J Membrane Biol (2014) 247:981–996
DOI 10.1007/s00232-014-9694-4

Solution Behavior and Crystallization of Cytochrome bc_1 in the Presence of Amphipols

Delphine Charvolin · Martin Picard · Li-Shar Huang · Edward A. Berry · Jean-Luc Popot

Received: 22 January 2014 / Accepted: 26 May 2014 / Published online: 19 June 2014

Abstract Detergents classically are used to keep membrane proteins soluble in aqueous solutions, but they tend to destabilize them. This problem can be largely alleviated thanks to the use of amphipols (APols), small amphipathic polymers designed to substitute for detergents. APols adsorb at the surface of the transmembrane region of membrane proteins, keeping them water-soluble while stabilizing them bio-chemically. Membrane protein/APol complexes have proven, however, difficult to crystallize. In this study, the composition and solution properties of complexes formed between mitochondrial cytochrome bc_1 and A8-35, the most extensively used APol to date, have been studied by means of size exclusion chromatography, sucrose gradient sedimentation, and small-angle neutron scattering. Stable, monodisperse preparations of bc_1/A8-35 complexes can be obtained, which, depending on the medium, undergo either repulsive or attractive interactions. Under crystallization conditions, diffracting three-dimensional crystals of A8-35-stabilized cytochrome bc_1 formed, but only in the concomitant presence of APol and detergent.

Keywords Membrane proteins · Surfactants · Amphipathic polymers · Stability · Small-angle neutron scattering · Size exclusion chromatography

Abbreviations

A8-35	A specific type of amphipol (Tribet et al. 1996)
APol	Amphipol
AUC	Analytical ultracentrifugation
BR	Bacteriorhodopsin
C_8E_4	Tetraethylene glycol monooctyl ether
Cmc	Critical micellar concentration
CMP	Contrast match point
DDM	Dodecyl-β-D-maltoside
EDTA	Ethylene-diamine-tetra-acetic acid
EM	Electron microscopy
$FAPol_{NBD}$	Fluorescent (NBD-labeled) A8-35
KMES	2-Morpholino-ethane sulfonic acid potassium salt
MD	Molecular dynamics
MW	Molecular weight
MWCO	Molecular weight cutoff
NBD	7-nitrobenz-2-oxa-1,3-diazol-4-yl
NMR	Nuclear magnetic resonance
βog	Octyl-β-D-glucopyranoside
OmpX	Outer membrane protein X from *Escherichia coli*
PEG4k	4 kDa polyethylene glycol
SANS and SAXS	Small-angle neutron and X-ray scattering, respectively
SEC	Size exclusion chromatography

Research article destined to the special issue of *J. Membr. Biol.* on amphipols.

D. Charvolin · M. Picard · J.-L. Popot
UMR 7099, Centre National de la Recherche Scientifique/ Université Paris-7, Institut de Biologie Physico-Chimique, FRC 550, 13, rue Pierre-et-Marie-Curie, 75005 Paris, France

Present Address:
M. Picard (✉)
Faculté de Pharmacie, Laboratoire de Cristallographie et RMN Biologiques, Centre National de la Recherche Scientifique/ Université Paris Descartes UMR 8015, Sorbonne Paris Cité, 75006 Paris, France
e-mail: martin.picard@parisdescartes.fr

L.-S. Huang · E. A. Berry
Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse 13210, NY, USA

SERCA1a	Sarcoplasmic reticulum calcium pump from fast twitch muscle
tOmpA	Transmembrane domain of outer membrane protein A from *E. coli*
Tris	*Tris*-hydroxymethyl-amino-methane

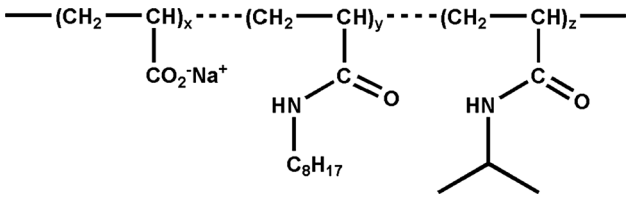

Fig. 1 Chemical structure of amphipol A8-35. $\langle x \rangle \approx 35$ %, $\langle y \rangle \approx 25$ %, $\langle z \rangle \approx 40$ %. From Ref. Tribet et al. (1996)

Introduction

Amphipols (APols) are small amphipathic polymers that can replace detergents at the transmembrane surface of solubilized membrane proteins (MPs) (Popot 2010; Popot et al. 2011; Tribet et al. 1996; Zoonens and Popot 2014). MPs are, as a rule, much more stable once trapped by APols than they are in detergent solutions (see e.g., Champeil et al. 2000; Dahmane et al. 2013; Feinstein et al. 2014; Pocanschi et al. 2013; Popot et al. 2011; Tifrea et al. 2011; Zoonens and Popot 2014). APols can also be used to fold to their native state MPs that have been either denatured (Dahmane et al. 2013; Pocanschi et al. 2006, 2013) or expressed under a non-native form (Bazzacco et al. 2012; Dahmane et al. 2009, 2011; Etzkorn et al. 2013; Leney et al. 2012), as well as to synthesize MPs in vitro (Bazzacco et al. 2012). APol-trapped MPs can be studied by most biochemical and biophysical approaches, e.g., nuclear magnetic resonance (NMR, reviewed by Planchard et al. 2014) or electron microscopy (EM) (Althoff et al. 2011; Arunmanee et al. 2014; Cvetkov et al. 2011; Flötenmeyer et al. 2007; Gohon et al. 2008; Huynh et al. 2014), while benefiting from the stabilizing effect of APols. The usefulness of APols as a tool for EM single-particle studies of MPs has recently been illustrated by the publication of the 3.4 Å resolution structure of a mammalian ion channel (Cao et al. 2013; Liao et al. 2013, 2014). Crystallizing MP/APol complexes, however, has remained a highly attractive but elusive goal.

In aqueous solutions, APols spontaneously assemble into small, well-defined globular particles resembling detergent micelles (Giusti et al. 2012; Gohon et al. 2006; Perlmutter et al. 2011). For A8-35 (Fig. 1), the best characterized APol to date, these particles have an average mass of ~40 kDa (Gohon et al. 2006) and comprise, on average, 9–10 A8-35 molecules (Giusti et al. 2014). The composition, dynamics, and structural and functional properties of MP/A8-35 complexes have been studied in particular detail for bacteriorhodopsin (BR) (Dahmane et al. 2013; Etzkorn et al. 2013; Gohon et al. 2008), the transmembrane domain of *Escherichia coli*'s outer membrane protein A (tOmpA) (Zoonens et al. 2005, 2007), *E. coli*'s OmpX (Catoire et al. 2009, 2010; Etzkorn et al. 2014; Perlmutter et al. 2014), and the sarcoplasmic reticulum calcium ATPase from fast twitch muscle (SERCA1a) (Champeil et al. 2000; Picard et al. 2006). In short, these studies show that well-defined complexes are formed, which, however, tend to be slightly larger than MP/detergent complexes (Catoire et al. 2010; Champeil et al. 2000; Gohon et al. 2008; Zoonens et al. 2005, 2007). NMR, EM, analytical ultracentrifugation (AUC), small-angle neutron scattering (SANS), size exclusion chromatography (SEC), and molecular dynamics (MD) studies indicate that APols specifically associate with the transmembrane surface of the protein, where they form a compact amphipathic layer providing the interface with the solution (Althoff et al. 2011; Catoire et al. 2009, 2010; Etzkorn et al. 2014; Gohon et al. 2008; Huynh et al. 2014; Liao et al. 2014; Perlmutter et al. 2014; Zoonens et al. 2005). The thickness of this layer, 1.5–2 nm, is only slightly larger than that of a detergent layer (Althoff et al. 2011; Gohon et al. 2008). SEC studies, however, indicate that the size distribution of tOmpA/A8-35 complexes is broader than that of tOmpA/detergent ones (Zoonens et al. 2007). In the simultaneous presence of APols and detergents, mixed APol/detergent particles and ternary MP/APol/detergent complexes form, the composition of the surfactant mixture being similar around the protein and in protein-free particles (Tribet et al. 2009; Zoonens et al. 2007). The biochemical stability of SERCA1a trapped in such ternary complexes is intermediate between that in pure detergent and that in pure A8-35 (Champeil et al. 2000). Of particular relevance this study, SEC measurements indicate that the Stokes radius (R_S) distribution of ternary tOmpA/A8-35/detergent complexes is narrower than that of tOmpA/A8-35 ones, even though their average value is comparable (Zoonens et al. 2007).

From a crystallographer's point of view, the situation can be summarized as follows. On the one hand, the stabilizing effect of APols, which is often considerable and is observed even in the presence of detergent, may offer rich perspectives to crystallize MPs or MP complexes that are unstable in detergent solutions without having to modify them genetically or to develop stabilizing binders. On the other, the polydispersity of tOmpA/A8-35 complexes suggests that the volume of the APol layer varies from one protein to the next, obviously a highly unfavorable factor.

Furthermore, the best characterized APol to date, A8-35, is a polyanion (Fig. 1). Electrostatic repulsion between APol layers can hardly be taken as an asset for crystallization attempts: no MP has ever been crystallized in the presence of a charged detergent (Privé 2007). This, however, may have several causes, among which the well-known destabilizing effect of charged detergents.

This study represents a first attempt at tackling this problem. As a model MP, we have chosen the cytochrome bc_1 complex extracted from beef heart mitochondria. Cytochrome bc_1 presents numerous advantages for such a study: the complex is abundant, colored, rugged, relatively easy to crystallize in a variety of conditions, and it features extended extramembrane domains that represent $\sim$4/5 of its mass (Berry et al. 2000). As a consequence, and despite the large number of α-helices and cofactors that compose its transmembrane region, crystals can form in which protein/protein contacts are solely established between extra-membrane surfaces and there is no or very little contact between surfactant layers. In this study, we have studied the formation, composition and dispersity of bc_1/A8-35 complexes, examined their solution behavior as a function of such variables as the presence of excess APol or the salt concentration, and carried out crystallization attempts both of binary bc_1/A8-35 complexes and of ternary bc_1/A8-35/detergent ones. The results confirm that crystallizing MP/APol complexes is not a simple affair, but indicate that it may be tractable.

Materials and Methods

Materials

Dodecyl-β-D-maltoside (DDM) was purchased from Anatrace; ethylene-diamine-tetra-acetic acid (EDTA), *Tris*-hydroxymethyl-amino-methane (Tris) and sodium chloride from Sigma; 2-morpholino-ethane sulfonic acid potassium salt (KMES) and 4 kDa polyethylene glycol (PEG4k) from Fluka. Centricon and Amicon devices were from Millipore, Bio-Beads SM-2from Bio-Rad, the Superose 6HR column from Amersham Biosciences. Water was purified on a Milli-Q academic system equipped with a Q-Gard1 cartridge and two Organex cartridges from Millipore (Saint-Quentinen-Yvelines, France) ("Milli-Q water"). DEAE Sepharose CL6B and size exclusion Sepharose CL6B were from Pharmacia (GE Healthcare). Lipoluma was from Lumac LSC.

