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Amphipols From A to Z

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Abstract

Amphipols (APols) are short amphipathic polymers that can substitute for detergents to keep integral membrane proteins (MPs) water soluble. In this review, we discuss their structure and solution behavior; the way they associate with MPs; and the structure, dynamics, and solution properties of the resulting complexes. All MPs tested to date form water-soluble complexes with APols, and their biochemical stability is in general greatly improved compared with MPs in detergent solutions. The functionality and ligand-binding properties of APol-trapped MPs are reviewed, and the mechanisms by which APols stabilize MPs are discussed. Applications of APols include MP folding and cell-free synthesis, structural studies by NMR, electron microscopy and X-ray diffraction, APol-mediated immobilization of MPs onto solid supports, proteomics, delivery of MPs to preexisting membranes, and vaccine formulation.

Keywords

membrane proteins, amphipathic polymers, membrane biophysics,
membrane biochemistry

INTRODUCTION

Membrane proteins (MPs) are notoriously more difficult to study in vitro than their soluble counterparts, mainly because of two factors: (a) the relative scarcity of most MPs and the difficulties encountered to overexpress them under a functional form, and (b) their frequent instability outside biological membranes. The complexity of a natural membrane is such that this environment is essentially impossible to mimic, even with artificial lipid bilayers, and much less so in aqueous solutions. Detergents are traditionally used to screen the hydrophobic transmembrane (TM) surface of MPs and make them water soluble, but they tend to inactivate them. There are several reasons why detergents destabilize MPs, possibly including the loss of physical constraints provided by the

membrane environment. It is, however, our contention that a major factor is the competition of detergents with the protein-protein and protein-lipid interactions that stabilize the native structure of MPs (for a discussion, see Reference 67). This effect is compounded by the fact that MP/detergent complexes must be handled in the presence of free detergent micelles, because below the critical micellar concentration (CMC) of the detergent, entropic effects drive the aggregation of transmembrane surfaces (for a recent discussion, see Reference 89). Free micelles act as a hydrophobic sink into which lipids, cofactors, and subunits can disperse, which is a leading cause of MP inactivation. This view has been at the inception of the work summarized in the present review.

There are several possible ways to reduce or suppress the hydrophobic sink (for a discussion,

MP: membrane protein

Integral membrane protein: a protein that is in contact with the hydrophobic interior of a biological membrane to which it exposes an extensive transmembrane hydrophobic surface

Detergent: a surfactant with the ability to solubilize fats

see Reference 67). One of them is to replace detergents with compounds that would have such a high affinity for the transmembrane surface of MPs that they would remain associated to it even in the presence of vanishingly low concentrations of free micelles. This can theoretically be achieved by endowing a hydrophilic polymer with a multitude of hydrophobic chains that would act as many anchoring points. This consideration has led to the development of amphipols, a class of amphipathic polymers specially designed to complex MPs and keep them water soluble. Here the term amphipol (APol) refers to “an amphipathic polymer that is able to keep individual MPs soluble under the form of small complexes” (67).

Fifteen years have passed since the feasibility of this approach was first demonstrated (87). In the interim, much work has been devoted to learning to control the synthesis of these molecules and to understanding their physical chemical properties, to investigating the nature and properties of the complexes they form with MPs, and to developing their applications. As will be shown, the latter have extended well beyond the initial idea of enabling the biochemist and biophysicist to carry out basically the same studies as in detergent solution, but under improved conditions. Some APols are by now commercial. The thirty-odd publications that have appeared to date about the properties and uses of APols provide their potential users with a solid body of data to help them to exploit applications that have been validated and to develop novel ones. Previous reviews have discussed the advantages and disadvantages of APols versus other novel approaches to stabilizing MPs and studying them *in vitro* (12, 35, 67). Two other reviews have focused on APols and cover the field up to 2003 (68, 79).

Over the years, several types of APols have been designed, synthesized, and tested. Their study is at various stages of development. In the present review, we refer mainly to the following four types of APols: (a) A8–35 (**Figure 1a**), the first APol to be validated, whose solubility is due to the presence of carboxylates (87); (b) phosphorylcholine-

based APols (PC-APols) (**Figure 1b**) (25, 26); (c) glucose-based, nonionic APols (NAPols) (**Figure 1c**) (5, 6, 71, 83); and (d) sulfonated APols (SAPols) (**Figure 1d**) (23, 64). Other types of APols are described in earlier reviews (67, 68, 79).

CHEMICAL STRUCTURE AND SOLUTION PROPERTIES OF AMPHIPOLS: A SPECIAL CLASS OF AMPHIPATHIC POLYMERS

Using APols efficiently depends on a good understanding of the way these polymers are designed and how they behave in aqueous solutions. In the present section, we summarize the chemical structure and solution behavior of the best field-tested APols and we describe the labeled or functionalized APols that have been developed and tested thus far.

Design, Synthesis, and Solution Properties of Amphipols

APols are amphipathic polymers, carrying both many hydrophilic and many hydrophobic groups (**Figure 1**). Being amphipathic, they belong, along with detergents, to the general class of surfactants. APols present a distinctive feature compared to most other amphipathic polymers: They self-assemble into well-defined particles comprising a few macromolecules [e.g., four molecules, on average, for 9- to 10-kDa A8–35 (32), three for 25-kDa NAPols (6)]. This results from the favorable combination of a short backbone chain length and an appropriate number and size of hydrophobic segments (so that the equilibrium size of the hydrophobic core can be reached with the amount of hydrophobic groups carried by just a few chains, while avoiding the otherwise typical formation of large networks of interconnected polymers due to hydrophobic self-association), flexibility (allowing a small radius of curvature of the interface with the solution), and a limited polydispersity in composition (to avoid large populations of chains with too few or too many hydrophobic groups compared to the optimum). In practice, macromolecules

Critical micellar concentration (CMC): the concentration of a small surfactant at which its molecules start to organize into micelles

Amphipathic polymer: a polymer comprising both hydrophobic and hydrophilic moieties

Amphipol (APol): an amphipathic polymer that can keep membrane proteins water soluble in detergent-free solutions as small individual entities by adsorbing onto their transmembrane surface

A8–35: a particular type of anionic amphipol

PC-APol: phosphorylcholine-based APol

NAPol: nonionic amphipol

SAPol: sulfonated APol

Surfactant: a compound that adsorbs at the air/water interface and thereby lowers the surface tension of water

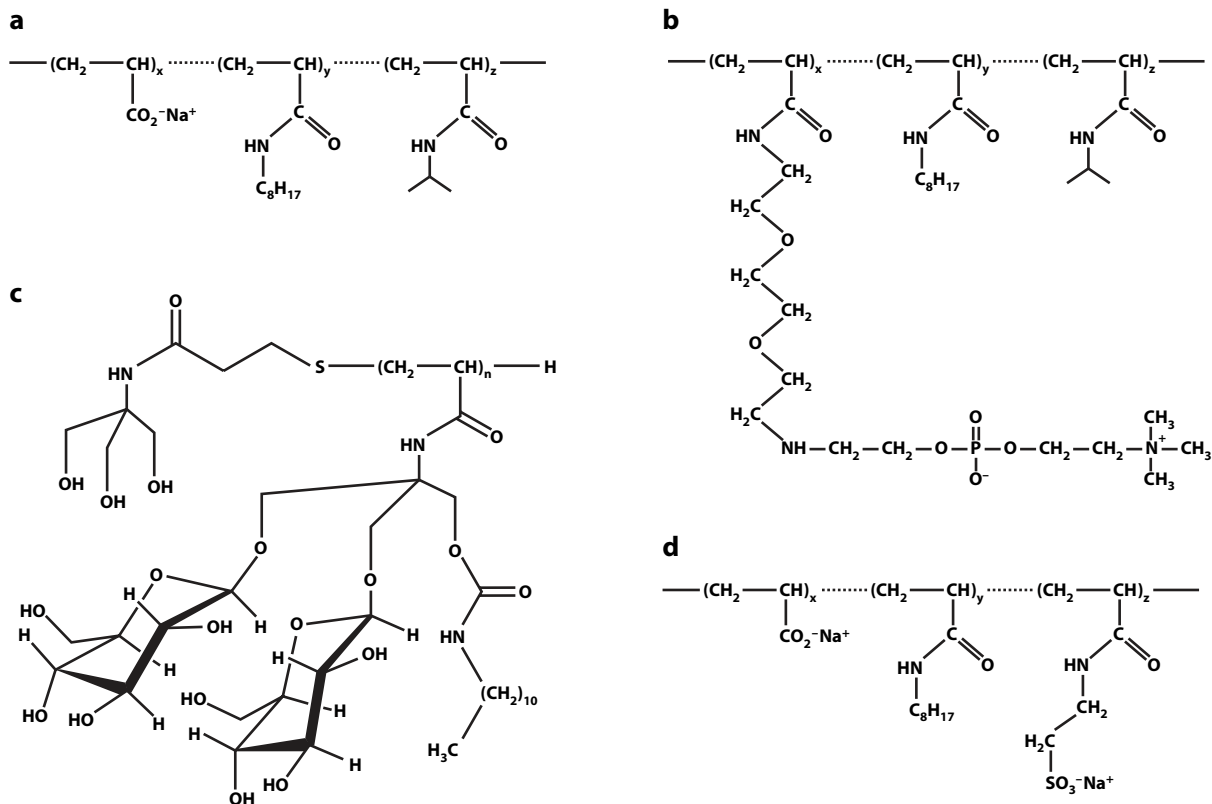


Figure 1

Chemical structures of four types of amphipols. (a) A polyacrylate-based APol, A8–35 (87); (b) a phosphorylcholine-based APol, C22–43 (26); (c) a nonionic, glucose-based APol (71); and (d) a sulfonated APol (23).

reaching or approaching such properties belong to one of the two following types: polysoaps, i.e., homopolymers made from amphipathic monomers bearing both alkyl and hydrophilic moieties, as is the case of homopolymeric NAPols (**Figure 1c**), or copolymers featuring a high density of hydrophobic monomers with short alkyl side groups (C_8 - C_{12}) separated by one or a few hydrophilic monomers (all other APols described to date). Both types of APols typically contain a few tens of alkyl groups per chain.

When APols were initially devised, their expected mode of interaction with MPs was the primary concern (68, 87). In order to efficiently trap and keep water-soluble individual MPs, it was thought that polymers ought to be small

and highly flexible and to remain soluble despite the presence of multiple hydrophobic chains. The hydrophobic chains should be densely spaced (they are statistically ~ 1 nm apart in A8–35) to avoid the formation of extended hydrophilic loops, which would interfere with many types of biophysical approaches. Chemically, this implied that the hydrophobic chains be relatively short (C_{18} chains were considered unsuitable) and that they be interspersed with highly polar groups. Polar groups are charged groups in most of the APols validated to date (23, 25, 26, 36, 59, 64, 87), hydroxyl groups in early NAPols (70), and glucose moieties in the most recently developed NAPols (5, 6, 71, 83). All the polymers that are efficient as APols self-assemble into small, compact, well-defined

globular assemblies resembling detergent micelles. This behavior is a result of the constraints initially imposed on their chemical structure.

