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Amphipols: polymeric surfactants for membrane biology research

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Abstract. Membrane proteins classically are handled in aqueous solutions as complexes with detergents. The dissociating character of detergents, combined with the need to maintain an excess of them, frequently results in more or less rapid inactivation of the protein under study. Over the past few years, we have endeavored to develop a novel family of surfactants, dubbed amphipols (APs). APs are amphiphilic polymers that bind to the transmembrane surface of the protein in a noncovalent but, in the absence of a competing surfactant, quasi-irreversible manner. Membrane proteins complexed by APs are in their native state, stable, and they remain water-soluble in the absence of detergent or free APs. An update is presented of the current knowledge about these compounds and their demonstrated or putative uses in membrane biology.

Key words. Membrane proteins; surfactants; detergents; amphipols.

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Solubilization and inactivation of membrane proteins by detergents

Integral membrane proteins are involved in such essential cell functions as bioenergy transduction, transmembrane transfer of nutrients and drugs, signal detection and cell-to-cell communication, adhesion, tissue formation and so on. They comprise about one-third of the proteins encoded in the genome of eukaryotic cells, and a majority of the targets of currently marketed drugs. A detailed knowledge of their structure, function and dysfunction is essential to a wide range of biomedical and biotechnological applications. In vitro studies of integral membrane proteins, however, are severely complicated by their insolubility in water, to the point that these proteins represent only ~0.2% of currently available high-resolution structures. The insolubility of membrane proteins is due to the highly hydrophobic character of those protein surfaces that, in situ, are in contact with the membrane interior. Detergents classically are used to handle them in aqueous solutions. Detergents are small amphiphilic molecules that mix well with lipids and, as a result, can partition into biological membranes and, under favorable circumstances, solubilize them. Thereby, membrane components become dispersed into lipid/detergent mixed micelles and protein/detergent complexes, the latter usually retaining protein-bound lipids. Detergent molecules adsorb cooperatively onto the transmembrane surface of the protein and form a monolayer-like assembly, which is in rapid equilibrium with aqueous monomers and protein-free micelles [1, 2]. Membrane proteins aggregate and, in general, precipitate when the concentration of free detergent falls under its critical micellar concentration (CMC), an indication that the detergent layer has come apart and the protein transmembrane surface has become exposed to water. Physical stability of the solutions is ensured by keeping the concentration of free detergent above the CMC, but this frequently entails biochemical instability.

The instability of detergent-solubilized proteins is a major problem in membrane biology. Its origin is seldom studied in detail, but in most well-characterized instances it results from the dissociating character of detergents: the very property that enables them to extract the protein from its environment makes them prone to interfere with intra- or intermolecular interactions that stabilize it. A frequent observation is that newly solubilized proteins that are reasonably stable when kept in the solubilization supernatant inactivate when they are moved to a fresh detergent solution, as occurs in the course of purification. This phenomenon usually can be traced to stabilizing components, such as lipids or hydrophobic cofactors, partitioning into detergent micelles. Yet free micelles need be present since detergents cannot be used under their CMC[1]. Some types of functional and/or structural studies can be carried out after the protein has been reinserted into artificial lipid vesicles, which very often stabilizes it. However, an isotropic solution is generally needed, be it for purification or for biophysical studies. Classical protective measures are to limit the amount of free micelles, to supplement them with lipids or cofactors and/or to transfer the protein, once solubilized, to a less dissociating detergent. These countermeasures, however, are often imperfect, and they generate their own problems (see e.g. 3–5).

This difficult situation has prompted the development both of 'protein-friendly' detergents (see [1, 5]) and of alternative media based, for instance, on the use of non-detergent surfactants and/or nonmicellar phases (reviewed in [5]). Over the past few years, we have been exploring two original approaches. The first one relies on diminishing the miscibility of the surfactant and lipids, so that micelles will not act as sinks. The difficulty is for the surfactant to remain able to prevent protein aggregation. It appears that these seemingly incompatible requirements can be met by hemifluorinated surfactants, which are designed to mix poorly with lipids while adsorbing efficiently enough onto the transmembrane surface of the proteins [5–8]. The second concept is to do away altogether with free micelles. This implies such a high affinity of the surfactant for the surface of the protein that protein/surfactant complexes remain stable in the presence of very low concentrations of free surfactant, which means that the dissociation rate constant has to be vanishingly small. One way to achieve this result, as discussed in this review, is to engineer multi-point attachment between a polymeric surfactant and the protein.

Rationales for the design of amphipols

Amphipols (APs) are amphipathic polymers specially designed to keep membrane proteins soluble [9]. The association properties of amphipathic polymers and their adsorption onto hydrophobic surfaces have been extensively studied by physical chemists (see e.g. [10], and references therein). The molecules studied are often very

[1] In principle, no micelles are present at the cmc of the detergent. Thus it is theoretically possible to work with membrane protein/detergent complexes in the absence of micelles by keeping the free detergent concentration exactly at the cmc or slightly below (the chemical potential of detergent in a mixed detergent/protein/lipid complex has to be lower than in a pure detergent micelle). However, as the cmc is a function of temperature, ionic strength and the presence of other solutes in the medium, the precise concentration to be used at each step would have to be determined empirically. The practice has been rather to use detergents at slightly above the cmc so that micelles are present and buffer the detergent activity at the cmc, whatever that may be under a particular set of experimental conditions.

big, typically hundreds or thousands of kDa, with a long hydrophilic, highly charged backbone to which a few very hydrophobic (e.g. octadecyl) groups are attached. When bound to a mineral particle, or a droplet of oil, they make it hydrophilic and water soluble. Because of the strong electrostatic repulsion between polymer-coated particles, very high loads can be obtained without precipitation. In industry, such polymers are used for instance in the manufacturing of inks or paints.