Decylubiquinone was from Sigma. The reduced form was prepared as follows: 300 µl of the 100 mM ethanolic quinone solution were mixed with 200 µl of 0.1 M sodium dihydrogen phosphate (NaH_2PO_4). The mixture was then reduced by adding a small amount of dithionite, sealing, and waiting overnight. The resulting colorless solution was extracted three times with 0.2 ml of hexane. The combined extracts were dried under nitrogen and the quinol dissolved in 300 µl ethanol/acetic acid 50:1 v/v.

Buffers

Tris (pH 8) and Tris (pH 8.5)–NaCl buffer contained 20 mM Tris/HCl, pH 8 (resp. 8.5), 100 mM NaCl and 5 mM EDTA. NaP buffer contained 20 mM NaH_2PO_4/Na_2HPO_4, pH 8. B4 buffer contained KMES 100 mM pH 6.4, 0.1 g l^{-1} DDM, 10 % PEG4k, 0.5 mM EDTA. All buffers were prepared in Milli-Q water.

Amphipols

The synthesis and characterization of the three APols used in this study have been described previously: A8-35 in Refs. Gohon et al. (2004, 2006), [^{3}H]A8-35 in Ref. Gohon et al. (2008), and 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled A8-35 ($FAPol_{NBD}$) in Ref. Zoonens et al. (2007). APol stock solutions were prepared by weighing 100 mg APol and adding 1 ml of pure water or buffer. They were usually kept at 4 °C until complete use but could also be frozen.

Size Exclusion Chromatography

SEC analyses were performed at 4 °C on an Äkta Explorer 100 FPLC device (Pharmacia, GE Healthcare) using a Superose 6HR 10/30 column and a multiple-wavelength UV-visible detection system. Runs were carried out at a flow rate of 0.3 ml min^{-1}. Prior to analysis, the column was equilibrated with Tris (pH 8 or pH 8.5)–NaCl buffer, the same buffer being supplemented with 0.2 mM DDM for cytochrome bc_1/DDM samples. For analytical chromatography, 100 µl samples were generally loaded on the column at a protein concentration of $\sim$2 g l^{-1}. The absorbance was monitored at 220, 280, and 415 nm. For preparative chromatography, 100–200 µl samples were injected at protein concentrations of 15–19 g l^{-1} and 0.5 ml fractions were collected.

Preparative Ultracentrifugation

50–100 µl of protein samples at 15–28 g l^{-1} were layered onto 2 ml 20–40 % linear sucrose gradients in Tris (pH 8 or pH 8.5)–NaCl buffer. They were centrifuged at 4 °C in a Beckman TL100 ultracentrifuge (TLS55 rotor) for 15–16 h at 44,000 rpm (150,000×g) to separate bc_1/APol complexes from free APol (Fig. 2a), at 55,000 rpm (200,000×g) in all other cases. Fractions of 120 µl were collected with a Hamilton syringe.

Small-Angle Neutron Scattering

The contrast match point (CMP) of PEG4k was estimated from its chemical formula to be ~17 % D_2O. Cytochrome bc_1 preparations in DDM and in A8-35 were prepared as described previously, and then transferred to NaP buffer in 17 % D_2O by two successive dialyses lasting, respectively, 22 and 18 h. Samples were concentrated to 30 g l^{-1} with Centricon devices of molecular weight cutoff (MWCO) 100 kDa. SANS measurements were performed at the Institut Laue Langevin (ILL, Grenoble, France) on beam line D22. Experiments were carried out at 20 °C in 1.0 or 2.0-mm path-length quartz cuvettes (Hellma, France), using neutrons with a wavelength of 10 Å. The CMP of PEG4k was experimentally checked using 5 % PEG4k solutions in H_2O/D_2O (0, 40, 67, and 100 % D_2O), as well as 1:1 v/v mixtures of PEG4k and 9, 13, and 17 % D_2O solutions, in order to enhance the signal close to the CMP. The percentage of D_2O (v/v) in each sample was checked by measuring neutron transmission. The experimentally determined CMP, 15.3 % D_2O, is close enough to 17 %, making it unnecessary to adjust the percentage of D_2O in cytochrome bc_1 preparations. The concentration of PEG4k and salt in each bc_1 sample was adjusted while diluting the sample from 30 g l^{-1} to its final protein concentration.

Protein Concentration Measurements

Protein concentration was determined from the absorption at 562 nm of samples reduced with dithionite, using a molar extinction coefficient $\varepsilon_{562} = 69{,}700$ l mol^{-1} cm^{-1}. Cytochrome bc_1 concentrations are expressed as the molarity of cytochrome c_1.

Preparation of Mitochondria

Mitochondria were prepared as described in Ref. Smith (1967). Briefly, after fat and connective tissues had been trimmed from the beef heart, tissues were homogenized in a blender and adjusted to pH 7.5 as rapidly as possible. A first centrifugation was run to remove residual muscle tissue and lipid granules, while a second one pelleted the membranes. Pellets were washed and finally resuspended and homogenized in as small a volume of KPi 50 mM, EDTA 0.5 mM as possible, in order to keep the protein concentration as high as possible. 50 ml aliquots were stored at −80 °C.

Cytochrome bc_1 Purification

The purification of cytochrome bc_1 was carried out as described in Ref. Berry et al. (1991). It is based on DDM extraction at a 1:1 DDM/protein mass ratio, anion-exchange chromatography on DEAE Sepharose CL6B with a 260–500 mM NaCl gradient, and SEC on Sepharose CL-6B. Pooled fractions from the last column were adjusted to 100 µM bc_1 before titrating with B4 precipitant solution to get rid of contaminants and aggregated materials. Those were pelleted and discarded, after which the supernatant was precipitated (or, more precisely, crystallized) upon addition of 0.2 additional volumes of precipitant buffer B4. Precipitation occurs instantly, but the whole solution is incubated for several hours in the cold room. This pre-crystallization step permits to obtain the protein in a reproducible state of detergent and buffer composition and to stabilize it: Small aliquots of this preparation could be stored at −80 °C for several months.

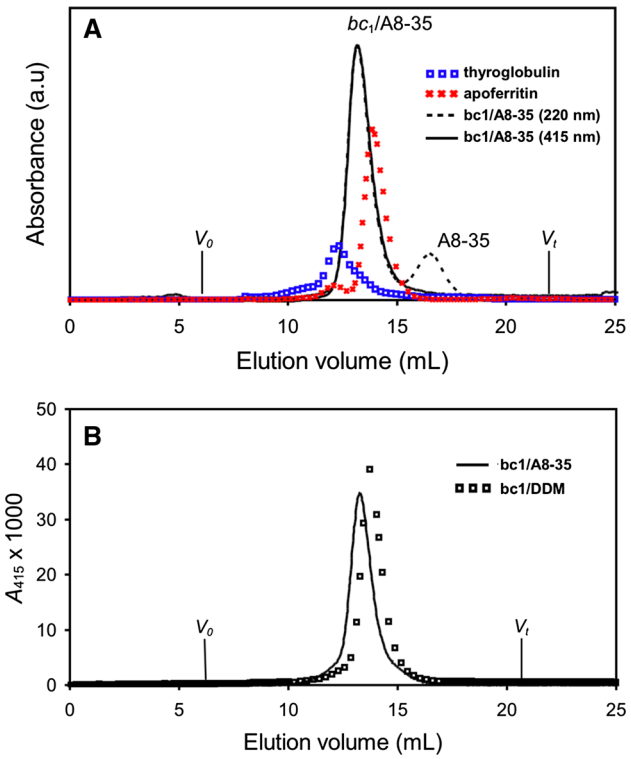

Fig. 2 Size exclusion chromatography analysis of cytochrome bc_1/A8-35 and cytochrome bc_1/detergent complexes. **a** Injection onto a Superose 6HR 10/30 column of 100 µl of A8-35-trapped cytochrome bc_1 (bc_1/A8-35 1:1.5 w/w). Elution profiles were recorded at 220 and 415 nm. The elution profiles of two markers, thyroglobulin ($M_W = 670$ kDa) and apoferritin ($M_W = 440$ kDa), are shown for comparison. Protein concentration was ~2 g l^{-1}. **b** Comparison of the elution profiles of bc_1/A8-35 and bc_1/DDM complexes (100 µl each), analyzed at 415 nm. The equilibration and running buffer for all experiments was Tris (pH 8.5)–NaCl buffer, supplemented in the last case with 0.2 mM DDM. V_0, excluded volume; V_t, total volume

Preparation of Cytochrome bc_1/Amphipol Complexes

The pre-crystallized preparation in detergent was thawed and pelleted in a bench centrifuge at 13,000 rpm, and the

pellet resuspended in buffer Tris (pH 8 or pH 8.5)–NaCl supplemented with 0.2 mM DDM. For some purification batches, redissolution was slow. In such cases, the resuspended sample was dialyzed overnight against Tris (pH 8.5)–NaCl buffer in order to eliminate any possible remaining trace of PEG4k. At this step, the concentration of cytochrome bc_1 was $\sim$20 g l^{-1}. Trapping was achieved by adding A8-35 in a 1:1.5 protein/A8-35 mass ratio from a 100 g l^{-1} solution in water and incubating for ½ h at 4 °C. The detergent was removed by adding wet Bio-Beads (20× the mass of detergent present in the solution), incubating for 3 h at 4 °C under slow agitation, and then diluting 5–10× in buffer and incubating overnight. The sample was then concentrated using 100 kDa MWCO Centricon or Amicon devices.

Evolution of Dispersity as a Function of Time

Samples of cytochrome bc_1 in DDM and of bc_1/A8-35 complexes, separated or not from free A8-35, were prepared as described above, in a quantity sufficient to conduct several injections at 2 g l^{-1} protein on the size exclusion column. Samples were diluted at 2 g l^{-1} on day 0 and were kept either at 4 °C in a refrigerator or at 20 °C in a thermostated incubator. Each sample was vortexed before 100 µl were taken and injected on the column at 4 °C. Peak areas were integrated using elution volume intervals of 7.7–17 ml for cytochrome bc_1 in DDM and 8–24 ml and 8–17 ml, respectively, for bc_1/A8-35 complexes containing, or not, free APol. Chromatograms were normalized to identical areas to make up for small differences in the volumes injected.