To prepare APols, various synthetic routes can be followed, which include (a) radical copolymerization of hydrophilic and hydrophobic monomers (25, 70, 83); (b) radical polymerization of a suitable monomer (e.g., an acrylic monomer) followed by random chemical modification of the homopolymer thus obtained (6, 23, 59, 87); and (c) homopolymerization of an amphipathic monomer (71).

A8-35 (**Figure 1a**) is by far the most extensively studied APol to date. Its behavior in aqueous buffers has been examined by size exclusion chromatography (SEC), dynamic and static light scattering, equilibrium and sedimentation velocity analytical ultracentrifugation (AUC), small-angle neutron scattering (SANS), inelastic neutron scattering (INS), and molecular dynamics (MD) (33, 34; F. Giusti, C. Tribet, M. Tehei, G. Zaccà & J.N. Sachs, unpublished data). From a practical point of view, the most important solution properties of A8-35 can be summarized as follows. At and above pH 7, most carboxylates are ionized in aqueous solutions, conferring A8-35 a high solubility in water ($>200 \text{ g}\cdot\text{liter}^{-1}$) (34). A8-35 molecules, whose average molecular weight varies between 9 and 10 kDa depending on the average length of the polyacrylic acid used for the synthesis, self-assemble into particles averaging $\sim 40 \text{ kDa}$ (33). The particles are monodisperse, much more so than the molecules that form them. Individual particles, therefore, comprise a variable number of molecules depending on the latter's size. On average, A8-35 particles comprise approximately four molecules and 75-80 octyl chains (33).

Investigations of A8-35 and PC-APol C22-43 in water by neutron and light scattering (33) and by isothermal titration calorimetry (26) failed to detect dissociation of the particles down to $0.1 \text{ g}\cdot\text{liter}^{-1}$. Down to this concentration, the hydrodynamic radius and molar mass of A8-35 particles are independent of both polymer concentration (33) and chain length polydispersity (C. Tribet &

F. Giusti, unpublished data). Mixtures of fluorescently labeled A8-35 samples have been studied in an attempt to determine the concentration at which individual chains start associating, which gives rise to a Förster resonance energy transfer (FRET) signal. The critical aggregation concentration thus evaluated is extremely low: $\sim 0.002 \text{ g}\cdot\text{liter}^{-1}$ in 100 mM NaCl, pH 8.0 (F. Giusti & C. Tribet, unpublished data).

A8-35 particles bind a maximum of $\sim 1.2 \text{ g}$ water per g polymer; their radius of gyration is $\sim 2.4 \text{ nm}$, and their Stokes radius is $\sim 3.15 \text{ nm}$ (33). Their operational specific volume (φ') in 100 mM NaCl is $0.87 \text{ mL}\cdot\text{g}^{-1}$ and their contrast match point (the percentage of D_2O at which their neutron scattering length density is the same as that of the solvent and the particles become invisible to neutrons) is 23.5% D_2O (34). The shape of SANS curves indicates that the particles are globular and present a sharp interface with water (33), consistent with the results of recent MD simulations (J.N. Sachs, unpublished data). The latter approach has revealed a few interesting features that had remained inaccessible experimentally, such as the intermingling of the APols chains that constitute the particles or the presence of water molecules in their center, probably a result of steric constraints that oppose the formation of a compact hydrophobic core (67; J.N. Sachs, unpublished data). The comparison of INS data collected on unlabeled versus partially deuterated A8-35 reveals backbone dynamics similar to that of CH_2 groups in lipids or free polymers (M. Tehei & G. Zaccà, personal communication).

Because the aqueous solubility of A8-35 depends on the presence of ionized carboxylates, it is highly sensitive to their protonation, as well as to the presence of multivalent cations, in particular Ca^{2+} ions; MP/A8-35 complexes are similarly affected (19, 25, 33, 34, 64). A8-35 therefore should not be used below pH 7 (preferentially at $\text{pH} \geq 7.5$) nor in the presence of millimolar concentrations of Ca^{2+} (Mg^{2+} is better tolerated; 64). Depending on the experiments at hand, a minor degree of aggregation may or may not be acceptable.

Critical aggregation concentration: the concentration of a polymer at which its molecules start to self-assemble

FAPol_{NBD}: A8-35 grafted with NBD

Stability: refers either to physical (colloidal) stability, i.e., particles remain water-soluble and do not aggregate nor precipitate, or to biochemical stability, i.e., MPs remain in their native state and, when measurable, functional

It can, however, become critical in radiation scattering experiments, where the presence of even minute amounts of small oligomers is highly detrimental (32). In NMR studies, working above pH 7 accelerates the exchange of solvent-exposed amide protons.

These experimental constraints have stimulated the development of alternative APols, such as PC-APols, NAPols, and SAPols (**Figure 1b-d**), which are insensitive to low pH and to the presence of Ca²⁺ ions (23, 25, 64). In aqueous buffers, SAPols and glucosylated NAPols form small, compact, globular, well-defined particles whose size and dispersity are comparable to those of the particles formed by A8-35 (6, 23, 83). As for A8-35, the size of NAPol particles seems insensitive to the length of the constitutive polymers (6).

Labeled and Functionalized Amphipols

The chemistry of APols offers rich opportunities for labeling and functionalization. To date, these modifications have been applied only to A8-35 or its close congener A8-75 (which differs from A8-35 by the absence of isopropyl grafts; 87), but they could be readily extended to other APols. Isotopically labeled APols include ¹⁴C-labeled A8-75 and ³H-labeled A8-35, which have been used mainly to follow the distribution of polymers during fractionation experiments and to quantify their binding to MPs (32, 68, 88). A form of A8-35 in which the polyacrylic acid chain is hydrogenated but the isopropyl and octyl groups are perdeuterated (34) has been heavily used for SANS, AUC, and NMR experiments (16, 18, 32-34, 99). Four APols functionalized with a fluorophore have been synthesized, namely a form of A8-75 carrying a naphthyl group (94) and three forms of A8-35 carrying a 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group (FAPol_{NBD}) (100), a rhodamine group, or a fluorescein one (F. Giusti, unpublished data), respectively. They have been used particularly in FRET experiments (100) and for observing the distribution of APols in lipid vesicles (94), in

MP crystals (**Figure 6**), and in cells and whole organisms (**Figure 8**). A biotinylated version of A8-35 has been used to mediate the immobilization of APol-trapped MPs onto solid supports (21).

FORMATION, STRUCTURE, AND PROPERTIES OF MEMBRANE PROTEIN/AMPHIPOL COMPLEXES

A vast body of information about MP/APol complexes is available. In this section, we examine how these complexes are formed; what is known of their composition, structure, and solution behavior; what effects APols have on the stability and functional properties of the MPs they complex; which methodological constraints they impart; which opportunities they offer; and how APol-trapped MPs can be transferred to other environments, be they detergent micelles or lipid bilayers.

Trapping Membrane Proteins with Amphipols

Because APols are poorly dissociating surfactants, they are not efficient at solubilizing biological membranes, even though they partition into them (19, 65). As a result, APols are generally unable to directly extract MPs from cell membranes, even though there are a few exceptions (68).

Usually, MPs are first extracted and purified in detergent by standard methods and then transferred to APols (87). In a typical trapping experiment, MPs in detergent solution are supplemented with aliquots from a concentrated stock solution of APols in water. The sample is briefly incubated, during which time APols mix with the detergent both in solution and at the hydrophobic transmembrane surface of MPs (89, 100). The MP/detergent/APol complexes thus formed can also incorporate endogenous lipids that had been solubilized along with MPs, or lipids added afterward, to increase the protein's stability and/or activity (see below). It is possible that, at this stage, lipids that had been

displaced by the detergent rebind (see below). The detergent concentration is then lowered below the CMC, usually by one of the following two methods:

- The solution is diluted under the CMC of the detergent. In this case, the detergent is not physically removed from the solution, but most of it disperses in the aqueous phase as monomers and its concentration in the surfactant layer surrounding the protein drops. This method is sufficient, for instance, to restore native-like allosteric properties to the nicotinic acetylcholine receptor (nAChR), possibly due to lipid rebinding (54). It is also useful to screen trapping conditions, by modulating the MP/APol ratio used and determining by ultracentrifugation, for each condition, which fraction of the protein remains soluble after dilution of the detergent under its CMC (see below).
- Polystyrene beads (Bio-Beads SM2) are added to the sample. Detergent molecules adsorb to the beads, which are removed by low-speed centrifugation (99). The use of Bio-Beads has the advantage of not diluting the protein, and it is usually preferred for preparative protocols. APols (at least A8–35, but this is likely to hold for all APols) do not adsorb significantly to the beads, presumably because their particles do not enter the beads' narrow anfractuositities (98).

Whichever procedure is used, residual detergent molecules, if present, can be removed by dilution/concentration cycles, dialysis, or preparative SEC using surfactant-free buffer (32, 99).

The amount of APols required for efficient trapping is an important parameter that must be established early in any study. The optimal MP/APol ratio depends on the protein of interest and can be determined by carrying out quick trapping tests using a concentration range of APols, dilution below the CMC of the detergent, and ultracentrifugation (25, 83, 87). If the concentration of APols is too low, the protein

aggregates and pellets upon ultracentrifugation [note that with low-CMC detergents such as dodecylmaltoside (DDM), precipitation can be slow and incomplete even in the absence of APols and well below the detergent's CMC]. Upon increasing the APol/protein ratio, a first threshold is reached where most of the protein is kept soluble but MP/APol complexes appear heterogeneous upon SEC or AUC. It is only above a second threshold that MP/APols complexes become homogeneous. Further increasing the APol/MP ratio is useless and can be detrimental: It may increase the viscosity of the sample after concentration (e.g., in solution NMR studies) and compromise the stability of the protein (because it recreates the hydrophobic sink that the use of APols aims to abolish). The minimal mass ratio of A8–35 to MP for a small, deeply membrane-embedded protein such as bacteriorhodopsin (BR) is ~ 3 g APol per g BR (32). Trapping experiments are usually carried out at a somewhat higher ratio, 5 g APol per g BR. This ratio exceeds by a factor of ~ 2.5 the amount of APol that actually binds to BR, which is ~ 2 g APol per g BR (32). The same requirement for a moderate excess of APols is observed with MPs that, like the cytochrome *bc₁* complex, feature a much higher ratio of mass to transmembrane surface and therefore bind much less APols per g protein (D. Charvolin, unpublished data).

There are at least two rationales to explain this need for an excess of APols over that amount that actually binds to the protein. The first one, hypothetical, is that the protein may select, in a population of heterogeneous APol molecules, those that contribute to form MP/APol complexes with the lowest free-energy level. The second rationale is well documented: Because APols are not strongly dissociating, they do not prevent the formation of small MP oligomers unless present in some excess over that amount actually bound by the proteins (32, 100).

