. The crystals obtained from A8-35-trapped BR diffract to high resolution, and the structure was solved to 2 Å. There is essentially no difference between BR structures obtained from these crystals and from crystals grown from the protein solubilized in detergents (Fig. 4d).

The approach to MP crystallization explored in the present work could well be very general. Indeed, on the one hand, crystallization in meso has proven applicable to a large variety of MPs (Cherezov 2011; Caffrey 2011). On the other, APols have proven very useful both to stabilize MPs in aqueous solution (reviewed in Popot et al. 2011) and to produce them, either by assisting their folding from a denatured state, such as that obtained by solubilization of inclusion bodies (Dahmane et al. 2009, 2013; Banères et al. 2011; Bazzacco et al. 2012; Leney et al. 2012; Pocanschi et al. 2013; Mary et al. 2014), or by acting as the host medium during cell-free synthesis (Bazzacco et al. 2012). Combining APol-assisted production and/or stabilization of MPs with in meso crystallization offers a promising new route to MP crystallization.

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