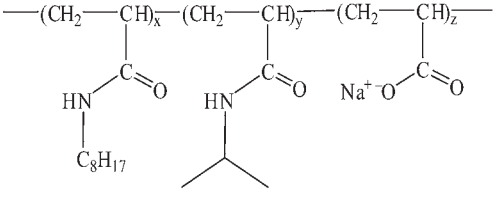
Our rationales for the design of amphipathic polymers adapted to membrane protein studies were the following [9]:

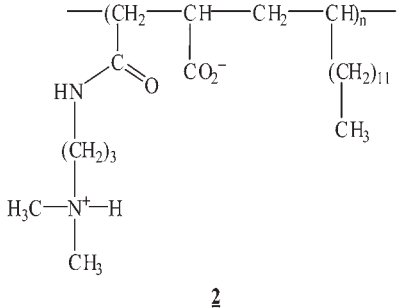
1) The basic idea was that they should bind to the surface of the protein by multiple attachment points, so as to diminish the dissociation rate and render the association quasi-irreversible; this implied that the molecules be big enough and soluble enough to carry many hydrophobic chains.
2) APs had to be much smaller than the amphipathic polymers ordinarily used by physical chemists, with a size commensurate with that of protein transmembrane regions (typically a few nanometers).
3) They ought to be highly flexible, so as to be able to adapt to the very small radius of curvature of protein surfaces and to their irregularities; in physical terms, their persistence length, which is the distance past which a polymer chain keeps no memory of its initial direction, had to be sufficiently short.
4) To favor formation of a thickly packed layer around the transmembrane region of the protein, rather than a fluffy one, we used closely spaced hydrophobic groups, separated by short loops; for the polymer to remain sufficiently soluble, the hydrophobic groups would be kept short.
5) From the point of view of organic chemistry, it was desirable to keep the synthesis of APs relatively simple; applications to structural biology, in particular, which may consume hundreds if not thousands of milligrams of material, would be jeopardized by a complicated and costly synthesis; amphipols of course also ought to be chemically stable for extended periods (weeks or months) in biological buffers.

Amphipol structure

Polyacrylate-based APs

The concept of APs was initially implemented using hydrophobically derivatized polyacrylates [9] (fig. 1, **1**). At variance with, for instance, a polypeptide chain (which otherwise would provide an attractive way to prepare well-defined APs), a polyacrylate backbone does not contain any conjugated bonds, and it does not form hydrogen bonds with itself. This makes it highly flexible. The extension and flexibility of polymer chains are often characterized by two complementary parameters: (i) the average radius of gyration, $\langle R_g \rangle$, and (ii) the persistence length, L_p (length of a segment along the chain that would require an energy of ~1 kT to be curved by a radius equal to its own length). The size and persistence length of polyelectrolytes such as polyacrylate-based APs depend





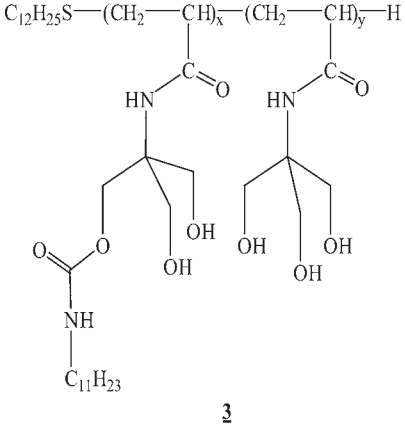


Figure 1. Structure of amphipols. **1**, polyacrylate-based, anionic AP; for amphipol A8-35, with which most of the results discussed in the present review have been obtained, $x \simeq 0.25$, $y \simeq 0.40$ and $z \simeq 0.35$; for an average chain, this corresponds respectively to ~17, ~28 and ~25 monomers, randomly distributed [9]. **2**, PMAL-B-100, an AP that is zwitterionic at neutral and basic pH and cationic at acidic pH [15]. **3**, a nonionic AP obtained by random cotelomerization of hydrophilic and hydrophobic monomers derived from THAM [19].

on intrachain hydrophobic attraction, electrostatic repulsion, and therefore on charge density and ionic strength. A lower ionic strength is expected to result in significant swelling of the polymer coil, due both to electrostatic stiffening (longer persistence length due to the repulsion between successive monomers) and to increasing excluded volume around monomers (repulsion between monomers that can be distant along the chain but become close in space in the coiled polymer).