All the ~ 30 polytopic and bitopic MPs tested thus far, which differ widely in their origin, secondary structure, topology, size, function, and oligomeric state, form water-soluble

complexes with APols (67, 68). This reflects the fact that the process of association is a physical phenomenon that does not depend on details of the protein's transmembrane surface, but simply on it exposing a large enough hydrophobic area for APols to adsorb onto. To our knowledge, no attempts have been reported yet to stabilize monotopic or lipid-anchored MPs with APols.

Composition, Structure, Dynamics, and Solution Properties of Membrane Protein/Amphipols Complexes: Methodological Opportunities and Constraints

MP/A8–35 complexes have been studied by SEC, AUC, small-angle X-ray scattering (SAXS), SANS, FRET, solution NMR, and INS. Their properties have been reviewed recently (67), and only the most salient facts are recalled here. Small MPs with limited extramembrane regions such as BR (27 kDa; seven transmembrane helices plus, in most experiments, bound lipids) bind proportionately more A8–35 (~2 g per g protein) (32) than do large complexes with extended extramembrane domains such as cytochrome *bc*₁ (490 kDa; 22 transmembrane helices; ~0.11 g A8–35 per g protein) (D. Charvolin, unpublished data). The amount of A8–35 bound to these two proteins roughly corresponds to half a dozen molecules of average molecular weight. Small transmembrane domains such as single α -helices can be expected to bind approximately one particle of APols, i.e., in the case of A8–35, ~40 kDa. The A8–35 layer is compact (1.5 to 2 nm thick) and does not form an extended corona (32). In SEC, SAXS, SANS, AUC, or NMR experiments, MP/A8–35 complexes indeed behave like compact, globular particles (67). The only MP/APol contacts detected to date are with the transmembrane surface of the protein (18, 99).

Preparing homogeneous MP/A8–35 complexes requires some care (32, 100), and it is difficult to totally avoid the presence of minor fractions of small oligomers, a nuisance in radiation scattering experiments (32). Even oligomer-

free preparations do not appear as narrowly distributed as MP/detergent complexes, unless some detergent is added (100). A tentative interpretation is that the large size and small numbers of adsorbed APol molecules make it impossible for the system to finely tune the volume of the adsorbed layer to that corresponding to the lowest free energy, generating differences in the exact amount of APol bound from one MP to the next. Detergent molecules would provide the small change needed to optimize the adjustment. Polydispersity is generated when the complexes are separated from the extra, free polymer present at the end of a trapping experiment (32, 100). The latter oligomerization is reversible upon adding back APols (100).

Conditions that provoke the aggregation of the polymer, such as, for A8–35, working at $\text{pH} \leq 7$ or adding Ca^{2+} ions, also cause MP/A8–35 complexes to aggregate (17, 25, 32, 64, 99). If either of these conditions is essential to the experiment considered and monodispersity is required, other APols must be used.

Upon trapping with APols a mixture of MPs, e.g., the supernatant of solubilized membranes, MPs are trapped under the association state (e.g., monomers, oligomers, and supercomplexes) that was theirs in the detergent solution, and they retain bound lipids (1, 5, 31, 32, 54, 90). APols do not interfere with most purification techniques, such as SEC (6, 19, 32, 100), sucrose gradient fractionation (54, 87), or immobilized ligand (24) or metal (100) affinity chromatography. In the last case, however, it seems that polyhistidine tags fused too close to the transmembrane region may interact less efficiently with the column than in detergent solution, probably because of steric and/or electrostatic effects (100). Ion-exchange chromatography should be restricted to MP/NAPol complexes, as is isoelectrofocusing.

APols do not interfere with most light spectroscopy techniques (for a UV-visible absorbance spectrum of A8–35, see Reference 100), including fluorescence spectroscopy (100; M. Opačić, unpublished data), circular dichroism (CD) (30, 66, 96), and synchrotron radiation circular dichroism (23; T. Dahmane,

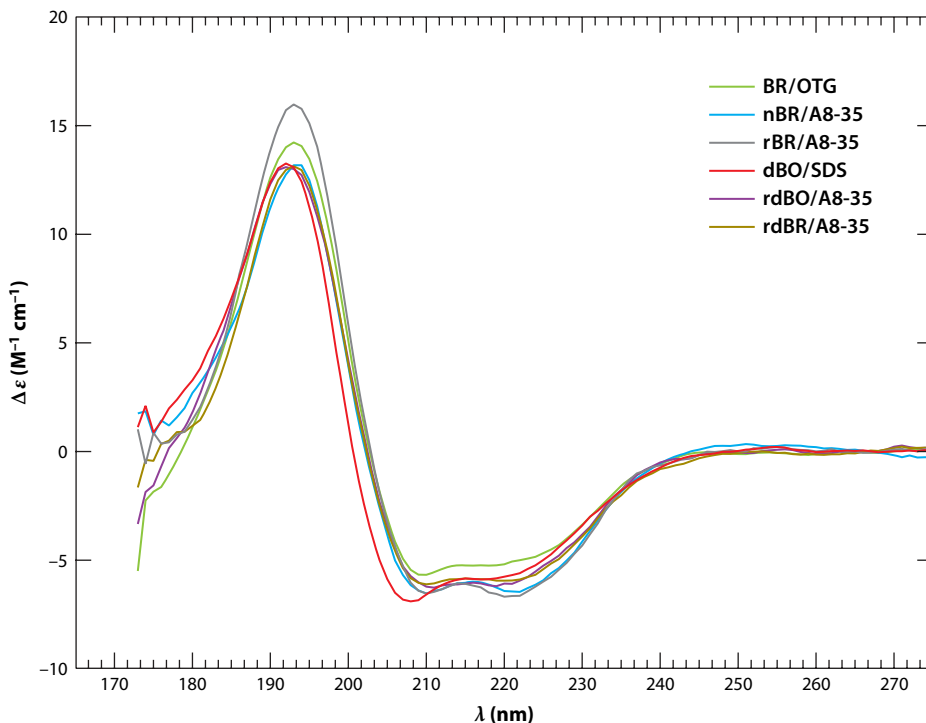


Figure 2

Synchrotron radiation circular dichroism (SR-CD) spectra of denatured and A8-35-refolded bacteriorhodopsin (BR). The spectra correspond to the following samples: BR/OTG (octylthioglucoiside), native BR (nBR) solubilized from purple membrane (PM) in OTG (along with PM lipids); nBR/A8-35, nBR transferred from OTG to A8-35 (1:5 BR/APol mass ratio); rBR/A8-35, BR refolded in A8-35 from sodium dodecylsulfate (SDS)-solubilized PM; dBO/SDS, bacterio-opsin (BO) delipidated and separated from retinal in organic solvents (dBO) and transferred to SDS; rdBO/A8-35, dBO refolded in A8-35 in the absence of retinal; rdBR/A8-35, dBO refolded in A8-35 in the presence of retinal. SR-CD spectra were recorded at 25°C on the 3m-nim Bessy and DISCO SOLEIL beamlines (29, 74). They extend down to ~175 nm, clearly resolving the 190 nm π - π^* transition of the exciton split originating from peptide bond electrons. Data from Reference 23 and T. Dahmane, M. Zoonens & F. Wien, unpublished data.

M. Zoonens & F. Wien, unpublished data) (Figure 2). Attempts have been made to apply Fourier transform infrared spectroscopy to measuring the rate of $^1\text{H}/^2\text{H}$ exchange at the amide bonds of A8-35-trapped BR. However, all current APols contain amide bonds, and the amide II region of protein spectra (1590–1510 cm^{-1}) is obliterated by APol signals (Y. Gohon & E. Goormaghtigh, unpublished data).

Functionality of Amphipol-Trapped Membrane Proteins

Functional and/or ligand-binding studies have been carried out with a dozen different

APol-trapped MPs. These include the calcium ATPase (SERCA1a) from the sarcoplasmic reticulum (SR) (19, 64), the nAChR (21, 54), the BR (5, 23, 32, 66), the transporter enzyme II mannitol (EII^{mdl}) (M. Opačić, J.-L. Popot & J. Broos, unpublished data) and OmpF (68), both from *Escherichia coli*, as well as half a dozen G-protein-coupled receptors (GPCRs) including rhodopsin (68), the leukotriene receptors 1 and 2 (BLT1 and BLT2), the 5HT_{4(a)} serotonin receptor, the CB1 cannabinoid receptor 1, and the GHSR-1a ghrelin receptor (16, 24 and J.-L. Banères, unpublished data). The data indicate that most proteins are still

SERCA1a:
fast-twitch skeletal
muscle sarcoplasmic
reticulum
Ca²⁺-ATPase

functional after transfer to APols and that the binding of small ligands, toxins, and antibodies is unimpaired compared to that observed in biological membranes.

BR, an archaeobacterial light-driven proton pump that accumulates in the so-called purple membrane of *Halobacterium salinarium*, accomplishes its entire photocycle after trapping in A8-35 (32) or in NAPols (5). Compared with the kinetics observed in purple membrane, the first steps of the photocycle are accelerated whether BR is solubilized in detergent or trapped in APols. The last steps feature similar kinetics in APol and in purple membrane, whereas in detergent solution the return to the fundamental state is slower. The kinetics of the photocycle is the same whether BR has been trapped in its native state or refolded in A8-35 from sodium dodecylsulfate (SDS)-solubilized purple membrane, i.e., in the presence of purple membrane lipids (66). On the contrary, when BR is refolded in A8-35 in the absence of lipids, late kinetics is similar to that in detergent solution (23). These observations suggest that the restoration of native-like protein-lipid interactions upon transferring BR from detergent to APol might be responsible for the recovery of purple membrane-like kinetics (23).

The allosteric equilibrium between the resting and desensitized states of the nAChR is strongly perturbed when receptor-rich postsynaptic membranes from the electric organ of *Torpedo marmorata* are solubilized in detergent (20). Membrane-like equilibrium and transition kinetics are recovered when the solubilized preparation is supplemented with A8-35 and diluted below the CMC of the detergent (54). As for BR, one possible explanation is that transfer to APols allows lipids to rebind to critical allosteric sites at the transmembrane surface of the receptor.

Recent studies of five GPCRs folded in APols indicate that their affinity for their ligands is similar in the membrane and in APols. However, as previously observed with rhodopsin (68), G protein activation by A8-35-trapped BLT1 is significantly slowed down compared to that observed in lipid/detergent

mixtures. In contrast, when folded in NAPols, BLT1 catalyzes GDP→GTP exchange on the $G_{\alpha i}$ subunit with kinetics similar to those in lipid/detergent mixtures, suggesting that electrostatic repulsion may account for the slowing down observed in the presence of A8-35 (J.-L. Banères, unpublished data).