Determination of Cytochrome bc_1 Activity in the Presence of A8-35 vs. Detergent

Electron transfer activity was measured as described by Trumpower and Edwards (1979), with minor modifications, in a mixture containing 40 mM sodium phosphate, 0.5 mM EDTA, 3 mM sodium azide, pH 7.4, 50 µM cytochrome *c* (horse heart, from Sigma) as electron acceptor and $\sim$60 µM decylubiquinol as electron donor. The reaction was initiated by adding the enzyme. The APol-trapped sample was prepared at pH 8 in 20 mM Tris/HCl, 100 mM NaCl, whereas the DDM-solubilized enzyme was prepared in MOPS/NaCl, pH 7.4. Samples containing $\sim$5 pmol of bc_1 were diluted into the assay mix. Reduction of cytochrome *c* was followed at ambient temperature ($\sim$22 °C) over a period of typically 30 s in an Aminco DW2 spectrophotometer. Millimolar extinction coefficients of 25 (reduced minus oxidized at 550–535 nm) for cytochrome *c*, and 70 (fully reduced at 562–600 nm) for the bc_1 complex were used. A unit of activity is defined as 1 µmol of cytochrome *c* reduced per minute and the turnover number as the number of moles of cytochrome *c* reduced per mole of cytochrome bc_1 complex per second.

Determination of Bound A8-35

The trapping procedure was carried out as described above, after mixing a solution of [^{3}H]A8-35 (Gohon et al. 2004) at 100 g l^{-1} with one of unlabeled A8-35 at the same concentration in a 1:9 ratio (v/v). Free A8-35 was separated from bc_1/A8-35 complexes either by SEC or by sucrose gradient centrifugation. The fractions containing cytochrome bc_1 were pooled, washed free of sucrose, and concentrated by cycles of centrifugation and dilution in a Centricon device with a MWCO of 100 kDa. The concentration of cytochrome bc_1 was determined spectroscopically as described above. Radioactivity was measured on a Beckman type LS 1801 liquid scintillation counter, after diluting a small volume (<100 µl) of each fraction in 3 ml of Lipoluma scintillator. The values given are the average of triplicate measurements. A calibration curve relating A8-35 to radioactivity was built using three different dilution series, ranging from 0.01–1 g l^{-1}, each series being prepared independently.

Crystallization of Cytochrome bc_1/A8-35/Detergent Ternary Complexes

Pre-crystallized material was pelleted and resolubilized in Tris/HCl pH 8 buffer supplemented with increasing amounts of A8-35 (from 0.04 to 0.3 g per g of protein). The protein concentration was typically 20 g l^{-1}. The material was briefly ultracentrifuged (20 min at 100,000×*g*) before being used to set up crystallization drops, either as such or after separation from free APol by SEC. Crystallization trials were carried out at 4 or 20 °C by the hanging-drop vapor diffusion technique using 24-well culture plates. Crystallization conditions were screened using either the commercial sparse matrix screen MembFac (Hampton Research) or a more specific screen derived from the crystallization conditions already established for detergent-solubilized cytochrome bc_1, adjusted by increasing the concentrations of NaCl and/or PEG4k. Crystals and spherulites grown in the presence of $FAPol_{NBD}$ were observed on a fluorescent microscope (IM-35 Zeiss inverted microscope). Crystals were tested for diffraction on a Micro7 RIGAKU X-ray home source equipped with Osmic mirrors (MSC) and with a MAR345 detector, as well as on beam line ID14-1 of the synchrotron X-ray source at the ESRF (Grenoble, France).

Results

Preparation and Characterization by SEC of Cytochrome bc_1/A8-35 Complexes

DDM-solubilized cytochrome bc_1 was supplemented with various concentrations of A8-35 (see below) and incubated for 5 min at 4 °C. The detergent was removed by adsorption onto Bio-Beads SM-2 and the preparation concentrated on a 100 kDa MWCO membrane. In order to determine the optimum amount of A8-35 needed for trapping, several protein/A8-35 ratios from 0.02 to 1.5 (w/w) were tested and the resulting samples analyzed by SEC. Ratios below 1:1 resulted in polydisperse bc_1 preparations, while those from 1:1 to 1:1.5 yielded monodisperse ones. A value of 1:1.5 was therefore generally used. The samples thus obtained showed two main peaks when analyzed by SEC (Fig. 2a). The first and major peak absorbs both at 220 and at 415 nm, the latter being a characteristic absorbance wavelength of cytochrome bc_1; it elutes between the peaks of apoferritin ($M_W = 440$ kDa) and of thyroglobulin ($M_W = 670$ kDa). Considering that the M_W of dimeric cytochrome bc_1 is equal to 486 kDa, this peak corresponds to cytochrome bc_1/A8-35 complexes. The second peak, smaller and only visible at 220 nm, corresponds to free A8-35 particles, whose mass is ~40 kDa (Gohon et al. 2006).

Figure 2b shows a comparison between a chromatogram of cytochrome bc_1 in DDM and after trapping with A8-35, analyzed at 415 nm. The two peaks are very close in size and position. Both peaks have the same width, indicating that the dispersity of cytochrome bc_1 is conserved when the protein complex is transferred from DDM to A8-35. The positions of the peaks, however, are slightly different, bc_1/A8-35 complexes eluting 0.4 ml earlier than cytochrome bc_1/DDM ones. This corresponds to a difference in hydrodynamic radius of <1 nm. It could be due to the A8-35 belt around the transmembrane region of cytochrome bc_1 being slightly thicker than the DDM one. It is more likely, however, to originate from repulsive interactions between the particles and the column. In the cases of BR/A8-35 and tOmpA/A8-35 complexes, indeed, Stokes radii determined by SEC are abnormally large as compared to those estimated by SANS, AUC, or NMR (for a discussion, see Refs. Gohon et al. 2008; Zoonens et al. 2007).

Washing the Excess of Free A8-35

Freshly prepared cytochrome bc_1/A8-35 samples were fractionated by ultracentrifugation in 20–40 % sucrose gradients. Three gradients were run in parallel (Fig. 3). In a control experiment, 50 µl of a mixture of A8-35 and [^{3}H]A8-35 (10 g l^{-1} overall) were layered on a surfactant-free gradient to check on the migration of free A8-35

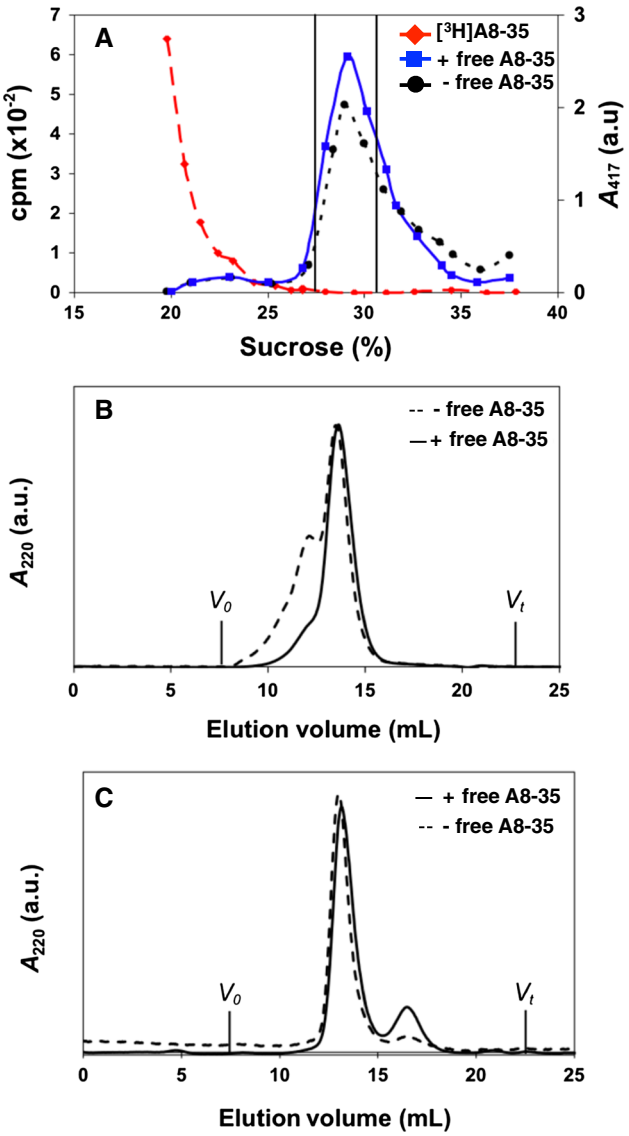

Fig. 3 Separating free A8-35 from cytochrome bc_1/A8-35 complexes. **a** Fractionation by ultracentrifugation in 20–40 % sucrose gradients. All gradients were made in Tris (pH 8.5)–NaCl buffer. Cytochrome bc_1/A8-35 complexes were layered onto either an APol-free gradient or a gradient containing 0.1 g l^{-1} free A8-35 and centrifuged for 16 h at 150,000×*g* at 4 °C. The concentration of cytochrome c_1 in each fraction was determined from its absorbance at 417 nm. As a control, free [^{3}H]A8-35 was layered onto an APol-free gradient, centrifuged under the same conditions, and the radioactivity of the fractions counted. **b** The three most concentrated fractions from the gradients in (**a**) (between *vertical bars*), containing or not free A8-35, were pooled, washed free from sucrose by dialysis, and injected onto a size exclusion column under the same conditions as in Fig. 2 (APol-free Tris (pH 8.5)–NaCl buffer). Elution profiles were analyzed at 220 nm. **c** A sample of bc_1/A8-35 complexes was injected onto a size exclusion column equilibrated with 0.2 g l^{-1} A8-35 in buffer Tris (pH 8.5)–NaCl buffer so as to separate the complexes from the excess of free A8-35. The bc_1/A8-35 peak was collected, concentrated, and reanalyzed on the same column in the same buffer, but for the absence of free APol. Data in **b** and **c** have been normalized to approximately the same maximal absorbance at 220 nm. V_0, excluded volume; V_t, total volume

particles. A second surfactant-free gradient received 50 µl of cytochrome bc_1/A8-35 complexes, at a protein concentration of 17 g l^{-1}, while an identical sample was layered onto a third gradient, containing 0.1 g l^{-1} free A8-35. The dispersity of the bc_1/A8-35 complexes collected from the gradients was checked by SEC, a method that efficiently resolves them from free A8-35 particles (Fig. 2a).

In keeping with SEC data, sucrose gradient profiles (Fig. 3a) show that cytochrome bc_1 migrates essentially as one species corresponding to a major band, which, under our centrifugation conditions, peaks at ~29 % sucrose. The distribution, however, tends to trail toward the bottom of the tube, especially when no free A8-35 is present in the gradient, indicating that part of the material forms larger aggregates. In the gradient onto which [^{3}H]A8-35 alone had been deposited, the radioactivity remained in the top fractions, showing that the procedure efficiently separates free APol particles from bc_1/APol complexes.