Experimental studies of the persistence length of highly charged polyacrylates are based on viscometry or light-scattering measurements of chain extension [11, 12]. It appears difficult in practice to clearly distinguish between the contributions of electrostatic stiffening and excluded volume to the observed effects of ionic strength. If these effects are entirely attributed to stiffening, an apparent value of the persistence length, L'_p, can be determined. Because this analysis neglects excluded volume effects, L'_p exceeds the true value of L_p. L'_p is usually written as:

$$L'_p = L'_i + L'_e$$

where L'_i is the apparent intrinsic persistence length (i.e. the asymptotic value at infinite ionic strength) and L'_e the electrostatic component. L'_i characterizes the rigidity of the chain under an imaginary uncharged form and includes rotameric, steric and secondary structure features. For polyacrylates, estimates of L'_i vary from 1.4 nm [11] to 2.7 nm [12], and L'_e has been found to vary as the reciprocal square root of ionic strength. For copolymers of acrylamide and sodium acrylate, for instance, the following empirical relation is obtained [12]:

$$L'_p\,(\text{nm}) = L'_i + 35\ \xi/C_s^{0.5}$$

with C_s the salt concentration (in mM) and $\xi = l_b/b$ (Manning charge parameter), where b is the intercharge spacing along the chain and l_b the Bjerrum length ($l_b = q^2/\varepsilon \cdot k_B \cdot T \simeq 0.72$ nm at 25 °C in water). For highly charged polyelectrolytes, b is taken equal to l_b and $\xi \simeq 1$ [12]. For derivatized polyacrylates in which two out of three carboxylates have been neutralized (i.e., purely hydrophilic polymers with the same charge density as amphipol A8-35), and in 100 mM salt, which is at the lower end of salt concentrations generally used in membrane biochemistry, L'_e estimates are close to 3.5 nm [12] or 3.9 nm [11].

Estimates of L'_e obtained by the above approach are larger (by a factor of ~2) than the theoretical values of L_e calculated by widely accepted theories. In addition to the overestimation entailed, as mentioned above, by neglecting excluded volume effects, one must note that small errors in estimates of the actual charge density, length polydispersity and the 'end-effects' characteristic of short polymers all significantly affect the variations of apparent persistence length, as shown by Monte Carlo simulations [13]. The above estimates therefore must be considered as giving a (fair) order of magnitude of the persistence length of polyacrylate chains and the way it depends on ionic strength, rather than rigorous determination. Altogether, the persistence length of polyacrylates in biological solutions appears likely to be small enough not to prevent a close association between the derivatized polymer and membrane proteins, which is borne out by experiment (see below).

In most of our own work, the average length of the chain has been ~70 monomer units (see below), i.e. ~19 nm when fully extended. This corresponds to an average molecular mass of ~5 kDa for the underivatized polymer, ~8 kDa after derivation [9]. Similar molecules have been used (under the name OAPA-20) by C. R. Sanders and coworkers [14, 15]. Less extensive experiments have been carried out with longer chains (~300 monomers [9]) and with shorter ones (~25 monomers [16]). The solubility of polyacrylate-based APs can be improved by partial grafting with sugar groups [C. Prata and C. T., unpublished data].

It is difficult to synthesize polyacrylate molecules of perfectly defined length. Our starting material usually has a polydispersity of ~2 (polydispersity is defined as the ratio of the weight average molecular weight over the number average moledular weight, i.e. $(\bullet\ n_i M_i^2/\bullet\ n_i M_i)/(\bullet\ n_i M_i/n_i)$, where n_i stands for the numer of molecules of molecular mass M_i in a given volume of solution). Upon size exclusion chromatography (SEC) analysis, the width of the peak at half-height covers the range from 2 to 16 kDa. One way to improve APs certainly lies in limiting this considerable polydispersity. This can be achieved either upon synthesis, e.g. by resorting to living radical telomerization or to ionic polymerization, or a posteriori by fractionation. Our experience indicates that both fractionation and telomerization yield final products with trapping properties similar to those of more polydisperse preparations, but a detailed comparison remains to be done. An interesting question that remains open to this day is the extent to which membrane proteins may select, from the mixture of AP molecules they are exposed to, those they have the highest affinity for, in which case it could turn out to be counterproductive to aim for perfectly defined APs.

Amphipathy was conferred by derivation with fatty amines. In most of our experiments, this was done with octylamine, but dodecylamine was also tested, less extensively, with satisfying results [17, and unpublished data]. The degree of derivation is a critical parameter. APs that succeed in maintaining membrane proteins soluble have to be highly derivatized. Our own experiments have generally aimed for 25% derivation with octylamine. Too high a level of derivation yields insoluble polymers, while too low levels lead to poor results when it comes to trapping membrane proteins. The charge density along the

polymer can be lowered by amidation of a further 40% of the carboxylates with isopropylamide. This does not seem to have much of an effect on the ability of the polymers to keep membrane proteins in solution [9], but there are some indications that a lower charge density may favor the biochemical stability of the trapped proteins [9, 18] (see below). The two APs with which most experiments have been carried out to date (fig. 1, **1**) have final molecular masses around 8 kDa, 25% derivation with octylamine, and, depending on whether they have been further derivatized with isopropylamine, either 75 or 35% free carboxylates. They are dubbed A8-75 and A8-35, respectively, where 'A' stands for 'acrylate', '8' for the average molecular mass, in kDa, and the last figure for the percentage of free carboxylates. On average, a single molecule of A8-35 contains ~70 acrylate units, ~17 of which carry an octyl chain, ~25 a free carboxylate and the rest an isopropyl. The various types of units are randomly distributed. In this nomenclature, the OAPA-20 molecules used by Sanders and co-workers [14] would be called A8–80.