Diacylglycerolkinase (DAGK) was fully active when transferred to APol PMAL B-100 (59). Other, less extensive functional studies with such MPs as the maltose transporter from *E. coli* or photosystems I and II are reviewed in Reference 68. The binding of antibodies by APol-trapped MPs is discussed below.

A particularly interesting case is that of SERCA1a, which has been studied in some detail (19, 64) and has been reviewed previously (67, 68). In brief, several APols (A8-35, PMAL C-12, PMAL A C-12, and SAPols) both protected SERCA1a against inactivation and slowed down the overall turnover (ATP hydrolysis) and individual steps in the catalytic cycle (release of trapped Ca^{2+}) (19, 64). Intermediate effects are observed in mixtures of APols and detergent. Trivial mechanisms, such as sequestering of Ca^{2+} or interference with ATP binding, do not account for the inhibition (19). Our current hypothesis about the origin of this phenomenon relies on the kinetics of reorganization of APols around membrane proteins when the latter undergo conformational changes that affect their transmembrane surface. Small (subnanometric) conformational changes, such as those that affect or may affect BR (40) and the nAChR (22, 39), can probably be accommodated by displacements of the APol's alkyl chains, which are expected to be rapid (submicrosecond) and to not involve a high free energy of activation. Larger interfacial movements (nanometer), such as those undergone by the transmembrane helix bundle of SERCA1a upon transiting between the E1 and E2 states (61), may cause a reorganization of the polymer's backbone, which could entail a higher free-energy penalty in APol than in detergent and thereby slow down the enzymatic cycle. As discussed below, this mechanism (called the Gulliver effect; 19, 68) may well contribute

to the protective effect of APols against MP denaturation.

Biochemical Stability of Amphipol-Trapped Membrane Proteins

As a rule, transferring a MP from a detergent solution to APols improves its stability, often dramatically (67, 68). In order to understand the underlying mechanism(s), one should pay attention to the volume of nonmonomeric surfactant to which the protein is exposed to distinguish effects that merely result from a decrease of the hydrophobic sink from those that reflect the interactions of the protein with the surfactant layer that surrounds its transmembrane region.

The effects of trapping a fixed quantity of BR with increasing amounts of APols have been examined in detail (23). Native BR was extracted from purple membrane along with

purple membrane lipids (32). Upon trapping it with A8-35 at BR/APol mass ratios ranging from 1:5 to 1:50, ternary BR/lipids/APol complexes formed. Control samples were stored in either 18-mM or 25-mM octylthioglucoside. Under the experimental conditions used, the volume of the hydrophobic sink was roughly comparable in 18- or 25-mM octylthioglucoside and at BR/A8-35 ratios of 1:10 or 1:20, respectively. Even at 40°C, BR is highly stabilized by A8-35, denaturing by <10% over a week (Figure 3). Under the same conditions, the protein in octylthioglucoside is totally inactivated in less than a day. Whereas a large excess of APols (ratio 1:50) is well tolerated at 4°C and at room temperature, at 40°C it negatively affects the stability of BR, approximately one-third of which is denatured after 6 days (Figure 3). This effect is most likely a consequence of delipidation, which is favored by increasing the volume of the hydrophobic sink (23). Inactivation is nevertheless slow compared

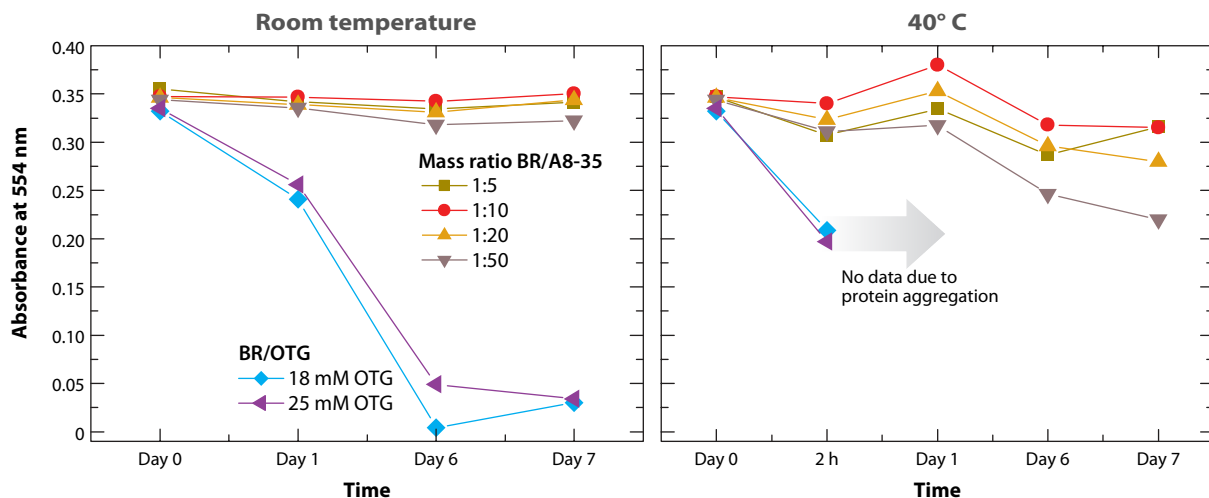


Figure 3

Time stability of bacteriorhodopsin (BR) in amphipol A8-35 versus octylthioglucoside (OTG). BR was extracted with OTG from *Halobacterium halobium* purple membrane (PM), along with PM lipids, trapped in A8-35 (32) at various BR/A8-35 mass ratios, and stored in the dark either at room temperature or at 40°C in a buffer containing 100 mM NaCl and 20 mM sodium phosphate, pH 7.0 ([BR] = 0.22 g·liter⁻¹). Its absorbance at 554 nm, which is proportional to the concentration of the holoprotein, was followed as a function of time. Control samples were kept in 18- or 25-mM OTG (total OTG concentration, including bound detergent). These two concentrations correspond to roughly the same mass concentration of free detergent as that of free A8-35 particles in the samples trapped respectively at 1:10 and 1:20 BR/A8-35 mass ratios. The absence of data points in OTG at 40°C past 2 h is due to the aggregation of the protein, accompanied by complete bleaching. Adapted from Reference 23.

LTB₄: leukotriene B₄
MOMP: major outer
membrane protein
from *Trachomatis*
pneumoniae

to that in octylthioglucoside. Thermostability is also improved: A8-35-trapped BR stands well being exposed at 60°C for 20 min, whereas BR in octylthioglucoside denatures at 40°C (23). BR/A8-35 preparations at a mass ratio of 1:5 (i.e., with ~3 g free APol per g BR) do not exhibit any denaturation even after six months of storage at 4°C (32). BR/A8-35 complexes can be frozen and thawed without denaturing the protein (32), but they cannot be lyophilized (Y. Gohon, unpublished data).

BLT1, one of the receptors of leukotriene B₄ (LTB₄), is significantly stabilized by A8-35. After 25 days at 4°C, no loss of activity is observed whether or not lipids are present. Under the same conditions, BLT1 kept in foscholine-16/asolectin solution loses about half of its activity over the same period. In detergent/lipid mixed micelles, BLT1 denatures at ~27°C, whereas it is stable up to ~35°C when trapped in pure A8-35 and up to ~39°C in the presence of A8-35 plus lipids (24).

Data about the relative ability of various APols to stabilize MPs remain scattered, but they seem to point in at least one direction: The less charges APols bear, the more stabilizing they are. Thus, BR is more stable when trapped in A8-35 than in A8-75, which is a similar polyacrylic acid-derived APol that carries ~75% free carboxylate groups rather than ~35% (87; C. Tribet, unpublished data). Similarly, SERCA1a is more stable in A8-35 than in SAPols, which also carry ~75% of charged groups (64). Cytochrome *b_{6f}*, a highly detergent-sensitive complex (13), is not very stable in A8-35 (5, 87), but much more so in NAPols (5, 70). The stability of BR in SAPols is much greater in the presence of 100 mM NaCl than in its absence (23). A simple rationale for these effects is that, as may be the case for detergents, electrostatic repulsion between net charges carried by the surfactant bound to a MP tends to favor the formation of particles with a small radius of curvature. This would drive the opening of the protein's structure or, in the case of a multisubunit assembly such as cytochrome *b_{6f}*, fragmentation.

Little information is available on the stability of APol-trapped β -barrel MPs. On the one hand, A8-35-trapped OmpA, a monomeric protein, denatures at lower urea concentrations than in the detergent lauryldimethylaminoxide, perhaps a consequence of electrostatic repulsion between the protein and the APol at pH 10 (J.H. Kleinschmidt, unpublished observations). On the other hand, the major outer membrane protein (MOMP) from *Chlamydia trachomatis*, a trimeric porin, is considerably thermostabilized (by >30°C) when transferred from Zwittergent 3-14 to A8-35 (M.J. Cocco, unpublished data). Thus, whether APols stabilize β -barrel MPs seems to depend on the protein, the method of denaturation, or both.

The study of SERCA1a has yielded highly valuable information on the mechanisms underlying the protective effects of APols (19, 64). Three points are particularly worth noting. First, the mere addition of A8-35 to a solution of sarcoplasmic reticulum in C₁₂E₈ or DDM markedly improves the stability of the ATPase, even though it increases the volume of the hydrophobic sink, which should drive delipidation (19). This is yet another indication that an APol environment is intrinsically less denaturing than a detergent environment. Second, as discussed above, trapping with APols reversibly inhibits the activity of the ATPase, to an extent that depends on the nature of the APol and on the presence or absence of detergent (19, 64). Third, there is a correlation between the degree of inhibition and the degree of protection against denaturation: The more stabilizing the environment is, the more inhibitory. Thus, SERCA1a surrounded by a mixture of A8-35 and C₁₂E₈ is both partially inhibited and partially stabilized (19), as is SERCA1a trapped in SAPols rather than in A8-35 (64). These observations suggest that the same mechanism may underlie the two phenomena. The high sensitivity of calcium-free SERCA1a to detergents (19 and references therein) can probably be attributed to the detergent prying apart the transmembrane helix bundle (57), which is stabilized by Ca²⁺ ions (86). One may speculate that the Gulliver effect

suggested above slows down both the natural conformational changes that take place during the enzymatic cycle and the nonnatural conformational excursions that open the structure and initiate denaturation, which could explain the correlation between the two effects.

Transferring MPs from Amphipols to Detergent Solutions or Membranes

APols bind to the hydrophobic surface of MPs in a noncovalent way, but, thanks to their multiple contact points, with an extremely slow dissociation rate. This makes their association with MPs permanent in the absence of a competing surfactant (68, 88, 89, 100), even at extreme dilutions (100). Nevertheless, APols do desorb from MPs in the presence of an excess of competing surfactant, be it free APols (88, 100), detergents (88, 89, 100), or lipids (59, 66).