The most concentrated bc_1 fractions from the APol-free and APol-containing gradients were pooled, dialyzed against a sucrose-free buffer, and analyzed by SEC. The profiles of elution at 220 nm confirmed that free APol had been either eliminated or reduced to a very low concentration (Fig. 3b). The sample fractionated in the presence of 0.1 g l^{-1} free A8-35 was closer to monodispersity than that from the APol-free gradient, which contained small bc_1 oligomers. This is consistent with previous observations with tOmpA (Zoonens et al. 2007), BR (Gohon et al. 2008), and OmpF (Arunmanee et al. 2014), indicating that complete removal of free APol induces the formation of MP/APol oligomers. In the case of tOmpA, this phenomenon was shown to be reversible upon back addition of free APol (Zoonens et al. 2007). Another crude preparation of bc_1/A8-35 complexes was centrifuged under similar conditions, but individual fractions from the gradient were this time analyzed separately by SEC. It showed that fractions collected from the top of the bc_1 band can be monodisperse even if there is no free APol in the medium, whereas the deeper they are taken in the gradient—past the major band—the more polydisperse they are, even in the presence of 0.1 g l^{-1} free APol in the gradient (data not shown).

Removal of free APol, or reduction of its concentration, can also be achieved by preparative SEC. In Fig. 3c, a sample of cytochrome bc_1 trapped in A8-35, containing ~9 g l^{-1} bc_1 and ~18 g l^{-1} A8-35, was injected onto a column equilibrated with a buffer containing 0.2 g l^{-1} free A8-35. The fractions containing the bc_1/A8-35 complex were collected, and reinjected onto an APol-free column. The resulting sample (Fig. 3c, dashed line) presented a very narrow distribution, better than that of the sample washed on a gradient containing 0.1 g l^{-1} free A8-35 (Fig. 3b, solid line), and very similar to that of the original unwashed sample (Fig. 3c, solid line).

Many experiments were carried out to separate free A8-35 from cytochrome bc_1/A8-35 complexes using protocols similar to those described here. They led to the general conclusion that getting rid of all free APol most often induced a partial aggregation of the sample, but that this phenomenon can be prevented by carrying out the separation procedure in the presence of a low concentration of free APol. The best results in terms of reproducibility, dispersity, and yield were obtained when layering bc_1/A8-35 complexes at ~15 g l^{-1} protein concentration (~22 g l^{-1} total A8-35) onto a gradient containing 0.2 g l^{-1} free APol and recovering only the top fractions of the major bc_1 band, which are also the most concentrated, the other fractions being potentially contaminated with aggregated material.

Composition of Cytochrome bc_1/A8-35 Complexes

Cytochrome bc_1 in detergent solution was trapped according to the usual protocol, using an APol mixture composed of 90 % unlabeled A8-35 and 10 % [^{3}H]A8-35, before being concentrated to 15 g l^{-1}. To determine the amount of bound A8-35 in the complexes, free APol was separated from the complexes and the ratio of radioactivity to protein concentration in the purified bc_1/A8-35 complexes determined. The separation was carried out either by sucrose gradient sedimentation or by SEC. In the first case, the bc_1/A8-35 samples were layered onto surfactant-free 20–40 % sucrose gradients buffered at pH 8.5 and separated from free A8-35 by overnight sedimentation. The gradients were fractionated and their absorbance at 553 nm (without reduction) and radioactivity content were determined for each fraction. The fractions of interest were pooled, washed from sucrose, and the concentration of protein (after reduction) and radioactivity were measured again. When using SEC, 150 µl of bc_1/A8-35 complexes were injected on the Superose 6 column and the eluate was fractionated. The low protein concentration in each fraction of the sucrose gradient led to imprecise measurements. Hence, protein concentration and radioactivity were measured on fractions corresponding to non-aggregated material after they had been pooled and concentrated on an Amicon device. The data (Table 1) indicate, quite reproducibly, that the amount of A8-35 bound to cytochrome bc_1 is the same at pH 8.0 and 8.5 and equal to 0.11 ± 0.014 g g^{-1}. This is much lower than the ratio measured for BR (~2 g g^{-1}) (Gohon et al. 2008), as expected from the very different distribution of masses of the two proteins between transmembrane and extramembrane domains (see "Discussion" section).

Table 1 Amount of bound A8-35 in cytochrome bc_1/A8-35 complexes

pH	Fractionation method[b]	A_{562} (1,000×)	[bc_1] (mg l^{-1})	^{3}H (cpm)[a]	[A8-35]$_t^{b,c}$ (mg l^{-1})	Bound A8-35 (g per g bc_1)
8	Sedimentation	244	851	4,256	111	0.13 ± 0.017
8	SEC	305	1,063	4,240	110	0.10 ± 0.013
8.5	Sedimentation	296	1,032	4,635	120	0.12 ± 0.016
8.5	SEC	297	1,035	4,090	106	0.10 ± 0.013

[a] Counts per minute; average of triplicate measurements

[b] bc_1/A8-35 complexes were separated from the excess A8-35 used for trapping by either ultracentrifugation or size exclusion chromatography (SEC); see "Materials and Methods" section

[c] [A8-35]$_t$ is the total concentration of A8-35, i.e., [[3H]A8-35] + [unlabeled A8-35]

Dispersity of Cytochrome bc_1/A8-35 Complexes as a Function of Storage Time

Two batches of protein were used to study the stability of preparations of cytochrome bc_1 either kept in 0.2 mM DDM or after trapping by A8-35 and to evaluate the effect of the presence of free A8-35 and of storage temperature on the evolution of the dispersity of bc_1/A8-35 complexes. A first batch was trapped in A8-35 and divided into three samples. Sample 1 was stored at 4 °C, sample 2 at 20 °C. Sample 3 was separated from free A8-35 on a 20–40 % sucrose gradient (150,000×g, 16 h). The bc_1 band was collected, washed free of sucrose by centrifugation in a Centricon device with a MWCO of 10 kDa, and stored at 20 °C. In another series of experiments, another batch of protein was divided into two aliquots. Sample 4 was left in DDM and kept at 4 °C, while sample 5 was trapped in A8-35 and separated from free A8-35 on a sucrose gradient, as described above. The band of cytochrome bc_1 was recovered, washed free from sucrose, and stored at 4 °C. Each sample was analyzed by SEC about every week (the resolution of the column degraded somewhat between the two series of experiments, leading to poorer chromatograms for samples 4 and 5).

At 4 °C, cytochrome bc_1 in DDM appeared to aggregate quite rapidly: A shoulder on the left side of the main peak appeared after a week and moved up to the dead volume of the column in the course of 2 weeks (Fig. 4a). On the contrary, A8-35-trapped cytochrome bc_1 stayed mostly monodisperse for more than a month (Fig. 4b). A small fraction of the material, however, degraded into a smaller form, leading to the formation of a small shoulder on the right side of the main peak, at an elution volume of ~15.5 ml. This position is close to that of catalase, a marker protein of 220 kDa used to calibrate the column, suggesting that the small form, which absorbs at 415 nm, is likely to be cytochrome bc_1 monomer. When free A8-35 was completely separated from complexes, a degree of aggregation appeared immediately after the separating procedure, as shown in the previous section. It did not evolve for about 3 weeks, suggestive of an equilibrium situation (Fig. 4c). Some further aggregation, however, developed afterward (not shown). When bc_1/A8-35 complexes were purified in the presence of a low concentration (0.2 g l^{-1}) of free A8-35, there was no trace of aggregation over at least 3 weeks, but a small shoulder at ~15 mL, presumably the bc_1 monomer, developed over the first 2 weeks (Fig. 4d).

At 20 °C (Fig. 5), A8-35-trapped cytochrome bc_1 could be considered as stable for about a week. After that, it started evolving toward aggregated states (Fig. 5a). In the absence of free APol, the aggregation was much more pronounced and rapid, appearing during the first week (Fig. 5b).

Taken together, these results demonstrate that A8-35 drastically improves the stability of cytochrome bc_1 as compared to that in DDM. The presence of free A8-35 visibly decreases aggregation, but it does not prevent monomerization, which, however, remains quite limited during at least 3 weeks, a favorable factor for crystallization attempts.

Solution Behavior of Cytochrome bc_1/A8-35 Complexes as a Function of Salt and PEG Concentrations

As already noted, a major drawback in using A8-35 for MP crystallization is the number of charges carried by the polymer. In the case of cytochrome bc_1, which binds ~53 kDa of A8-35, the complex is thus supplemented with about 135 negative charges, which may oppose the formation of protein/protein contacts and, thereby, prevent crystallization. We have therefore studied by neutron scattering the interactions between cytochrome bc_1/A8-35 complexes in solution, and their sensitivity to the presence of salt and polyethylene glycol (PEG). SANS experiments were conducted in the presence of PEG4k and NaCl and of 17 % D_2O, i.e., close to the measured CMP of PEG4k (15.3 % D_2O; see "Materials and Methods" section), in order to eliminate its contribution. Data were recorded for

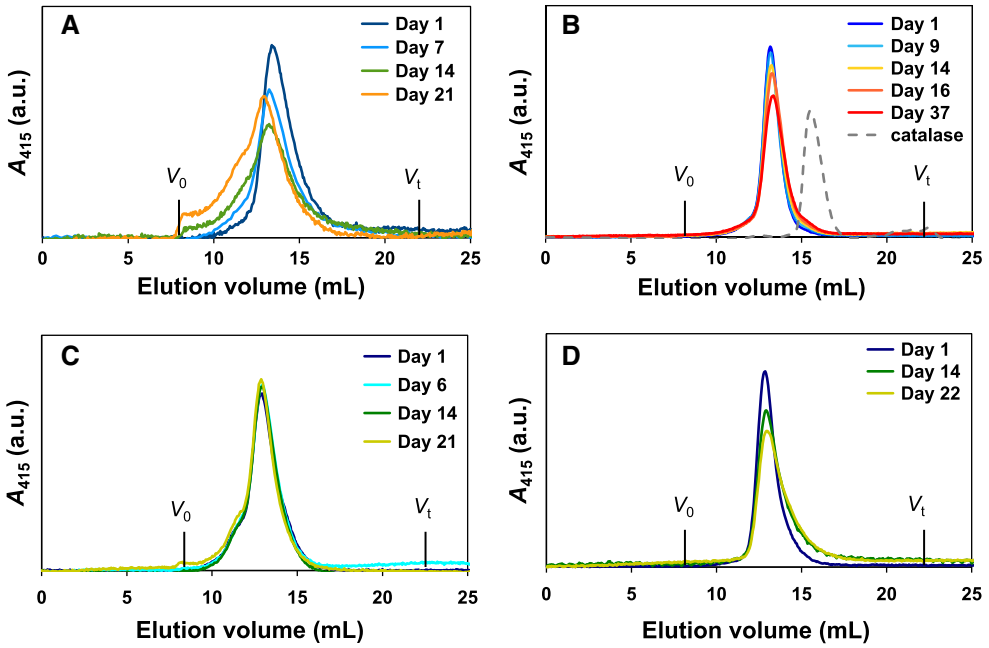

Fig. 4 Evolution of the dispersity of bc_1/surfactant complexes as a function of the time of storage at 4 °C under various conditions. Preparations were analyzed by SEC under the same conditions as in Fig. 2. **a** Evolution of cytochrome bc_1 complexes kept in 0.2 mM DDM. **b** Cytochrome bc_1/A8-35 complexes kept in the presence of the excess of A8-35 present at the time of trapping (1:1.5 bc_1/A8-35 mass ratio). The SEC profile of catalase (M_W = 220 kDa) is shown for comparison. **c** The same sample as in (**b**) after separation from free A8-35 by sucrose gradient sedimentation and elimination of the sucrose by centrifugation on an Amicon 30 kDa MWCO device. **d** The same sample as in (**b**) after separation from the excess of free A8-35 by sucrose gradient sedimentation in Tris (pH 8.5)–NaCl buffer containing 0.2 g l^{-1} A8-35 and elimination of the sucrose by centrifugation on an Amicon 30 kDa MWCO device. All profiles were recorded at 415 nm. Data have been normalized to the same total peak area. V_0, excluded volume; V_t, total volume

two samples of protein, one in DDM and the other after trapping by A8-35, at the same concentrations of salt and PEG. Because of the large size of the protein and of the presence of D_2O at a concentration close to the CMPs of both A8-35 (23.5 % D_2O; Ref. Gohon et al. 2004) and DDM (21.7 % D_2O; Ref. Timmins et al. 1994), scattering is dominated by the protein. Guinier representations (ln $I(Q)$ vs. Q^2) (Guinier and Fournet 1955) yielded a curved plot for the sample in detergent and a linear one for the sample in A8-35 (Fig. 6a). Therefore, in the presence of the same concentrations of salt and PEG4k, and at the same concentration of protein, bc_1 complexes aggregate in DDM and not after trapping by A8-35.