Strong-acid, nonionic and zwitterionic APs

Polyacrylate-based APs, which hitherto have been by far the most extensively studied, have the two characteristics of carrying a net charge and of being weak acids. The first feature prevents the use of membrane protein/AP complexes in some experimental situations, such as ion-exchange chromatography or isoelectrofocusing, and is likely to be a hindrance in others, such as crystallization. The second one limits their use to neutral or basic pH, since protonation of the carboxylates induces aggregation and precipitation. When monodispersity of the complexes is essential, as in crystallization attempts, it is even advisable not to work below pH 7.5 (see below). This is a problem also for nuclear magnetic resonance (NMR) applications, where it is useful to limit amide proton exchange by lowering the pH, or when working with proteins that are more stable at acidic pH. To alleviate these constraints, other types of APs have been experimented with.

The weak-acid character can be avoided by substituting sulfonate groups for carboxylates. The properties of polysulfonated APs are currently under study [F. Giusti and M. Zoonens, unpublished data]. The development of overall neutral APs is in a more advanced stage. We have shown that nonionic amphipathic polymers derived from tris(hydroxymethyl)-acrylamidomethane (THAM) (fig. 1, 3) can be used to stabilize membrane proteins in aqueous solutions in the same manner as polyacrylate-derived APs do [19]. The study of the complexes thus obtained is in progress. Others have successfully used APs that are zwitterionic at neutral and basic pH (fig. 1, 2) [15]. Preliminary experiments using hydrophobically derivatized pullulane (a polysaccharide) have also been reported [20].

AP synthesis and purification, and their importance for biochemical applications

A very important point needs to be stressed. Membrane biochemists are used to detergents. Detergents have their problems (many of them), but they usually equilibrate rapidly between phases and compartments. To quote a figure, the residency time of an octyl-β-D-glucopyranoside (C_8-G) molecule in a micelle is in the microsecond time range [21]. A biochemist having to deal with a membrane protein/AP preparation will instinctively think that any slow process in the system has to do with the protein. This is not always true. Polymers, because they are long molecules, do not necessarily rapidly find a path to their free energy minimum (see e.g., for adsorption phenomena, [22]). They present a hysteresis of their own and can become locked into undesirable states, e.g. aggregated ones. We have found out (the hard way) that two chemically identical preparations of APs, or different samples from the same batch after having been handled differently, can exhibit widely different aggregation properties, which in turn reflect on those of the complexes they form with membrane proteins [18]. This problem, which is briefly discussed in the next section and will be further documented in forthcoming articles, should be kept in mind by anyone working with APs, and appropriate controls carried out (see next section).

Solution properties of amphipols

Detailed studies of the solution properties of pure APs are available only for A8-35 [18]. A8-35 molecules auto-organize into small micelles, ~30 kDa in mass, with a relatively narrow size distribution: upon SEC, 'good' (see below) preparations of A8-35 exhibit a half-height peak width only slightly larger than that of a soluble protein of comparable mass, horseradish peroxydase [18]. A8-35 particles have been studied by SEC, small angle X-ray and neutron scattering (SAXS and SANS), quasi-elastic light scattering (QELS) and analytical ultracentrifugation (AUC) [18, and unpublished data]. The data are compatible with the existence of more or less spherical objects containing ~1.5–2 g of bound water per gram of AP. SANS measurements suggest that the particles do not feature a well-defined hydrophobic core. This may seem surprising. It becomes much less so, however, if one reflects that the distance that separates two alkyl chains along the polymer (statistically, eight C–C bonds, i.e. ~1 nm when the chain is fully extended) is similar to the length of an octyl chain, and significantly shorter than the Stokes radius of a particle (~3 nm according to SEC and SANS measurements). This prevents hydrophilic and hydrophobic groups from segregating completely. Because the size of the particles formed by pure A8-35 is not very different from that of small proteins, and because the

density of this polymer is relatively high (1.16 $g \cdot l^{-1}$; [C. Ebel, unpublished data) and not very different from that of membrane proteins (typically ~1.33 $g \cdot l^{-1}$), it is not straightforward to totally separate protein/A8-35 complexes from excess A8-35 when trapping small proteins such as the transmembrane domain of outer membrane protein A (tOmpA) or bacteriorhodopsin (BR), unless one resorts to affinity chromatography [18, and M. Zoonens and F. Zito, unpublished data].