The displacement of MP-bound APols by free APols was first evidenced upon centrifuging MPs trapped with [^{14}C]A8–75 through a sucrose gradient containing unlabeled A8–75 (88). Subsequently, the exchange rate was determined by measuring the decrease of the FRET signal between tOmpA (the transmembrane domain of *E. coli* OmpA) and FAPol_{NBD} upon addition of an excess of unlabeled A8–35 (100). At low ionic strength, the kinetics of exchange extends over tens of hours. In the presence of 100 mM NaCl, the exchange is essentially over after 10 min. These observations are consistent with a mechanism involving collisions between MP/APol complexes and free APol particles, probably followed by fusion, mixing, and fission. Such a mechanism is also suggested by many other observations: (a) The concentration of nonassembled A8–35 molecules is extremely low (see above), making it highly improbable that they can mediate the exchange between free and bound APol. (b) Retinal, a hydrophobic molecule that does not partition significantly in water, can be delivered from A8–35 particles to bacterio-opsin (the apoprotein) refolded beforehand in the same APol (23). (c) A8–35-trapped tOmpA reversibly oligomerizes upon elimination of free APol particles (100).

APols and detergents mix rapidly and efficiently (25, 26). Isothermal titration calorimetry measurements indicate that neutral detergents (DDM, octylthioglucoside, C₈E₄) partition in anionic (A8–35) or cationic (C22–43) APols, and their mixing is quasi-ideal (26). This result can seem surprising given that neutral and charged detergents do not mix ideally. Charged detergents are typically prone to form smaller assemblies than neutral detergents (and they facilitate the dispersion and denaturation of proteins) because of the coulombic repulsion between their ionized head groups. In ionic APols, however, the charged groups are covalently linked to one another. Covalent association balances the repulsion between vicinal ionic groups, which cancels the coulombic effects at short length scales.

When MP/FAPol_{NBD} complexes are mixed with a nonionic detergent (DDM, C₁₂E₈) in a stopped-flow instrument, the kinetics of displacement of MP-bound APols, as followed by FRET, is extremely rapid (<1 s) (100). The thermodynamics of the exchange of A8–35 and C22–43 for octylthioglucoside or C₈E₄ has been studied in detail by isothermal titration calorimetry (89). The exchange is isenthalpic and entropy-driven. Fluorescence and isothermal titration calorimetry data converge toward the conclusion that APols mix with MP/detergent complexes and MP-free detergent micelles nearly ideally, so that at equilibrium the layer of MP-bound surfactant and the free mixed micelles have almost the same composition (89, 100). This is an important observation because it implies that APols have no special affinity for MP transmembrane surfaces. Isothermal titration calorimetry studies also provide insights into the origin of the difference of behavior of MP/APol versus MP/detergent complexes at low surfactant concentration. Upon dilution of a solution of MP/detergent complexes below the CMC of the detergent, entropy favors the aggregation of the protein because it frees hundreds of detergent molecules as monomers. APols, on the contrary, stick to MPs even at extreme dilutions (100), because their desorption entails no

tOmpA:
transmembrane
domain of OmpA from
Escherichia coli

Folding: refers to a MP acquiring the 3D structure of the native protein, without having ever been correctly folded previously

significant entropy gain (for a discussion, see Reference 89).

APols can also be displaced by lipids. Two studies have described the spontaneous delivery of APol-trapped MPs to preformed lipid vesicles and black lipid films. In the first study, DAGK was delivered from complexes with the APol OAPA-20 to palmitoylcholine vesicles while retaining its ability to phosphorylate dibutyrylglycerol (59). The second study reported the transfer of two A8-35-trapped β -barrel MPs, OmpA and FomA, to black lipid membranes (66). After transfer, the two proteins formed channels with properties similar to those observed after reconstitution from a classical detergent solution.

At concentrations sufficient to deliver MPs, APols are neither cytolytic nor toxic when they are applied to living cells or injected into mice (68, 82; D. Bagnard, G. Crémel, L.M. de la Maza, unpublished observations). This opens onto extremely interesting basic and biomedical applications, where APols are used to deliver MPs, MP fragments, or other hydrophobic molecules to cell membranes.

There are two caveats to APol-mediated delivery of MPs to preexisting membranes. First, this type of transfer can be expected to be a high-risk event for the protein, as some of its hydrophilic extramembrane regions have to cross the membrane, which necessarily exerts strong distorting forces on the structure. DAGK, FomA, and OmpA are robust proteins, and in the experiments mentioned above (59, 66), it is not known which fraction of them actually adopt a transmembrane orientation without being denatured. This somewhat brutal approach is unlikely to result in high yields of functional insertion with more fragile proteins, unless they refold easily. Second, the APols with which the protein was initially trapped can be expected to remain associated with the target membrane while most likely diffusing away from the protein. When A8-35-refolded OmpA and FomA were applied to black lipid films, the properties of the channels they formed indeed appeared perturbed unless A8-35 was also added on the *trans* side

of the film (66). This suggests that adsorbed APol molecules affect the electrostatics and/or the internal pressure gradient of the lipid bilayer (66). Other studies have shown that APols by themselves can form pores in natural (19, 82) or artificial (51, 82, 92, 93) membranes. As shown below, when FAPol_{NBD}-trapped transmembrane peptides are delivered to COS cells, the polymer integrates the plasma membrane along with the peptides (see **Figure 8**). Vigilance regarding possible artefactual effects is therefore in order.

APPLICATIONS

The original idea behind the design of APols was that, by trapping MPs under a water-soluble form more stable than that in detergent solutions, APols would make it easier to purify them and to explore in vitro their structural and functional properties. As the study of APols progressed, however, it became apparent that their applications extended beyond this original concept. In the present section, we examine those applications of APols that have been tested and either validated or found problematic, along with an assessment of their prospects and/or limitations.

Folding Membrane Proteins to their Native State

Overexpressing MPs as inclusion bodies allows production of large amounts of protein, which is hard to achieve when directing them to cell membranes. However, it is difficult to fold to their native state the inactive MPs obtained in this way. To date, in vitro folding of MPs in detergents, detergent/lipid mixtures, or lipid vesicles has been achieved for a dozen of β -barrel MPs (14) and about as many α -helical MPs. The latter include BR (42, 69); the major light-harvesting complex II of higher plants (75); DAGK (37); the small multidrug transporter from *E. coli*, EmrE (58); and a few GPCRs (2-4, 47). Conditions for efficient folding tend to be highly idiosyncratic.

The feasibility of using APols to assist MP folding was first established using as models two β -barrel MPs, OmpA and FomA, from the eubacteria *E. coli* and *Fusobacterium nucleatum*, respectively, and an α -helical MP, BR (66). Following dilution of urea-unfolded OmpA or FomA into urea-free buffer in the presence of A8-35, both proteins developed β -sheet secondary structure, became protected from proteolysis by trypsin, and recovered the electrophoretic mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that is characteristic of the folded β -barrel (66). The folding yields were near 100%. Upon application of the refolded MPs to black lipid films, single-channel recordings showed the formation of functional pores (66). OmpA has also been refolded in NAPols, albeit with much slower kinetics (J.H. Kleinschmidt, unpublished data). tOmpA has been renatured in SAPols, and the recovery of the native structure has been demonstrated by solution NMR (23).

When a solution of denatured BR in SDS solution is supplemented with A8-35 in various mass ratios and dodecyl sulfate precipitated as its potassium salt (PDS), the purple color of native BR starts to develop within minutes. Following removal of PDS crystals by centrifugation and overnight dialysis, UV-visible absorption spectra indicate that BR renaturation is complete at BR/A8-35 wt/wt ratios of either 1:5 or 1:10. The synchrotron radiation circular dichroism spectrum of refolded BR is indistinguishable from that of the native protein (**Figure 2**) (23). Upon illumination, A8-35-refolded BR undergoes its full photocycle (23, 66). Similar results have been obtained in NAPols (5).

The ability of APols to induce and stabilize the native fold of MPs has been further explored using a series of GPCRs from *E. coli* inclusion bodies purified under denaturing conditions (in SDS solutions). Conditions initially established to refold BR were applied virtually without changes to folding the LTB₄ receptors BLT1 and BLT2, serotonin receptor 5-HT_{4a}, cannabinoid receptor CB1 (24), and

ghrelin receptor GHSR-1a (J.-L. Banères, unpublished data). Folding yields of 40%–70% were systematically achieved, based on ligand-binding experiments. For all five receptors tested, addition of exogenous lipids (in 1:5 mass ratio to the APol) significantly increased the refolding yields (e.g., from ~50% to ~70% for BLT1). The pharmacological properties of APol-folded receptors, including the K_d values for agonists, are close to those observed in either detergent solutions or membrane fractions. As observed for most APol-trapped MPs, GPCRs folded in A8-35 are significantly more stable than those kept in lipid/detergent mixtures (24). Folding and stabilization of the BLT2 receptor in A8-35 have made it possible to determine by solution NMR the structure of BLT2-bound LTB₄. The BLT1 and GHSR-1a receptors have also been folded in NAPols, with yields similar to those achieved in A8-35 (J.-L. Banères, unpublished data).

Cell-Free Synthesis of Membrane Proteins

In vivo overexpression of MPs has to navigate the Charybdis of limited expression under a functional form and the Scylla of poor folding yields of mass-produced but inactive protein. An alternative approach that has attracted much attention over the past few years is to express target MPs in vitro, using a cell lysate that contains the machinery for transcribing and translating genes carried by appropriate plasmids (84, 101). Cell-free synthesis presents the advantages of doing away with toxicity issues and of lending itself to straightforward labeling using limited amounts of labeled amino acids (48). MPs, however, will precipitate if synthesized in the absence of any surfactant. Three main strategies have been described: (a) expression in the absence of surfactant, followed by solubilization of the precipitate with a detergent; (b) expression in the presence of a detergent; and (c) expression in the presence of a lipid bilayer, in the form of either lipid vesicles or nanodiscs (15, 44, 46). The third approach

Refolding: refers to MPs that were initially obtained under their native state and have been denatured using urea, SDS, or organic solvents, prior to being brought back to their initial 3D structure

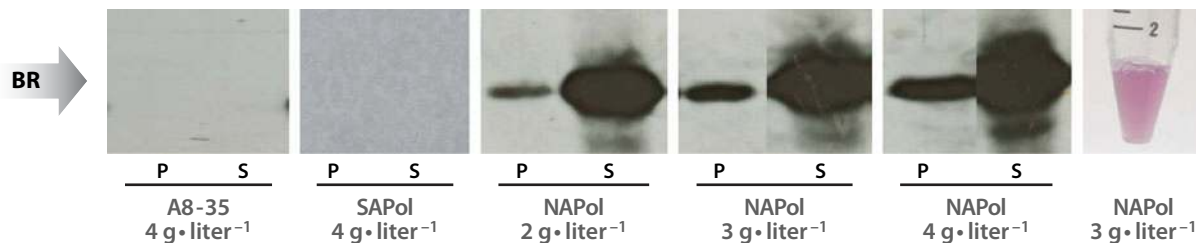


Figure 4

Amphipol-assisted cell-free expression of bacteriorhodopsin (BR). In vitro synthesis of polyhistidine-tagged BR in the presence of retinal and of A8-35, SAPols, or NAPols at the indicated concentrations. At the end of the synthesis, the samples were centrifuged at $16,000 \times g$ for 20 min. The proteins present in the pellet (P) and supernatant (S) were separated by SDS-PAGE on a 12% polyacrylamide gel and detected using an anti-His-tag antibody. Far right panel: BR after purification on a nickel column. The purple color of the solution indicates the presence of native BR (representing at least two-thirds of the protein present in the purified sample). Adapted from Reference 5.

suffers from two disadvantages: The amount of protein that can be thus produced tends to be limited by the small volume of lipids that can be made available, and not all MPs insert correctly into preformed bilayers in the absence of the translocon machinery (50). The first two approaches involve the use of detergents. Resuspending precipitated MPs does not always yield a functional protein (49). Expression in the presence of detergent is a priori a hazardous, and certainly not a universal approach: Given that many, if not most, fully folded MPs become unstable upon being exposed to detergents, it is to be expected that many of them will not reach their native state when synthesized in their presence. Indeed, while many MPs can be expressed in this way (81), demonstrations of their functionality are rare (7, 45).