Adding more PEG or NaCl to bc_1/A8-35 samples increased the bend of the low-angle region of the plot, indicating that interactions in solution had become attractive (Fig. 6b). This observation is consistent with a previous study, carried out using small-angle X-ray scattering (SAXS), showing that, at concentrations of bc_1/A8-35 complexes compatible with crystallization ($\sim$20 g l^{-1} protein), it is possible to cancel repulsive interactions between complexes by adding 200–300 mM NaCl (in the absence of PEG), and to create attractive interactions by further increasing the salt concentration (Popot et al. 2003). These results suggest that crystallization conditions could possibly be identified, provided that precipitant concentrations are increased as compared to the crystallization conditions used in detergent solution (Zhang et al. 1998).

Activity of Cytochrome bc_1/A8-35 Complexes in Solution

Electron transfer activities were measured spectroscopically (see "Materials and Methods" section) for a bc_1 complex either kept in DDM solution or trapped in A8-35. For identical concentrations of complex, the turnover numbers turned out to be very similar, 367 and 341 s^{-1}, respectively, suggesting that A8-35 does not affect the activity of the complex.

Attempts at Crystallizing Cytochrome bc_1/A8-35 Complexes

Solution measurements indicate that (i) cytochrome bc_1 trapped in A8-35 is more stable than in DDM solution; (ii) the two types of bc_1/surfactant complexes have very nearly the same size and dispersity; (iii) purification of bc_1/A8-35 complexes on sucrose gradients or by SEC in the presence of low concentrations of free A8-35 yields complexes that are monodisperse and remain so for weeks; (iv) repulsive

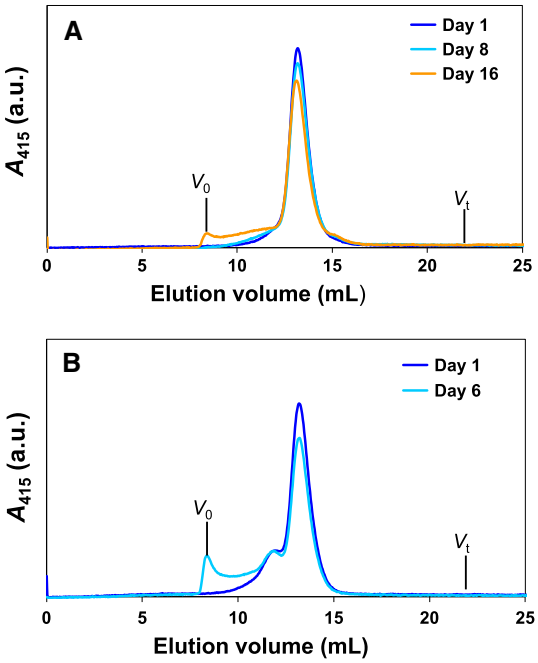

Fig. 5 Evolution of the dispersity of bc_1/A8-35 complexes as a function of the time of storage at 20 °C. Same conditions as in Fig. 4, except that samples were stored at 20 °C. **a** As trapped (bc_1/A8-35 1:1.5 w/w). **b** Same sample after complete elimination of free A8-35 by sucrose gradient sedimentation and of the sucrose by centrifugation on a Centricon 100 kDa MWCO device. All profiles were recorded at 415 nm. Data have been normalized to the same total peak area. V_0, excluded volume; V_t, total volume

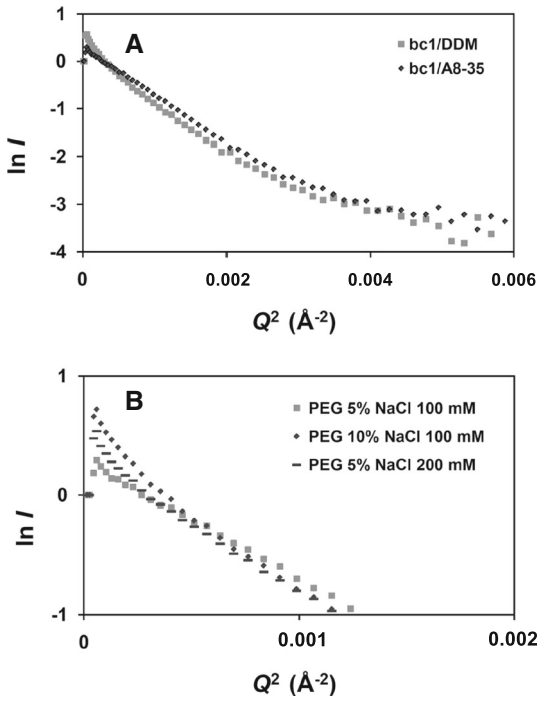

Fig. 6 Small angle neutron scattering by solutions of cytochrome bc_1/surfactant complexes. All solutions were made in NaP buffer containing 17 % D_2O, so as to eliminate most of the contribution to neutron scattering of PEG, DDM, and/or A8-35. The data are presented as Guinier plots (Guinier and Fournet 1955). **a** Cytochrome bc_1 (15 g l^{-1}) either in 0.2 mM DDM solution (*gray*) or after trapping in A8-35 (*black*) at a 1:1 w/w trapping ratio, in the presence of 5 % PEG4k and 100 mM NaCl. **b** Guinier plots of bc_1/A8-35 complexes (20 g l^{-1} bc_1) in the same buffer at various PEG and NaCl concentrations. All data were recorded at room temperature using 10 Å wavelength neutrons

electrostatic interactions between the complexes can be canceled by raising the ionic strength of the solutions; and (v) the bc_1/A8-35 complex retains its activity. On the basis of these observations, crystallization attempts were undertaken. They were carried out first by trapping cytochrome bc_1 in A8-35 according to the usual protocol, lowering or not the concentration of free A8-35 and then conducting vapor diffusion experiments. Many crystallization parameters were tested, using either commercial kits (Hampton Research: Crystal Screen Lite, PEG/ion and MembFac) or homemade ones, and varying such parameters as pH (6.4–8.5), protein concentration (5, 10, 20, or 35 g l^{-1}), temperature (4 or 20 °C), and drop volume (4–10 µl). This approach did not yield any positive results, leading most often to phase separation or gel formation, but never to good-looking precipitates nor crystals. A more gradual approach was therefore tested.

Crystallization of Cytochrome bc_1 in Ternary Complexes with Detergent and A8-35

As recalled in the "Introduction" section, ternary complexes form when MPs are put in the simultaneous presence of both APols and detergents (Tribet et al. 2009; Zoonens et al. 2007). These complexes have solution and biochemical properties that are intermediate between those of MP/APol and MP/detergent complexes in terms of charge and size dispersity, as well as of MP stability (Champeil et al. 2000; Zoonens et al. 2007). This suggests that APols could possibly be used as additives, to help crystallizing MPs whose stability in pure detergent solutions is insufficient. Furthermore, crystallizing ternary MP/APol/detergent complexes could provide insights as to how to crystallize MP/APol ones. The strategy followed was to start from conditions that reproducibly yield bc_1 crystals in detergent solution and to progressively supplement the samples with increasing concentrations of A8-35, while adjusting crystallization conditions at each step. The original procedure consists in thawing a frozen aliquot of pre-crystallized bc_1/DDM complexes, pelleting it, resolubilizing it in a buffer to a concentration of ~20 g l^{-1} bc_1, and setting hanging drops at 4 °C against a reservoir solution containing 30 mM sucrose, 0–30 mM octyl-β-D-glucoside (βOG), 10 % PEG4k, 100 mM KMES, pH 6.4, and 0.5 mM EDTA (Zhang et al. 1998). Drops are prepared by

mixing 2 µl of the protein with 2 µl of the reservoir solution and therefore contain both DDM and, if applicable, βOG. Because the charges introduced by the addition of A8-35 to the samples were expected to be a hindrance to crystallization, two approaches were attempted to control their effect. The first one was to try to take advantage of the ability of low concentrations of divalent cations to screen (and presumably cross-link) the negative charges carried by the carboxylate groups of A8-35 (Picard et al. 2006). The second one, based on the observation that repulsive interactions between PM/A8-35 complexes can be turned into attractive ones at higher salt and/or PEG concentrations, was to progressively increase the concentration of precipitants along with that of A8-35, so as to prevent the inhibition of crystal growth.

Attempts at Crystallizing Ternary Complexes in the Presence of Divalent Cations

The pre-crystallized material was resolubilized in a buffer containing A8-35, so that the solution could be directly used for setting up the crystallization trays. Note that even though no detergent was added, some DDM was present in the solution, brought by the pre-crystallized material. Assuming cytochrome bc_1 to bind about the same amount of DDM (~260 molecules per dimer; Ref. Breyton et al. 1997) as cytochrome b_6f, whose transmembrane region is very similar (Stroebel et al. 2003), and to retain it upon crystallization, this can be estimated to ~0.27 g DDM per g bc_1. Initial experiments were carried out with low ratios of A8-35 to bc_1 (0.04 g per g). Trials were conducted in the presence of increasing, millimolar concentrations of Ca^{2+}. They yielded showers of crystals within 24 h, with a good reproducibility. Attempts at controlling nucleation by modulating the protein and PEG concentrations were unsuccessful. Other kinds of objects were observed when the APol to protein mass ratio was increased to 0.3 g g^{-1} and the incubation temperature of the trays was raised from 4 to 20 °C. Still, objects were small. Exposed to an X-ray source, they did not show any diffraction spots, whether from salt or from protein crystals.