Polydispersity is a major concern when preparing APs. It falls, roughly, in two categories. First, A8-35 preparations contain a few percents (in mass) of very large particles (MDa range). These are not a concern in most experiments because they are a small minority and do not associate with membrane proteins [18]. The trouble starts with particles that are only slightly larger than the minimal size. These particles are found in many AP preparations, and upon protein trapping, they do associate with proteins and yield polydisperse protein/AP complexes. This may not be too much of a hindrance for some applications, such as when exploring the environment of membrane proteins or their spectroscopic or ligand-binding properties, but it is a major hurdle in others, in particular crystallography or solution-state NMR. Extensive investigations have led us to the conclusion that polydispersity is not an intrinsic property of some batches of APs that would be chemically different. It actually depends on the way the polymers have been purified and handled. Polydispersity, for instance, develops over time if an aqueous solution of A8-35 is kept for several days at pH ″ 7 [C. Prata and C. Tribet, unpublished data). More details will be given elsewhere. From a practical point of view, suffice it to say that it is advisable, whenever monodispersity is a concern, to check, e.g. by SEC or AUC, on the size and dispersity of the particles formed by the pure polymer under the conditions to be used, and not to let the pH of the solutions fall below ~7.5.

If the complexity of the solution behavior of A8-35 is any indication, any rational development of APs will depend on rather extensive studies of the physicochemical properties of each new type of molecule. This is a harsh constraint because these studies are rather laborious and, to the biochemist, unrewarding. Short-cutting them, however, will most likely lead to unsatisfying if not irreproducible results. As of now, much less information is available about APs other than A8-35. Preliminary observations indicate that upon SEC nonionic, THAM-derived APs behave as particles whose size is commensurate with A8-35 particles and PMAL-B-100 as somewhat larger ones [D. Charvolin, Y. Gohon and F. Giusti, unpublished data].

Effects of amphipols on biological and artificial membranes

APs are not detergents, or, more exactly, they are only very weak ones. Biological membranes as a rule are not solubilized by soaking them in a solution of APs [9, 17]. This basic observation, however, must be qualified. First, APs do interact with membranes. They permeabilize the sarcoplasmic membrane to calcium ions [17] and form pores in black lipid membranes [A. Ghazi, personal communication]. Giant (tens of micrometers) egg phosphatidylcholine unilamellar vesicles exposed to A8-35 (called 5-25C8-40C3 in that paper) first produce filaments and buds before breaking up into small vesicles, whereas in the presence of A8-75 (called 5-25C8), they take up polyedral shapes and show evidence for lateral segregation of their components [23]. Upon extended incubation (hours), A8-35 is able to disperse large (tens of nanometers) unilamellar dipalmitoylphosphatidylcholine/dipalmitoylphosphatidic acid vesicles into large (~10 nm in radius) mixed micelles [24]. A8-35 also dissolves lipid monolayers at the air-water interface [M. Flötenmeyer and K. Leonard, unpublished data), but not monolayers of fluorinated lipids [J. Dietrich and C. Vénien-Bryan, personal communication]. Certain proteins, such as the maltose transporter from *Escherichia coli* [M. Zoonens and H. A. Shuman, unpublished data] or the human insulin receptor overexpressed in Chinese hamster ovary cells [G. Crémel, personal communication], can be directly extracted by A8-35. Hydrophobically modified pullulane has been reported to extract proteins from *E. coli* and *Pseudomonas fluorescens* outer membranes [20].

Because APs are not strong detergents, they can potentially be used to deliver membrane proteins to preformed membranes. This has been best documented in a study of the delivery of amphipol-trapped diacylglycerol kinase (DAGK) to lipid vesicles [14]. Fibroblasts and mouse embryonic stem cells survive prolonged exposure (days) to concentrations of APs up to 0.05–0.1 $g \cdot l^{-1}$, i.e. higher than those they would need be exposed to in a protein delivery experiment [J. Barra, personal communication]. This is an interesting novel experimental situation, because unlike protein/detergent complexes, which are destabilized by dilution below the CMC of the detergent, a protein/AP complex injected into a cell culture medium or the buffer that bathes a black film will remain physically stable until it interacts with the target membrane. Upon interaction, it is likely that the polymer associates with and diffuses over the surface of the membrane, favoring the insertion of the surfactant-depleted protein. Needless to say, the insertion process must subject proteins to strong distorting forces, and only the toughest of them can be expected to survive this step and remain functional. Nevertheless, APs clearly open an interesting new avenue to reconstitution or cell culture experiments.

In keeping with the low toxicity of APs in cell culture, pure APs or protein/AP complexes can be injected into mice (to the level of 50 µg per injection) without any visible pathogenic effect. According to ELISA tests and immunoreplicae, antibodies are raised against the trapped proteins, but not against APs [C. Leclerc, Y. Pierre and Y. Gohon, unpublished data).