It is therefore of great interest to examine whether less aggressive surfactants are compatible with cell-free synthesis (11, 62, 63, 72). Both A8-35 and SAPols, however, inhibit the synthesis of MPs (Figure 4), without affecting that of a control soluble protein, the green fluorescent protein (63). It may be that these APols, both of which are polyanions, inhibit translation by simultaneously binding to MP hydrophobic segments as they appear out of the ribosome tunnel and interacting with positively charged patches at the surface of the ribosome. This hypothesis is supported by the observation that excellent results are obtained when BR is expressed in

vitro in the presence of NAPols: Not only is the yield of synthesis ($\sim 0.4 \text{ g}\cdot\text{liter}^{-1}$ in the presence of $3 \text{ g}\cdot\text{liter}^{-1}$ NAPol) higher and more reproducible (5) than that observed in the presence of DDM (63), but a majority of BR ($\sim 90\%$) is present in a soluble form (Figure 4) and remains so over several months (5), whereas in DDM it tends to precipitate (63). BR expressed in the presence of NAPols and retinal folds to a greater extent (at least two-thirds) than in detergent, as shown by the appearance of the purple color characteristic of the holoprotein (Figure 4) (5). Preliminary data indicate that NAPol-assisted cell-free synthesis also applies to GPCRs, as exemplified by the leukotriene receptor BLT1 (E. Billon-Denis, F. Zito, J.-L. Banères, unpublished data).

NMR

Early NMR experiments were aimed at better characterizing MP/APol complexes and at exploring the resources and limitations of NMR to study their structure and dynamics. Three small, well-characterized β -barrel proteins from the outer membrane of either *E. coli* (tOmpA and OmpX) or *Klebsiella pneumoniae* (KpOmpA) were chosen as models. These three proteins, whose 3D structures are known, have been extensively studied by solution NMR, mostly in the presence of detergent. NMR studies of their complexes with

KpOmpA:
transmembrane
domain of OmpA from
Klebsiella pneumoniae

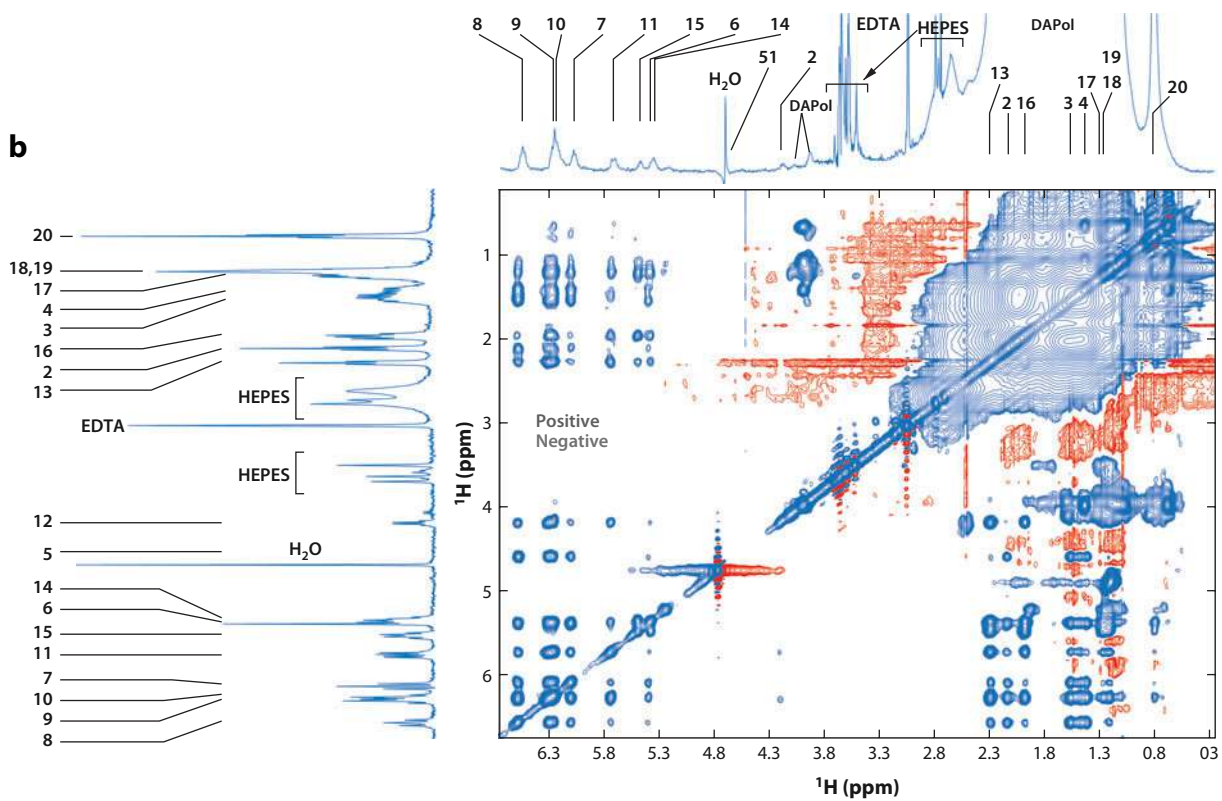
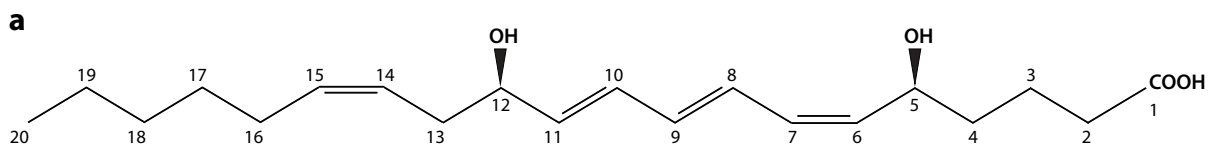
APols showed them to either retain or regain their native fold when associated to A8-35 (17, 99; M. Renault & A. Milon, personal communication), to SAPols (23), or to NAPols (5). The complexes appear slightly bigger than MP/detergent complexes, based on estimates of the overall correlation time, τ_c . For instance, the correlation time of A8-35-trapped OmpX (31 ns) (17) lies between that of OmpX in solutions of dihexanoylphosphatidylcholine (24 ns) (52) and that of OmpX inserted into small isotropic bicelles (35 ns) (53). The slightly larger size of MP/APol complexes compared to those formed with the most favorable detergents does not preclude obtaining high-resolution spectra nor achieving enough sensitivity to perform 3D experiments. Indeed, 3D ^{15}N -edited $[\text{}^1\text{H}, \text{}^1\text{H}]$ -HSQC-NOESY spectra have been collected to explore dipolar interactions between KpOmpA and A8-35 (M. Renault & A. Milon, personal communication). Comparative 2D $[\text{}^{15}\text{N}, \text{}^1\text{H}]$ -TROSY spectra of BR in detergent solution, in nanodiscs, and in A8-35 have been recently published (73).

A drawback of A8-35 for NMR studies is the need to work at $\text{pH} > 7$ in order to avoid aggregation. A high pH renders the observation of exchangeable protons more difficult, if not impossible in some cases, when the chemical exchange becomes too fast (17, 99). This has been one of the incentives leading to the development of pH-insensitive APols, such as SAPols and NAPols, both of which make it possible to record NMR spectra under acidic conditions (5, 23). These new APols, however, suffer from other disadvantages: The purification of SAPols is demanding, which currently limits the amounts that can be conveniently produced (23), and NAPols, due to their complex chemical structure (**Figure 1c**), cannot be easily perdeuterated.

NMR spectroscopy has been used to examine the organization of MP/A8-35 complexes, yielding an increasingly detailed view of protein-polymer interactions. A general description of the distribution of the alkyl chains was first obtained, taking advantage of the variation of dipolar environment experienced

by the amide protons of tOmpA upon trapping with either A8-35 or its deuterated homolog (99). Two further studies exploited intermolecular ^1H - ^1H or ^{13}C - ^1H dipole-to-dipole cross-relaxation phenomena. They yielded a qualitative detection of spatial proximities between ^{13}C atoms in the side chain of aromatic residues of OmpX and ^1H nuclei of A8-35 (18) and the identification of hydrophobic contacts between specific amide protons of KpOmpA and octyl and isopropyl chains of A8-35 (M. Renault & A. Milon, personal communication). The three studies showed that contacts with the alkyl chains of A8-35 are confined to the transmembrane, hydrophobic surface of the proteins. $^1\text{H}/^2\text{H}$ exchange measurements show that some of the amide protons of the membrane-spanning region of OmpX exchange much more readily than others, which likely reflects the dynamics of the barrel (17).

Application of solution NMR to GPCRs is rare, mainly due to the difficulty of producing sufficient amounts of functional receptors (10 and references therein). APol-assisted folding of BLT2 has made it possible to solve by NMR the structure of its natural ligand, LTB_4 , in its receptor-bound state. Perdeuterated BLT2 receptor was overexpressed in *E. coli* as inclusion bodies and folded to its native state (24) in deuterated A8-35. The high-resolution structure of the BLT2-bound leukotriene was derived from transferred nuclear Overhauser effect signals (16). The ligand undergoes a drastic reorganization upon binding, switching from an elongated structure with many coexisting rotamers to a highly constrained conformation (**Figure 5**). This information is of great interest from a pharmacological point of view, because it opens new perspectives in BLT-targeted drug design (no antagonists of this LTB_4 -mediated proinflammatory pathway are available today for clinical use). It also contributes to a detailed analysis of the molecular events that lead to receptor activation. Because the ability of APols to fold and stabilize MPs seems quite general, the same strategy can probably be transposed to many other systems.