Omitting PEG (but maintaining the calcium concentration in the drop in the millimolar range) led to the formation of larger, reddish and globular objects, hereafter called spherulites, along with small bipyramidal transparent objects (Fig. 7a). The latter appeared upon X-ray diffraction to be salt crystals. The reddish color of the spherulites indicated that they contain cytochrome bc_1, but they did not diffract. They appeared after 1 week, very reproducibly, mainly in the conditions described in the legend to Fig. 7 but also at higher A8-35/bc_1 ratios (1.5 g g^{-1}). To determine whether spherulites contained APols, experiments were repeated in the presence of NBD-labeled A8-35 ($FAPol_{NBD}$) (Zoonens et al. 2007). Spherulites readily formed (Fig. 7b), under the same conditions as with plain A8-35. Observation under a fluorescence microscope confirmed that they indeed contain $FAPol_{NBD}$ (Fig. 7c). Efforts were made to turn spherulites into crystalline, X-ray-diffracting object, by modulating the nature and concentration of divalent cations, pH, temperature, PEGs, or trying additives from the MembFac screen (Hampton Research), but to no avail.

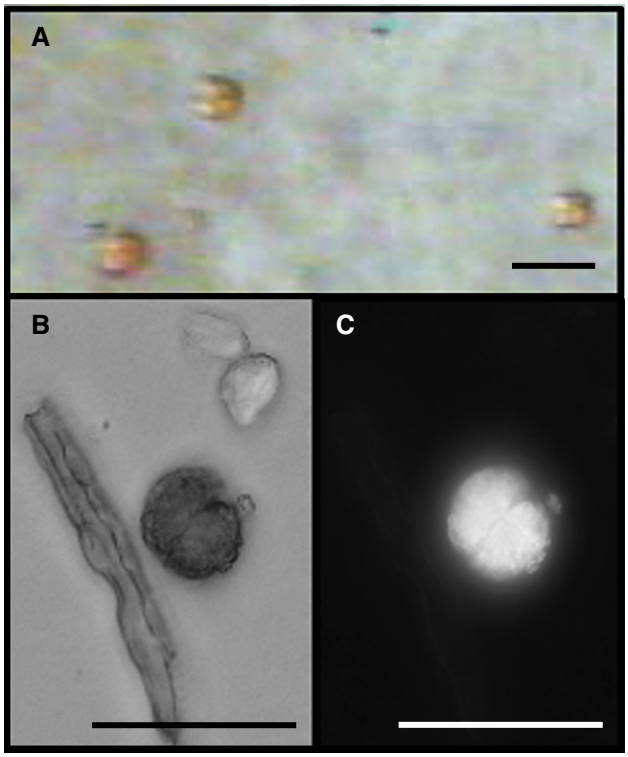

Fig. 7 Spherulites obtained with cytochrome bc_1/A8-35/DDM ternary complexes. **a** A picture taken 7 days after resuspending a sample of bc_1/DDM complexes using 0.3 g A8-35 per g bc_1. Reservoir: 30 mM sucrose, 100 mM KMES pH 6.4, 1 mM Ca^{2+} and 5 mM Mg^{2+}; sample: 35 g l^{-1} bc_1. No PEG4k, no βOG, incubation at 20 °C. **b**, **c** Spherulites obtained in the presence of $FAPol_{NBD}$, observed under white light (**b**) and by fluorescent microscopy (**c**); black and white photographs. Reservoir: 30 mM sucrose, 100 mM KMES, pH 6.4, 5 mM Ca^{2+} and 5 mM Mg^{2+}; sample: 35 g l^{-1} bc_1. *Bars* 100 µm

Crystallization of Ternary Complexes in the Absence of Divalent Cations

The pre-crystallized protein was this time resuspended in the presence of increasing concentrations of A8-35 and incubated on ice overnight. In initial experiments, no detergent was added to the DDM present in the pre-crystallized material. On the following day, the sample was centrifuged, so as to pellet aggregated material, and the supernatant used to set up crystallization drops. Conditions

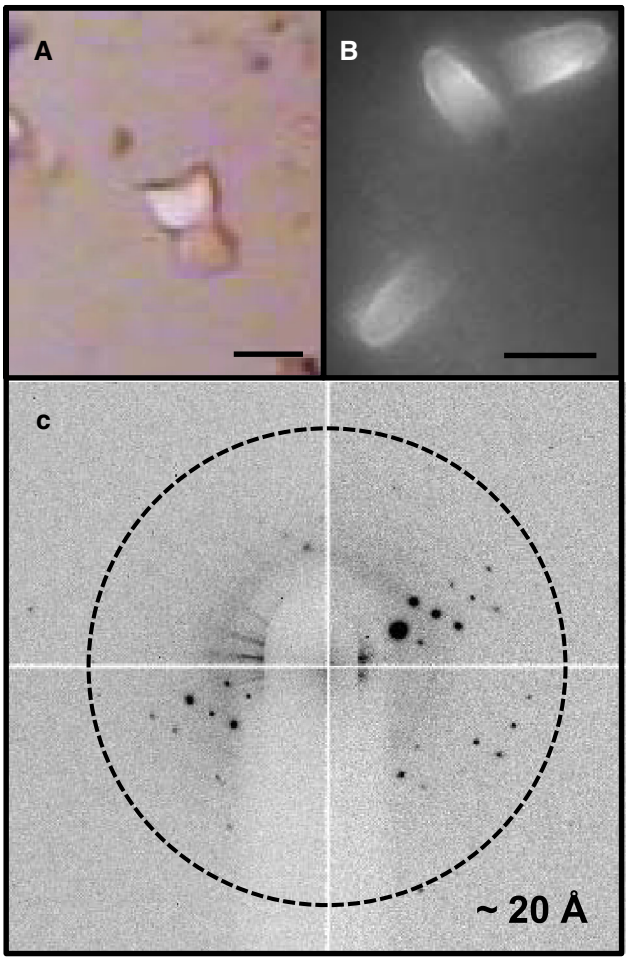

Fig. 8 Crystallization of cytochrome bc_1/A8-35/detergent ternary complexes. Adapted from Fig. 6 in Popot et al. (2011). **a** Crystals were obtained under the following conditions: sample: 17 g l^{-1} bc_1, 0.3 g A8-35 per g protein; reservoir: 12 % PEG4k, 100 mM NaCl, 10 mM βOG. Samples were incubated 10 days at 20 °C. *Bar* 100 μm. **b** Crystals obtained under the same condition as in (**a**), but after replacing plain A8-35 by fluorescent A8-35 ($FAPol_{NBD}$), observed by fluorescence microscopy. *Bar* 100 μm. **c** Diffraction pattern recorded on a synchrotron light source (ESRF, beamline ID14-1)

were those used for crystallizing bc_1/DDM complexes (Zhang et al. 1998), except for the absence of detergent in the resolubilization buffer. The sample was mixed 1:1 with the reservoir solution (typically 2 + 2 μl). The concentration of NaCl was varied from 60 to 120 mM and that of PEG4k from 4 to 12 %. Hits were readily obtained: nucleation, good-looking precipitates, and even tiny crystals. To optimize their size, βOG was added in the reservoir up to a maximum of 15 mM (critical micellar concentration, cmc ≈ 20 mM). Reddish crystals were obtained after 10 days from trays incubated at 20 °C. They had a wedge shape and often grew head to tail, up to 120 μm long (Fig. 8a). They were obtained quite reproducibly for an APol/protein ratio of 0.3 g g^{-1}. As previously, crystals were grown in the presence of $FAPol_{NBD}$, and fluorescence microscopy confirmed that they did contain APols (Fig. 8b). A dozen of crystals were tested on a synchrotron X-ray source. They diffracted, with spacings compatible with their being protein crystals (Fig. 8c), but the resolution limit was quite poor (∼20 Å). These results should be considered as preliminary, since circumstances made it impossible to carry out attempts to improve freezing conditions and crystal handling. Crystals were also obtained under the exact same conditions as above (NaCl 60–120 mM, PEK4k 4–12 %), but in the absence of βOG. In that case, however, crystal formation turned out to be irreproducible, and crystals diffracted to even poorer resolution limits.

Discussion

Whereas their well-established stabilizing character would seem to make APols useful agents for crystallizing MPs, attempts to do so have long been frustrating, and they remain only partially successful. In this study, we have tried to identify factors that may contribute to progressing toward this goal, using cytochrome bc_1 trapped by APol A8-35 as a model system.

Complexation of cytochrome bc_1 by A8-35 took place efficiently under conditions similar to those described earlier for other MPs, e.g., the tOmpA (Zoonens et al. 2005) or BR (Gohon et al. 2008; Tribet et al. 1996; Zoonens et al. 2014). As observed for these proteins, efficient retention into solution following dilution of the detergent under its cmc required that the bc_1 be incubated with A8-35 at APol/protein mass ratios higher than that eventually measured in the final complexes. The latter, however, was much lower for cytochrome bc_1 (∼0.11 g bound A8-35 per g protein) than for BR (∼2 g per g) (Gohon et al. 2008). This is consistent with the different distribution of masses between transmembrane and extramembrane regions: In BR (27 kDa), the transmembrane region represents ∼75 % of the mass of the protein, i.e., ∼20 kDa, against only ∼22 % (∼107 kDa) for the bc_1 complex (486 kDa in its native dimeric form). It is surprising, however, that the bc_1 dimer, with 26 transmembrane α-helices, should bind about the same amount of A8-35 (∼53–54 kDa) as monomeric BR, which contains only 7 helices + bound lipids. Given that these measurements are difficult to carry out with good accuracy (if only because MPs tend to oligomerize when separated from free APols; see below and Refs. Arunmanee et al. 2014; Gohon et al. 2008; Zoonens et al. 2007), they clearly have to be taken as provisional.

Solutions studies of bc_1/A8-35 complexes showed them to be essentially monodisperse. Upon SEC, they migrated as though they were slightly larger than bc_1/detergent

(DDM) ones. These two features have been observed previously for SERCA1a (Champeil et al. 2000), BR (Gohon et al. 2008), tOmpA (Zoonens et al. 2005, 2007), and OmpX (Catoire et al. 2010). In the case of cytochrome bc_1, a large MP with a relatively narrow transmembrane "waist," it seems likely that the earlier elution has more to do with interactions with the column than with an actual increase of hydrodynamic radius (for a discussion of these effects, see Refs. Gohon et al. 2008; Zoonens et al. 2007). Detailed studies have shown that, upon SEC, tOmpA/A8-35 complexes appear more broadly distributed than tOmpA/C_8E_4 ones, suggesting that the amount of bound surfactant varies more from one complex to another than it does in detergent solution (Zoonens et al. 2007). No such observation was made here. This is not too surprising: Because a large fraction of the surface of tOmpA is covered with surfactant and the protein has only small extramembrane domains, differences in the amount of bound APol from particle to particle may visibly affect the R_S distribution. With cytochrome bc_1, most of the bulk of which is extramembrane, slight variations of the amount of APol surrounding the transmembrane "waist" of the protein are unlikely to strongly affect its apparent R_S, which is more sensitive to its overall length.