Keeping membrane proteins soluble with amphipols

In a typical trapping experiment, a purified protein in detergent solution is supplemented with an amount of AP (usually in the range of 1–10 g per gram of protein) that largely exceeds the binding capacity of the protein (typically 0.2–1 g/g; see below, table 2). The concentration of the detergent is then lowered below its CMC. This can be achieved in a variety of ways. Often, the solution is first diluted below the CMC, after which detergent monomers can be eliminated by adsorption onto Bio-Beads, by dialysis or by running the protein/AP complexes onto a sucrose gradient or a molecular sieve [9, 17–19, 25–27]. Depending on the protocol chosen and the size of the protein, excess AP may or not be more or less efficiently removed at that stage, which may affect the behavior of the protein/AP particles (see below). In some cases, Bio-Beads have been added directly to the ternary mixture without prior dilution below the CMC [17, 18, and D. Charvolin, Y. Gohon and M. Zoonens, unpublished data]. It is also possible to attach protein/detergent complexes onto an affinity column [14, 15, and M. Zoonens and H. A. Shuman, unpublished data] or a BIAcore chip [28] and carry out the exchange of surfactant on the immobilized protein (fig. 2). The dispersity of the final particles may depend on the protocol chosen [17].

An important observation is that APs bind to proteins even in the presence of detergent micelles. The formation of such ternary complexes is essential to the success of trapping [25]. Although its understanding is likely to be critical to that of the properties of the final complexes, the succession of events that lead from a protein/detergent complex to a protein/AP one has not yet been studied in detail. It is not known, for instance, what the kinetics of formation of the ternary complexes are, nor whether their composition evolves over extended incubation, nor has it been determined whether additional binding of APs takes place upon lowering the detergent concentration below its CMC nor during its final elimination.

Once formed, and in the absence of competing surfactants, the association between APs and membrane proteins is extremely stable. Experiments in which complexes of membrane proteins and radioactive APs have been centrifuged for several hours onto gradients containing surfactants or not have shown: (i) the absence of a measurable loss of radioactive AP when the gradient contains neither AP nor detergent; (ii) partial AP exchange in the presence of unlabeled AP and (iii) desorption of most of the bound AP if the gradient contains detergent above its CMC [25]. Extensive washing of OmpF/AP complexes adsorbed onto BIAcore chips with surfactant-free solutions revealed no AP desorption either over tens of minutes (fig. 2) (Q. Hong, J.-L. Popot and J. Lakey, unpublished data). Whether complexes that are washed for hours or days with surfactant-free buffer release some of the bound APs remains, however, an open question. A limited amount of desorption, difficult to detect with the approaches used thus far, could indeed account for the observed tendency of cytochrome *bc*1/A8-35, BR/A8-35 and tOmpA/A8-35 complexes to form small, soluble aggregates following extensive removal of free AP [D. Charvolin, Y. Gohon and M. Zoonens, unpublished data, and 18]. Similar observations have been made with the Ca^{2+}-ATPase, some of the latter data suggesting that this process may be modulated by the presence or absence of bound lipids [17]. It is, however, difficult, in the case of the Ca^{2+}-ATPase, to sort out aggregation phenomena that are due to the physical properties of the particles from those that may be subsequent to protein denaturation. The extensive removal of APs from protein/AP complexes upon exposure to detergents above their CMC [25] offers an alternative route to reconstitution of

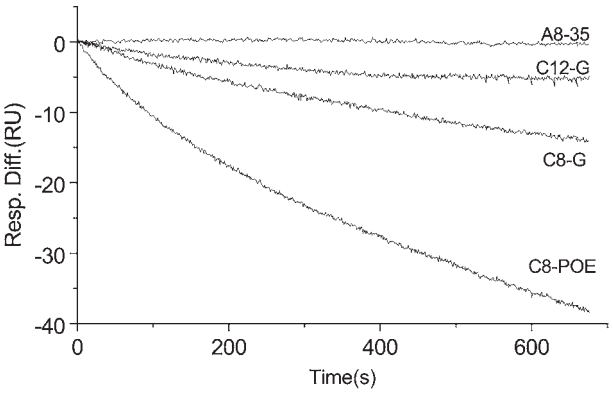


Figure 2. Dissociation rates of surfactants from immobilized OmpF porin. An OmpF mutant (D183C) with a single cysteine in a periplasmic turn was labeled with biotin maleimide and immobilized, at the level of 150 resonance units (RU), on the surface of a BIAcore SA chip bearing covalently attached streptavidin. The buffer contained 10 $g \cdot l^{-1}$ octylpolyoxyethylene (C_8-POE), 10 mM HEPES, 150 mM NaCl and 3.4 mM EDTA, pH 7.4. The chip was washed with a 10 $g \cdot l^{-1}$ solution (or, in the case of amphipols, a 1 $g \cdot l^{-1}$solution) of either amphipol A8-35, dodecyl-β-D-glucopyranoside (C_{12}-G), C_8-G or C_8-POE, and the exchange of surfactant followed by surface plasmon resonance (SPR) at a flow rate of 5 $\mu l \cdot min^{-1}$. Once a stable baseline had been achieved, i.e. after 30–60 min (zero RU value on this graph), the solution was replaced with surfactant-free buffer ($t = 0$) and the dissociation of surfactant monitored by SPR. The signal from a blank surface bearing only streptavidin was subtracted from the rough data, so that the data shown represent the evolution over time of the amount of surfactant that is actually bound to OmpF. (Q. Hong, J.-L. Popot and J. Lakey, unpublished data).

Table I. A list of integral membrane proteins whose complexes with amphipols have been studied in some detail (others are mentioned in the text).