Immobilizing Membrane Proteins onto Solid Supports for Ligand-Binding Studies

The development of sensitive detection methods and the implementation of high-throughput technologies increasingly resort to the immobilization of functional proteins onto surfaces. On the one hand, protein arrays combined with optical detection provide an efficient mode of high-throughput screening, with a particular potential in diagnostics (95). On the other hand, surface-sensitive techniques such as surface plasmon resonance (97) and fluorescence microscopy provide information regarding the kinetics of interaction, in addition to the classical thermodynamic parameters (91).

Because of the poor stability of purified MPs in detergent solutions, many current studies are carried out in native (41, 55) or reconstituted lipid membranes (9, 78), which entails a series of technical constraints: nonspecific binding to other proteins and/or lipids, the difficulty of working out and standardizing protocols for MP reconstitution and for the immobilization of membrane fragments or vesicles, and the impossibility to access simultaneously the extracellular and intracellular domains of MPs.

Reversible or irreversible affinity tags are generally used to interface MPs with surfaces without disturbing the protein's function. Tags are typically added by fusing to the target MP a short peptide or a cargo-protein (38, 85). This approach is not applicable to proteins extracted from native tissues. In that case, the protein is chemically modified, usually on NH_2 or SH groups.

APols provide a novel, highly versatile way to immobilize MPs. Its advantages over the above approaches are many:

- Trapping with functionalized APols does not alter the structure of the protein, and it leaves its N and C termini free for protein-ligand interactions or for other modifications.
- The protein can be trapped with a functionalized APol after its purification, during folding, or during cell-free synthesis.
- Although the MP/APol association is highly stable, it can be displaced by detergents or other APols. Affinity tags can thus be added or removed as desired.
- Because MPs are typically surrounded by at least five to six molecules of APols, several functional groups can be combined in a controlled stoichiometry, by the simple device of trapping the protein with a mix of appropriately functionalized APols.
- The MP benefits from the stabilization afforded by APols.
- The MP can (and, as a rule, must) be handled in surfactant-free solutions, which facilitates the handling of fluidics and detection using surface-sensitive techniques such as surface plasmon resonance.

It should be noted, however, that the location of the functional groups in the APol belt cannot be controlled and vary from one protein to the next, as well as over time, which, depending on the experiment, can be considered either a liability or an asset.

Whereas several APols carrying affinity tags have been or are being synthesized, biotinylated A8–35 (BAPol) is the best characterized to

BAPol: biotinylated
A8–35

Figure 5

Amphipol-assisted folding and stabilization of a G-protein-coupled receptor allow NMR determination of the structure of a receptor-bound ligand. The perdeuterated BLT2 receptor of proinflammatory leukotriene B₄ (LTB₄) was expressed as inclusion bodies in *Escherichia coli*, solubilized in SDS, and folded in deuterated A8–35 (DAPol). The structure of BLT2-bound LTB₄ was determined by analyzing transferred nuclear Overhauser effect signals. (a) Chemical structure of LTB₄. (b) Dipolar interactions in an LTB₄/[u-²H,¹⁵N]BLT2/DAPol sample, 2D NOESY spectrum (mixing time $\tau_m = 0.5$ s, $\nu_H = 600$ MHz, 25°C). The corresponding 1D ¹H spectrum is shown above the 2D spectrum. The 1D spectrum of free LTB₄ in solution is displayed on the left side. Numbers refer to the protons annotated on the chemical structure of LTB₄ in panel a. (c) Three-dimensional structure of LTB₄ bound to BLT2 (hydrogen atoms are light gray, oxygen atoms are red; and carbon atoms are assigned a different color for each conformer). Adapted with permission from Reference 16. Copyright 2010 American Chemical Society.

date. As described above, trapping with APols as a rule does not interfere with ligand binding. The immobilization of BAPol-trapped MPs of various sources and sizes to streptavidin-coated chips or beads does not impair their function, e.g., the binding of a toxin and of a small acetylcholine analog to the nAChR, or the photocycle of BR, nor does it prevent the specific recognition of antibodies by tOmpA, BR, and cytochromes bc_1 and b_{cf} (21). The binding of antibodies and/or toxins to APol-trapped MPs has also been observed for OmpF (68) and MOMP (M.J. Cocco & L.M. de la Maza, unpublished data). APol-mediated MP immobilization thus appears as a promising system for diagnostic assays, as well as for functional investigations of ligand and protein interactions with MPs, with potential applications in drug screening. Prospects include the use of more sophisticated supports (electrodes, nanoparticles, and other nano-objects) and of other tagged APols. Histidine-tagged (F. Giusti & P. Kessler, unpublished data) and oligonucleotide-carrying APols (C. Le Bon & F. Giusti, unpublished data), for instance, would provide for the reversibility of the immobilization and, in the latter case, targeting, with a strong potential for the development of protein arrays. Tagged NAPols would reduce the probability of electrostatically driven nonspecific binding.

X-Ray Crystallography

The application of APols to MP crystallography would provide fascinating perspectives. This goal, however, may seem a priori hard to reach, because MP/APol complexes appear more polydisperse than MP/detergent complexes. In the case of charged APols, this difficulty is compounded by the need to achieve the right balance between minimizing the electrostatic repulsion between APol layers and preventing aggregation. Over the past few years, however, considerable progress has been made toward identifying factors that favor the formation of monodisperse preparations of MP/APol particles. Furthermore, repulsive interactions between MP/A8-35 complexes can be cancelled

or even turned to attractive interactions by appropriately modulating the ionic strength of the solutions (68).

Current attempts have focused on a model MP, mitochondrial cytochrome bc_1 , which combines the advantages of being sturdy, colored, relatively easy to crystallize, and of featuring large extramembrane domains that provide anchoring points for the formation of well-ordered 3D contacts while keeping protein-bound surfactant layers away from each other (8). A8-35 was added to purified, detergent-solubilized cytochrome bc_1 , and the detergent adsorbed onto polystyrene beads. Crystallization trials were carried out by vapor diffusion. Under the many crystallization conditions tested, complete elimination of the detergent precluded any crystals from forming. On the contrary, ternary cytochrome bc_1 /A8-35/detergent complexes did crystallize, in the presence of concentrations of salt and polyethylene glycol higher than is required for crystallization from detergent solution (D. Charvolin, M. Picard, E.A. Berry & L.-S. Huang, unpublished data). The red color of the crystals indicated that they do contain cytochrome bc_1 (**Figure 6a**). The presence of APols in the crystals was established by growing them in the presence of FAPol_{NBD} (**Figure 6b**). A few of these crystals have been tested on a synchrotron X-ray source and proved to diffract, with unit cell dimensions consistent with their being protein crystals. The current resolution of the patterns, however, is poor (>20 Å) (**Figure 6c**). It is not known whether this reflects an intrinsic property of the crystals, arises from problems with handling and/or freezing them, or is due simply to the limited number of trials that could be carried out. There are several possible reasons why cytochrome bc_1 /A8-35/detergent complexes crystallize whereas bc_1 /A8-35 ones do not. One of them is that the presence of detergent lowers the electrostatic charge density of the surfactant layer, and it may allow for easier charge redistribution. Another is that, as observed with tOmpA (100), ternary complexes may be more closely monodisperse than binary ones.

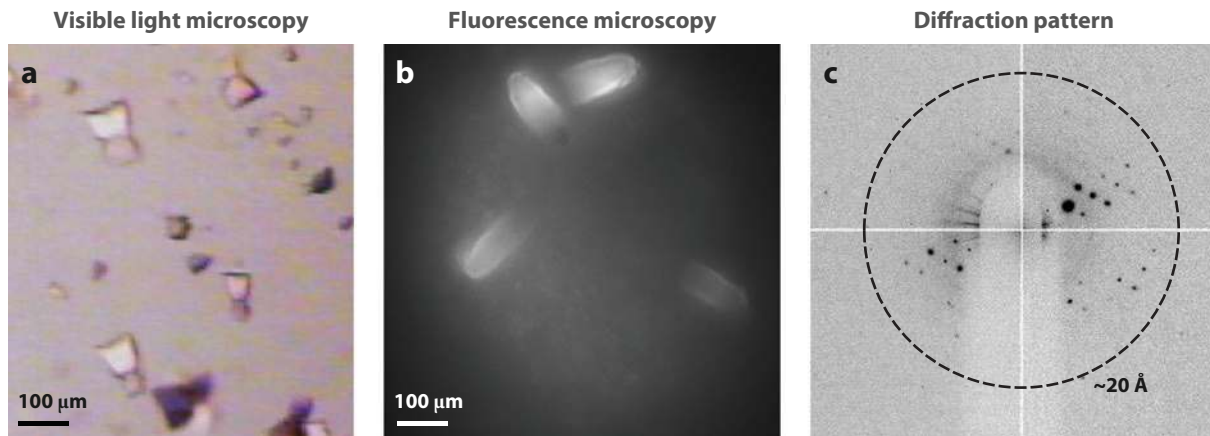


Figure 6

Crystals of ternary cytochrome *bc*₁/A8-35/detergent complexes. (a) Crystals obtained in the presence of A8-35, observed by visible light microscopy. (b) Crystals obtained in the presence of an NBD-labeled analog of A8-35, observed by fluorescence microscopy. (c) Diffraction pattern (collected at the ESRF facility in Grenoble) of one of the crystals shown in panel b. (From D. Charvolin, M. Picard, E.A. Berry and L.-S. Huang, unpublished data). Abbreviation: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

Although these experiments were interrupted, they show that crystallizing APol-trapped MPs is indeed feasible. The advent of NAPols opens new, exciting perspectives in this field, because their use would eliminate the electrostatic repulsion that is, presumably, the major handicap in using charged APols such as A8-35.

Electron Microscopy

APol-trapped MPs have been observed by scanning transmission electron microscopy (EM) (90), by transmission EM after negative staining (1, 28, 32, 67 and references therein) and by cryo-EM (1, 28). Because APols are mild surfactants, they may be particularly well suited to single-particle EM studies of fragile MP supercomplexes. Supercomplexes of the mitochondrial respiratory chain are stable and soluble in A8-35 after removal of the digitonin used for solubilization, and present NADH-dehydrogenase and cytochrome *c* oxidase activity after purification (1). Images of supercomplex B (consisting of one copy of complex I, two of complex III, and one of complex IV) adsorbed onto a continuous carbon support film were recorded under cryo-conditions

(**Figures 7a,b**). The particles exhibited similar views as in digitonin (80). A 3D volume (**Figure 7c**) was calculated by the random conical tilt method, revealing many details

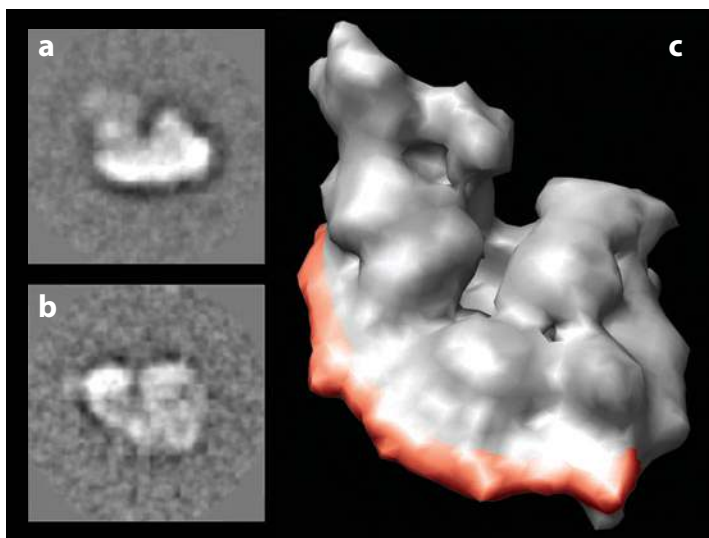


Figure 7

Cryo-electron microscopy (cryo-EM) reconstruction of mitochondrial supercomplex B (I₁III₂IV₁) trapped in APol A8-35. (a,b) Typical 2D projections from cryo-EM representing side (a) and top (b) views. (c) 3D reconstruction as seen slightly tilted from the matrix side. The red fringe indicates the likely distribution of A8-35. See Reference 1.

not previously visible. In keeping with NMR data, A8-35 seems to form a belt along the transmembrane region of the supercomplex (Figure 7c) (1).