Replacing the detergent belt with an APol one does not affect the electron transfer activity of the bc_1 complex. Note that we can rule out that an artifactual acceleration of the reaction due to favorable electrostatic interactions between the positive charges of cytochrome *c* and the polyanionic polymer could somehow compensate a degree of inhibition. Indeed, cytochrome *c* is present in the reaction medium at a concentration well above the K_m, so binding of cytochrome *c* by the bc_1 complex is always saturated and cannot be the rate-limiting step. If the negative charge of A8-35 were to accelerate the binding of cytochrome *c* and lower its K_m, it would not speed up electron transfer.

The homogeneity of bc_1/A8-35 particles, an essential factor in crystallization attempts, can be affected by two additional factors: on the one hand, stabilizing biochemically the complexes, and, in particular, avoiding monomerization; on the other, preventing bc_1 dimers from forming higher order oligomers. As regards the first point, substituting DDM with A8-35 drastically improved the biochemical stability of cytochrome bc_1. Over the course of several weeks at 4 °C, however, a small degree of monomerization did develop. If previous experience with the homologous b_6f complex (Breyton et al. 1997), and unpublished observations) and with G protein-coupled receptors (Dahmane et al. 2009) is any guide, trapping some lipids along with the protein might be expected to improve stability. As regards oligomerization, observations with the bc_1 complex are reminiscent of those made with tOmpA (Zoonens et al. 2007), BR (Gohon et al. 2008), and OmpF (Arunmanee et al. 2014): Namely, the presence of a small excess of APol is needed to prevent bc_1 dimers from forming higher-order oligomers. We have previously attributed this effect to the poorly dissociating character of APols: Upon removal of free APol, APol-complexed MPs tend to form small aggregates, which in the case of tOmpA, redisperse upon adding back APols (Zoonens et al. 2007). The present observations with the bc_1 complex suggest that this may be a general phenomenon, which has been suggested to be potentially useful to control the formation of MP arrays (Arunmanee et al. 2014). Experiments that require or benefit from monodispersity, such as radiation scattering or crystallization attempts, should therefore be carried out in the presence of a slight excess of APols. In the case of cytochrome bc_1, this excess is much lower ($\sim$0.1 g free A8-35 per g of bound one) than in those of tOmpA (Zoonens et al. 2007) and BR (Gohon et al. 2008) ($\sim$1:1). Since the underlying mechanism is the competition between protein/protein and protein/APol interactions, the optimal ratio of free to bound APol can be expected to vary depending on the tendency of the protein to self-associate, as well as on the nature of the APol and buffer conditions.

SANS (this study) and SAXS (Popot et al. 2003) studies show that, as expected, interactions between the highly charged bc_1/A8-35 particles are repulsive at low salt concentration. They become attractive upon increasing the ionic strength and/or adding PEG. In the case of cytochrome bc_1, repulsive interactions disappear and attractive interactions develop around 300 mM salt in the absence of PEG. Under these conditions, Guinier plots become linear, which is compatible with perfect monodispersity and neutral interactions. It cannot be excluded, however, that the onset of association masks lingering repulsive interactions. These observations suggests, nevertheless, that conditions can be found where the charged character of A8-35 ceases to prevent bc_1 complexes from interacting one with another. The shape of the bc_1 complex facilitates the formation of crystals in which the dimers interact exclusively via their extramembrane domains, without bringing transmembrane regions into close proximity. Such is indeed the case of the crystals ($P6_522$) that are usually obtained from the procedure used here (Zhang et al. 1998). It seemed therefore reasonable to hope that crystallization may occur.

Our extensive attempts at crystallizing pure bc_1/A8-35 complexes nevertheless consistently yielded negative results. Attempts were conducted either blindly, by using crystallization kits designed either for soluble or for membrane proteins, or by searching the crystallization space around those conditions that give good crystals in detergent solution (Zhang et al. 1998), while varying such factors as the ionic strength, temperature, pH, PEG nature

and concentration, or the presence of traces of divalent cations. None of these approaches led to the formation of even "hopeful precipitates." Better success was achieved using a stepwise approach. Starting from conditions that consistently yield crystals in the presence of detergent, the detergent phase was progressively enriched (or poisoned) by supplementing it with increasing ratios of APols, while adjusting the crystallization conditions to as to keep obtaining crystals. This approach did lead to the formation of ternary crystals containing the protein (based on their color and unit cell dimensions), A8-35 (based on the fluorescence observed when crystals were formed in the presence of a fluorescently labeled derivative of A8-35) and detergent. The presence of detergent in the crystals was not directly established, but can be inferred from previous studies that show that, in mixtures containing a MP, detergent and APols, the composition of the surfactant layer adsorbed onto the protein reflects that of APol/detergent mixed particles (Tribet et al. 2009; Zoonens et al. 2007). On the basis of the mass ratio of bc_1/DDM in the starting material estimated above (see "Results" section), the amounts of A8-35 and βOG supplemented, and the assumption of a free distribution of the detergent between APol/detergent mixed particles, soluble bc_1/A8-35/detergent complexes and crystals, we estimate that the mass ratio of APol to detergent in the crystals most enriched in APols reached about 1:1.

Why ternary complexes crystallized whereas pure bc_1/APol complexes did not can have several causes. First, diluting A8-35 with neutral detergent effectively lowers the charge density of the surface of the surfactant layer, diminishing global electrostatic repulsion. Second, it offers a way for the system to locally avoid close approach between carboxylate groups of APol molecules adsorbed onto neighboring bc_1 dimers, since charged groups can move away from each other and be replaced by neutral detergent polar heads. Third, we have already mentioned that ternary tOmpA/A8-35/detergent complexes appear more homogeneous, upon gel filtration, than binary tOmpA/A8-35 ones (Zoonens et al. 2007). This effect is probably due to the fact that MPs bind only a limited number of APol molecules and that the latter are relatively large and heterogeneous. This may prevent the mixture from relaxing to a state where each MP is surrounded by exactly the same amount of surfactant. Detergents may provide the "small change" that helps adjusting the surfactant layer to about the same size from one particle to another, obviously a favorable factor for crystallization. Fourth, it is worth noting that some EM (Althoff et al. 2011) and MD (Perlmutter et al. 2014) studies suggest that MP-bound A8-35 layers are not smooth, but bumpy. The origin of this phenomenon is uncertain, and it has not been observed in more recent EM studies (Liao et al. 2013, 2014; for discussions, see Huynh et al. 2014; Zoonens and Popot 2014). If real, it may conceivably interfere with crystallization. It would be of interest to examine whether mixed APol/detergent belt exhibit or not the same feature.

Whereas crystals of ternary complexes were obtained, those that could be tested diffracted only to ~20 Å resolution. It is currently not known whether this represents an intrinsic limit or is simply due to the small number of crystals that could be examined and/or to difficulties in handling and/or freezing them. Nevertheless, the mere fact that 3D crystals of APol-trapped MPs could be obtained is an encouragement to pursue such attempts. Those could be carried out using either A8-35 or more recently developed APols with different chemical structures (Bazzacco et al. 2012; Dahmane et al. 2011; Diab et al. 2007a, b; Sharma et al. 2012). Of particular interest is the coming of age of totally non-ionic APols, whose latest versions have shown an excellent ability to trap and stabilize MPs (Bazzacco et al. 2012; Sharma et al. 2012). Whereas these molecules can be expected to form somewhat thicker protein-bound layers, due to the bulkiness of their glucose-based polar moieties, this hypothetical drawback may be more than balanced by the absence of electrostatic repulsion.

Finally, we note two other ways in which APols may help to obtain the crystallographic structure of MPs. First, it is now known that APol/detergent mixtures can (i) stabilize MPs as compared to pure detergent (Champeil et al. 2000) and (ii) yield 3D crystals (this study). APols therefore could possibly be used as additives, to stabilize MPs whose instability in pure detergent solution prevents crystals from growing to a sufficient size. Second, APols have proven an excellent medium in which to fold MPs to their native state (Bazzacco et al. 2012; Dahmane et al. 2009, 2011; Leney et al. 2012; Pocanschi et al. 2006), as well as to synthesize them in vitro (Bazzacco et al. 2012). They could therefore be used as mere tools to produce the folded protein, whereas crystallization would be attempted after transferring the latter either to a detergent, a bicelle, or a lipidic cubic or sponge phase. This approach has recently permitted to obtain crystals diffracting to <2 Å of BR transferred from A8-35 to a lipid 3D phase (Polovinkin et al. 2014).

Acknowledgments Particular thanks are due to A.-N. Galatanu and J.-C. Courant for participating in some of these experiments, to P. Hervé for the synthesis of [^{3}H]A8-35, to P. Timmins for his participation in the SANS measurements, to D. Picot and L. Barucq for testing crystals at the ESRF, to I. Gallay for help with the X-ray equipment at the IBPC, to J. Barra and L.J. Catoire for their precious help with the figures, and to Y. Gohon and M. Zoonens for useful comments on the manuscript. This study was supported by the Centre National de la Recherche Scientifique, Université Paris-7, the Human Frontier Science Program Organization (Grant RG00223-2000-M), E.U. Specific Targeted Research Project LSHG-CT-2005-513770 IMPS (*Innovative tools for membrane protein structural proteomics*),

and NIH grant R01DK44842. The stays of EAB in France were subsidized by awards from the France-Berkeley Fund and the French Ministère de l'Education Nationale et de la Recherche.