Protein name	Source	Function	Subunits	Overall mass (kDa)	Secondary structure	APs	Properties of complexes			References
							Soluble	Native	Functional	
Cytochrome b_6f	*Chlamydomonas reinhardtii*	redox pump	2 × 8	228	α	A, N	+	+	(+)	9, 19, 25, 26
Bacterio-rhodopsin	*Halobacterium salinarum*	light-driven H^+ pump	1	27	α	A, N	+	+		9, 18, 19
OmpF porin	*Escherichia coli*	pore	3 × 1	102	β	A	+	+	+	9, 25, 28[a]
Photosynthetic reaction center	*Rhodobacter sphaeroides*	light-driven redox pump	3	96	α	A	+	+		9
Rhodopsin	*Bos taurus*	G-protein-coupled receptor	1	39	α	A	+	+	±	U
tGpA	*[E. coli]*	?	2 × 1	~8	α	A	+	+		16, U
Ca^{2+}-ATPase	*Oryctolagus cunniculus*	calcium pump	1	110	α	A	+	+	±	17
Complex I	*Neurospora crassa*	redox pump	~35	1120	(α)	A	+	+		30[b]
Acetylcholine receptor	*Torpedo marmorata*	gated channel	2 × 5	535	(α)	A	+	+	+	27
tOmpA	*[E. coli]*	?	1	19	β	A, N	+	+		34, U
Maltose transporter	*E. coli*	ABC pump	4	150	(α)	A	+	+	±	U
Cytochrome bc_1	*Bos taurus*	redox pump	2 × 11	490	α	A, N	+	+		U
Diacylglycerol kinase	*[E. coli]*	kinase	3 × 1	40	(α)	A, Z	+	+	±, +	14, 15
Photosystem II reaction center	*Pisum sativum*	light-driven redox pump	5	103	α	A	+	+	+	U
Photosystem II reaction center	*Thermosyne-chococcus elongatus*	light-driven redox pump	2 × 17	550	α	A, N	+	+	+	U

[a] Q. Hong, J.-L. Popot and J. Lakey, unpublished data.
[b] M. Flötenmeyer et al., unpublished data.
Source of the protein: brackets indicate heterologous or ectotopic expression; tGpA: glycophorin A transmembrane region ; tOmpA: outer membrane protein A transmembrane region. *Secondary structure:* α-helix bundles (α) or β-barrels (β); parentheses indicate that no high-resolution structure is available. *APs:* 'A', anionic (most often A8-35); 'N', non-ionic, THAM-based; 'Z', zwitterionic. *Native state:* '+' indicates that there are strong indications (spectroscopic or others) that the protein or complex is in its native state; *Functional:* '+' means that at least one important function, such as ligand binding, has been tested and found native-like; '±' means that the AP-trapped protein was only partially functional (low activity, or not all tested functions present); '(+)' refers to the fact that cytochrome b_6f was tested in a medium containing detergent above its CMC (see text). *References:* the names of the authors of unpublished work ('U') are given in the text.

AP-trapped proteins that would not resist direct integration into preformed vesicles, namely that of exchanging APs for detergent before performing a classical reconstitution from detergent solution.

No data are available concerning the dynamics of protein-bound APs. It is reasonable to think that even though exchange with the solution may be very slow, adsorbed molecules are constantly moving and wriggling, with individual alkyl chains temporarily leaving the surface and rebinding to it, or crawling over the protein or protein-bound lipids. By analogy with the dynamics of detergent molecules in micelles [21], one may speculate that the residency time of most AP octyl chains at a given point of the protein surface is likely to be in the sub-microsecond range. Because of the dense crisscrossing of the surface by the polymers (statistically, anchoring points are about 1 nm apart), the question may be asked whether AP dynamics has an influence on the kinetics of protein conformational transitions that involve large-scale (nanometer) rearrangements of the transmembrane surface (see below).

The ability of APs to keep membrane proteins soluble in the absence of detergent micelles does not depend on any

Table 2. Composition of membrane protein/amphipol complexes, and a comparison with that of membrane protein/dodecylmaltoside ones.

Protein	MW kDa	Amphipols						Dodecylmaltoside Bound C_{12}-M	
		Amphipol type	salt mM	Bound amphipols			Bound lipids	# of C_{12}	g/g
				# of C_8	kDa	g/g			
$(b_6f)_2$	228	A8-75	20	103[a]	46	0.22	+[b]	260[c]	0.65
RC	102	A8-75	18	83[a]	41	0.41	?	148[d]	0.90
$(OmpF)_3$	96	A8-75	30	83[a]	41	0.43	?		
$(AChR)_2$	535	A8-35	120	330[e]	150	0.28	+[e]		
BR	27	A8-35	120	110–120[f]	50–55	1.8–2	+[f]	208[d]	4.1
tOmpA	19	A8-35	120	31[g]	14	0.75	–[g]		
$(bc_1)_2$	490	A8-35	120	110–142[h]	49–63	0.10–0.13	?		