Proteomics: 2D Gels and Mass Spectrometry

2D gel electrophoresis is a standard technique in proteomics. It is used, in particular, to study protein expression in cells. Single spots can be picked out from the gels and mass spectrometry performed to determine the mass of each protein and collect sequence information. In the first dimension, proteins are usually separated according to their pI by isoelectrofocusing in a pH gradient. APols that carry a net charge cannot be used for isoelectrofocusing, but NAPols can. The feasibility of this approach has been tested using NAPols whose solubility relies on the presence of either hydroxyl groups (70) or glucose moieties (71). In the second dimension, APols are expected to be displaced by SDS. [APols indeed do not affect SDS-PAGE patterns (59, 66, 90).] In brief, the results show that these two types of NAPols can indeed be used to run 2D gels (5; Y. Gohon, unpublished data) and the spots excised and subjected to mass spectrometry analysis (Y. Gohon, P. Bazzacco, E. Billon-Denis, C. Béchara, G. Bolbach & S. Sagan, unpublished data).

Matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) measurements have been performed on several APol-trapped MPs, namely OmpX (18), tOmpA, BR, and the cytochrome *bc*₁ and *b₆f* complexes (P. Bazzacco, C. Béchara, G. Bolbach & S. Sagan, unpublished data). Neither NAPols nor ionic APols are detected, probably due to their high polydispersity. No significant differences are observed between MP/APols and MP/detergent complexes. Thus, mass spectrometry could be used to check on the extent of isotopic labeling of A8-35-trapped OmpX (18). In preparations of A8-35-trapped and NAPol-trapped cytochrome *b₆f*, all but two of the eight subunits were detected, albeit with variable yields, as well as free heme and, for NAPol-trapped com-

plexes, some lipids (P. Bazzacco, C. Béchara, G. Bolbach & S. Sagan, unpublished data).

Altogether, these results indicate that A8-35 and NAPols are compatible with MALDI-TOF mass spectrometry analyses of both intact MPs and of their digests, and that NAPols are suitable to perform isoelectrofocusing and 2D gel electrophoresis. The stabilizing properties of APols ought to facilitate analyses of fragile MPs and MP complexes by both of these techniques.

Amphipols as Vectors for the Delivery of Membrane Proteins and Transmembrane Peptides

Upon injection of MP/A8-35 complexes into mice, no toxicity is observed. Antibodies are produced against MPs, not against APols (68). In cell cultures, A8-35 is not cytolytic, at least at the concentrations needed to deliver MPs to the plasma membrane (68). This has led to the examination of APols as vectors for immunization or other therapeutic purposes.

The transmembrane domain of bitopic receptors, which is made up of a single hydrophobic α -helix, plays an important role in their oligomerization (43, 56). Recent evidence indicates that peptides mimicking the transmembrane domain of neuropilin-1 (NRP1), a receptor controlling various biological effects ranging from cell migration to cell proliferation and cell death (76), are able to block its biological functions (77). In addition to helping to dissect signaling mechanisms, these peptides also present a therapeutic relevance in cancer. Preclinical studies have demonstrated that blocking NRP1 using transmembrane peptides in tumor cells or in the endothelial cells establishing the neovascular network supporting tumor growth largely reduces brain tumor expansion and dissemination both in vitro and in vivo (60). However, NRP1 transmembrane peptides are insoluble and prone to aggregation, which makes it difficult to ensure optimal distribution and efficacy in whole organisms. APols keep various hydrophobic peptides soluble (27, 30, 96), suggesting

that they could be used as solubilizing and vectorizing agents. An analysis has been undertaken of the biodistribution of APol-trapped transmembrane peptides applied to COS cells (**Figure 8a**). Confocal microscopy showed that the complexes can reach and enter the plasma membrane within minutes. Both peptides and APols are endocytosed after a few hours, leading to a total clearance of the plasma membrane in four days. To examine whether APols could be potentially useful delivery agents *in vivo*, mice bearing subcutaneous tumors were injected with FAPol_{NBD}. Histological examination of tumor slices revealed strong staining of intratumor blood vessels (**Figure 8b**) (G. Crémel & D. Bagnard, unpublished data).

Another potentially promising therapeutic use of APols is the formulation of vaccines. Even though MPs from pathogenic organisms represent *a priori* privileged targets for the immune system, their production and handling pose formidable obstacles in the way of manufacturing affordable vaccines. The ability of APols to fold overexpressed MPs and to stabilize them may help to circumvent these difficulties. Recent data indicate that vaccines formulated with MOMP/A8–35 complexes protect mice against *C. trachomatis* infection better than vaccines containing MOMP/detergent complexes, to a level approaching that observed upon vaccination with the live organism (L.M. de la Maza, unpublished data). It remains to be established whether this improvement results from the strong stabilization of MOMP by A8–35, from a different presentation of the immunogen to cells of the immune system, or from a combination of both mechanisms. However, given how general MP stabilization by APols is, it is probably worth examining the possibility of extending this approach to MP-based vaccines against other infectious diseases, possibly also against some forms of cancer. The development of therapeutic uses of APols is still at a preliminary stage. However, the above data suggest that APols may provide an interesting alternative to the use of detergents, liposomes, and nanoparticles whenever organisms are to be injected with MPs or transmembrane fragments thereof.

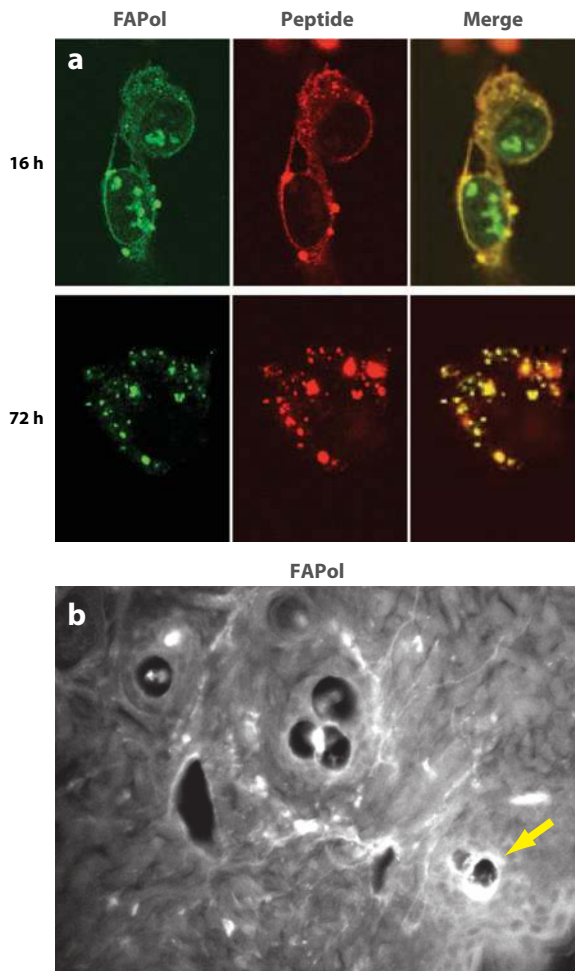


Figure 8

Using amphipols to deliver transmembrane peptides to cells and tissues. (a) Confocal microscopic analysis of the distribution of NBD-labeled A8–35 (FAPol_{NBD}; green) and a rhodamine-labeled neuropilin-1 transmembrane peptide (red) 16 and 72 h after application of peptide/FAPol_{NBD} complexes to COS-7 cells. Note that the distribution of the two labels does not totally overlap, indicating at least partial segregation. After 72 h, the plasma membrane has been cleared of both labels. (b) Distribution of FAPol_{NBD} in tumor slices. Mice bearing subcutaneous tumors (C6 glioma) were injected intraperitoneally with 100 μ L of 10^{-7} M FAPol_{NBD} 24 h before tissue collection. Vascular and perivascular staining demonstrate tissue penetration (yellow arrow). Unpublished data from G. Crémel & D. Bagnard. Abbreviation: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

CONCLUSION

Much is now known about what can and what cannot be advantageously done by substituting detergents with APols. Some properties of MP/APol complexes, e.g., the dynamics of

APol-trapped MPs, remain to be more fully explored. Many applications have been validated but not fully exploited, whereas the difficulties met by other applications, e.g., 3D crystallization, remain to be solved. Many more applications will appear and develop as novel APols become better characterized (nonionic APols in particular), as the variety of functions grafted

onto APols increases, and as more laboratories familiarize themselves with their use. It is our hope that the work invested by so many people over so many years to turn APols into practical, reliable tools will provide the community of MP specialists with enough solid data and incentives so that their use becomes more widespread.

SUMMARY POINTS

1. APols are a special class of amphipathic polymers designed to keep integral MPs water soluble in the absence of detergents.
2. Various types of APols have been tested and validated. They have in common the ability to assemble in aqueous solutions in the form of small, globular, well-defined particles.
3. APols make MPs water soluble by adsorbing onto their hydrophobic transmembrane surface. The APol layer is thin and remains stable even at high dilutions.
4. MP-adsorbed APols can be readily exchanged for detergents, lipids, or other APols.
5. Most MPs are much more stable as MP/APol complexes than they are in detergent solutions. Several mechanisms contribute to this effect.
6. APols, as a rule, do not interfere with ligand binding or with MP function. They may, however, slow down some transconformations or interactions.
7. APols are an excellent medium in which to fold MPs to their functional state, and some APols can be used for MP cell-free synthesis.
8. APols lend themselves to many applications, including structural studies, proteomics, MP immobilization, and delivery.

DISCLOSURE STATEMENT

P. Bazzacco, D. Charvolin, F. Giusti, B. Pucci, C. Tribet, and J.-L. Popot are coauthors of patents on amphipols and their applications.

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