References

Althoff T, Mills DJ, Popot J-L, Kühlbrandt W (2011) Assembly of electron transport chain components in bovine mitochondrial supercomplex $I_1III_2IV_1$. EMBO J 30:4652–4664

Arunmanee W, Harris JR, Lakey JH (2014) Outer membrane protein F stabilised with minimal amphipol forms linear arrays and LPS-dependent 2D crystals. J Membr Biol. doi:10.1007/s00232-014-9640-5

Bazzacco P, Billon-Denis E, Sharma KS, Catoire LJ, Mary S, Le Bon C, Point E, Banères J-L, Durand G, Zito F, Pucci B, Popot J-L (2012) Non-ionic homopolymeric amphipols: application to membrane protein folding, cell-free synthesis, and solution NMR. Biochemistry 51:1416–1430

Berry EA, Huang L-S, DeRose V (1991) Ubiquinol–cytochrome *c* oxidoreductase from higher plants. Isolation and characterization of the bc_1 complex from potato tuber mitochondria. J Biol Chem 266:9064–9077

Berry EA, Guergova-Kuras M, Huang L-S, Crofts AR (2000) Structure and function of cytochrome bc_1 complexes. Annu Rev Biochem 69:1005–1075

Breyton C, Tribet C, Olive J, Dubacq J-P, Popot J-L (1997) Dimer to monomer conversion of the cytochrome b_6f complex: causes and consequences. J Biol Chem 272:21892–21900

Cao E, Liao M, Cheng Y, Julius D (2013) TRPV1 structures in distinct conformations reveal activation mechanisms. Nature 504:113–118

Catoire LJ, Zoonens M, van Heijenoort C, Giusti F, Popot J-L, Guittet E (2009) Inter- and intramolecular contacts in a membrane protein/surfactant complex observed by heteronuclear dipole-to-dipole cross-relaxation. J Magn Res 197:91–95

Catoire LJ, Zoonens M, van Heijenoort C, Giusti F, Guittet E, Popot J-L (2010) Solution NMR mapping of water-accessible residues in the transmembrane β-barrel of OmpX. Eur Biophys J 39:623–630

Champeil P, Menguy T, Tribet C, Popot J-L, Le Maire M (2000) Interaction of amphipols with the sarcoplasmic reticulum Ca^{2+}-ATPase. J Biol Chem 275:18623–18637

Cvetkov TL, Huynh KW, Cohen MR, Moiseenkova-Bell VY (2011) Molecular architecture and subunit organization of TRPA1 ion channel revealed by electron microscopy. J Biol Chem 286:38168–38176

Dahmane T, Damian M, Mary S, Popot J-L, Banères J-L (2009) Amphipol-assisted *in vitro* folding of G protein-coupled receptors. Biochemistry 48:6516–6521

Dahmane T, Giusti F, Catoire LJ, Popot J-L (2011) Sulfonated amphipols: synthesis, properties and applications. Biopolymers 95:811–823

Dahmane T, Rappaport F, Popot J-L (2013) Amphipol-assisted folding of bacteriorhodopsin in the presence and absence of lipids. Functional consequences. Eur Biophys J 42:85–101

Diab C, Tribet C, Gohon Y, Popot J-L, Winnik FM (2007a) Complexation of integral membrane proteins by phosphorylcholine-based amphipols. Biochim Biophys Acta 1768:2737–2747

Diab C, Winnik FM, Tribet C (2007b) Enthalpy of interaction and binding isotherms of non-ionic surfactants onto micellar amphiphilic polymers (amphipols). Langmuir 23:3025–3035

Etzkorn M, Raschle T, Hagn F, Gelev V, Rice AJ, Walz T, Wagner G (2013) Cell-free expressed bacteriorhodopsin in different soluble membrane mimetics: biophysical properties and NMR accessibility. Structure 21:394–401

Etzkorn M, Zoonens M, Catoire LJ, Popot J-L, Hiller S (2014) How amphipols embed membrane proteins: global solvent accessibility and interaction with a flexible protein terminus. J Membr Biol. doi:10.1007/s00232-014-9657-9

Feinstein HE, Tifrea D, Sun G, Popot J-L, de la Maza LM, Cocco MJ (2014) Long-term stability of a vaccine formulated with the amphipol-trapped major outer membrane protein from *Chlamydia trachomatis*. J Membr Biol. doi:10.1007/s00232-014-9693-5

Flötenmeyer M, Weiss H, Tribet C, Popot J-L, Leonard K (2007) The use of amphipathic polymers for cryo-electron microscopy of NADH: ubiquinone oxidoreductase (complex I). J Microsc 227:229–235

Giusti F, Popot J-L, Tribet C (2012) Well-defined critical association concentration and rapid adsorption at the air/water interface of a short amphiphilic polymer, amphipol A8-35: a study by Förster resonance energy transfer and dynamic surface tension measurements. Langmuir 28:10372–10380

Giusti F, Rieger J, Catoire L, Qian S, Calabrese AN, Watkinson TG, Casiraghi M, Radford SE, Ashcroft AE, Popot J-L (2014) Synthesis, characterization and applications of a perdeuterated amphipol. J Membr Biol. doi:10.1007/s00232-014-9656-x

Gohon Y, Pavlov G, Timmins P, Tribet C, Popot J-L, Ebel C (2004) Partial specific volume and solvent interactions of amphipol A8-35. Anal Biochem 334:318–334

Gohon Y, Giusti F, Prata C, Charvolin D, Timmins P, Ebel C, Tribet C, Popot J-L (2006) Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8-35. Langmuir 22:1281–1290

Gohon Y, Dahmane T, Ruigrok R, Schuck P, Charvolin D, Rappaport F, Timmins P, Engelman DM, Tribet C, Popot J-L, Ebel C (2008) Bacteriorhodopsin/amphipol complexes: structural and functional properties. Biophys J 94:3523–3537

Guinier A, Fournet G (1955) Small-angle scattering of X-rays. Wiley, New York

Huynh KW, Cohen MR, Moiseenkova-Bell VY (2014) Application of amphipols for structure–functional analysis of TRP channels. J Membrane Biol. doi:10.1007/s00232-014-9684-6

Leney AC, McMorran LM, Radford SE, Ashcroft AE (2012) Amphipathic polymers enable the study of functional membrane proteins in the gas phase. Anal Chem 84:9841–9847

Liao M, Cao E, Julius D, Cheng Y (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. Nature 504:107–112

Liao M, Cao E, Julius D, Cheng Y (2014) Single particle electron cryo-microscopy of a mammalian ion channel. Curr Opin Struct Biol 27:1–7

Perlmutter JD, Drasler WJ, Xie W, Gao J, Popot J-L, Sachs JN (2011) All-atom and coarse-grained molecular dynamics simulations of a membrane protein stabilizing polymer. Langmuir 27:10523–10537

Perlmutter JD, Popot J-L, Sachs JN (2014) Molecular dynamics simulations of a membrane protein/amphipol complex. J Membrane Biol. doi:10.1007/s00232-014-9690-8

Picard M, Dahmane T, Garrigos M, Gauron C, Giusti F, le Maire M, Popot J-L, Champeil P (2006) Protective and inhibitory effects of various types of amphipols on the Ca^{2+}-ATPase from sarcoplasmic reticulum: a comparative study. Biochemistry 45:1861–1869

Planchard N, Point E, Dahmane T, Giusti F, Renault M, Le Bon C, Durand G, Milon A, Guittet E, Zoonens M, Popot J-L, Catoire LJ (2014) The use of amphipols for solution NMR studies of membrane proteins: advantages and limitations as compared to other solubilizing media. J Membr Biol. doi:10.1007/s00232-014-9654-z

Pocanschi CL, Dahmane T, Gohon Y, Rappaport F, Apell H-J, Kleinschmidt JH, Popot J-L (2006) Amphipathic polymers: tools to fold integral membrane proteins to their active form. Biochemistry 45:13954–13961

Pocanschi C, Popot J-L, Kleinschmidt JH (2013) Folding and stability of outer membrane protein A (OmpA) from *Escherichia coli* in an amphipathic polymer, amphipol A8-35. Eur Biophys J 42:103–118

Polovinkin V, Gushchin I, Balandin T, Chervakov P, Round E, Schevchenko V, Popov A, Borshchevskiy V, Popot J-L, Gordeliy V (2014) High-resolution structure of a membrane protein transferred from amphipol to a lipidic mesophase (under review)

Popot J-L (2010) Amphipols, nanodiscs, and fluorinated surfactants: three non-conventional approaches to studying membrane proteins in aqueous solutions. Annu Rev Biochem 79:737–775

Popot J-L, Berry EA, Charvolin D, Creuzenet C, Ebel C, Engelman DM, Flötenmeyer M, Giusti F, Gohon Y, Hervé P, Hong Q, Lakey JH, Leonard K, Shuman HA, Timmins P, Warschawski DE, Zito F, Zoonens M, Pucci B, Tribet C (2003) Amphipols: polymeric surfactants for membrane biology research. Cell Mol Life Sci 60:1559–1574

Popot J-L, Althoff T, Bagnard D, Banères J-L, Bazzacco P, Billon-Denis E, Catoire LJ, Champeil P, Charvolin D, Cocco MJ, Crémel G, Dahmane T, de la Maza LM, Ebel C, Gabel F, Giusti F, Gohon Y, Goormaghtigh E, Guittet E, Kleinschmidt JH, Kühlbrandt W, Le Bon C, Martinez KL, Picard M, Pucci B, Rappaport F, Sachs JN, Tribet C, van Heijenoort C, Wien F, Zito F, Zoonens M (2011) Amphipols from A to Z. Annu Rev Biophys 40:379–408

Privé GG (2007) Detergents for the stabilization and crystallization of membrane proteins. Methods 41:388–397

Sharma KS, Durand G, Gabel F, Bazzacco P, Le Bon C, Billon-Denis E, Catoire LJ, Popot J-L, Ebel C, Pucci B (2012) Non-ionic amphiphilic homopolymers: synthesis, solution properties, and biochemical validation. Langmuir 28:4625–4639

Smith AL (1967) Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small scale. Methods Enzymol 10:81–86

Stroebel D, Choquet Y, Popot J-L, Picot D (2003) An atypical haem in the cytochrome b_6f complex. Nature 426:413–418

Tifrea DF, Sun G, Pal S, Zardeneta G, Cocco MJ, Popot J-L, de la Maza LM (2011) Amphipols stabilize the *Chlamydia* major outer membrane protein and enhance its protective ability as a vaccine. Vaccine 29:4623–4631

Timmins P, Pebay-Peyroula E, Welte W (1994) Detergent organisation in solutions and in crystals of membrane proteins. Biophys Chem 53:27–36

Tribet C, Audebert R, Popot J-L (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions. Proc Natl Acad Sci USA 93:15047–15050

Tribet C, Diab C, Dahmane T, Zoonens M, Popot J-L, Winnik FM (2009) Thermodynamic characterization of the exchange of detergents and amphipols at the surfaces of integral membrane proteins. Langmuir 25:12623–12634

Trumpower BL, Edwards CA (1979) Purification of a reconstitutively active iron–sulfur protein (oxidation factor) from succinate–cytochrome *c* reductase complex of bovine heart mitochondria. J Biol Chem 254:8697–8706

Zhang Z, Huang L-S, Shulmeister VM, Chi Y-I, Kim KK, Hung L-W, Crofts AR, Berry EA, Kim S-H (1998) Electron transfer by domain movement in cytochrome bc_1. Nature 392:677–684

Zoonens M, Popot J-L (2014) Amphipols for each season. J Membrane Biol. doi:10.1007/s00232-014-9666-8

Zoonens M, Catoire LJ, Giusti F, Popot J-L (2005) NMR study of a membrane protein in detergent-free aqueous solution. Proc Natl Acad Sci USA 102:8893–8898

Zoonens M, Giusti F, Zito F, Popot J-L (2007) Dynamics of membrane protein/amphipol association studied by Förster resonance energy transfer. Implications for *in vitro* studies of amphipol-stabilized membrane proteins. Biochemistry 46:10392–10404

Zoonens M, Zito F, Martinez KL, Popot J-L (2014) Amphipols: a general introduction and some protocols. In: Mus-Veteau I (ed) Membrane protein production for structural analysis. Springer, New York