Data refer to the dimeric cytochrome b_6f complex from *C. reinhardtii*, the photosynthetic reaction center from *R. sphaeroides*, trimeric OmpF from *E. coli*, dimeric acetylcholine receptor from *T. marmorata*, monomeric bacteriorhodopsin from *H. salinarum* and the monomeric transmembrane domain of OmpA from *E. coli*. The amount of bound AP per particle was determined using radioactive amphipols (all proteins) and by SANS and AUC (BR, tOmpA). There is evidence that the amount of bound AP may depend on the ionic strength at which trapping was performed [25] and on the way the particles were separated from free APs [18]. AP binding is expressed in kDa per particle, as the mass of AP per mass of protein and as the number of AP octyl chains per particle. The presence or absence of lipids was ascertained using radioactive lipids (b_6f), chemical analysis (AChR, BR, tOmpA) and biophysical measurements (BR, tOmpA). The binding of C_{12}-M, which has a similar mass per alkyl chain, is shown for comparison. Data are from the following sources: *a* [25]; *b* [26]; *c* [3]; *d* [29]; *e* [27]; *f* [18]; *g* [34 and unpublished data]; *h* [D. Charvolin, unpublished data].

protein feature. As shown in table 1, proteins that have been shown to remain soluble under these conditions encompass the whole gamut of size, complexity, secondary structure, transmembrane mass distribution, subcellular localization and function.

It appears that with due caution, APs can be used to explore interactions between membrane proteins. Trapping with A8-35 of a mixture of BR monomers and oligomers in octylthioglucoside (C_8-TG) solution yielded protein/AP complexes whose polydispersity reflected that of the original mixture [18]. Trapping of a mixture of monomers and dimers of the nicotinic acetylcholine receptor (nAChR) in CHAPS resulted in a mixture of monomeric and dimeric nAChR/A8-35 complexes [27], while trapping of purified monomeric and dimeric forms of cytochrome b_6f yielded AP-trapped monomers and dimers [26]. When carried out under appropriate conditions, trapping of membrane proteins with APs therefore seems to 'freeze' the association forms they have in detergent solution. In keeping with this conclusion, trapping with either of the four original APs [9] of a supernatant of thylakoid membranes from *Chlamydomonas reinhardtii* solubilized with the detergent Hecameg yielded a mixture of protein/AP complexes that could be resolved by sucrose gradient fractionation into the complexes classically observed in Hecameg solution [C. Tribet and J.-L. Popot, unpublished data]. As discussed below, trapping of detergent-sensitive complexes is probably one of the most interesting applications APs can be put to.

Composition, structure and solution properties of membrane protein/amphipol complexes

Currently available quantitative data about the composition of protein/AP complexes are summarized in table 2 and illustrated in figure 3 by models showing what a large (cytochrome bc_1) and a small (tOmpA) protein trapped by APs may look like. Two points are particularly worth noting: (i) a given protein always binds much less AP (in mass) than detergent, the difference being, in some cases, as high as a factor of 3; (ii) lipids, whenever associated with the protein in detergent solution, become trapped, forming a ternary protein/lipid/AP complex. The lower mass of bound AP as compared with detergent has an important implication, namely that it translates into a smaller number of alkyl chains (table 2). Cytochrome b_6f, for instance, binds ~260 molecules of dodecylmaltoside (C_{12}-M) per dimer [3] and only ~100 A8-75 alkyl chains [25]; a photosynthetic reaction center, ~150 molecules of C_{12}-M [29] vs. ~80 A8-75 octyl chains [25]; BR and its associated lipids, ~280 molecules of C_8-TG [18] against ~120 A8-35 octyl chains [18]. This points to a different organization of the alkyl chains of the surfactant with respect to the hydrophobic surface of the protein and, possibly, a higher accessibility of the latter to water. As regards the trapping of lipids, it can have profound effects on the stability and functionality of the protein (see below). It provides also, potentially, a novel approach to investigating specific protein/lipid interactions: ternary complexes formed soon after solubilization, either by trapping from a crude detergent supernatant or, in favorable cases, upon direct solubilization by amphipols, can be purified in the absence of detergent and their composition analyzed.

The one series of experiments that has been carried out using radioactive detergent indicated that following separation of detergent monomers and AP-trapped cytochrome b_6f on surfactant-free sucrose gradients, detergent removal was extensive: from ~260 in detergent solution [3], the amount of bound [^{14}C]C_{12}-M fell below the detection threshold of ~10 molecules per b_6f dimer [25]. On the other hand, there is every reason to think that, whenever present and even well below their CMC, detergents associate with the AP layer, which can affect the functionality and stability of the protein (see below).

SANS analysis of BR/A8-35 particles indicates that as should be anticipated, the polymer lies in a peripheral position as compared with the protein [18]. Although the mass of AP that binds to a given protein is less than that of detergent, the polymer layer is likely to be about as thick as a conventional detergent layer because of its higher degree of hydration. Indeed, BR-adsorbed A8-35 still binds some 1.3–1.5 g of water per gram of polymer [18], which nearly makes up for the lower mass of bound surfactant. Data obtained by surface force measurements on A8-35 layers adsorbed onto macroscopic hydrophobic surfaces [A. Kumpelainen et al., unpublished data), electron microscopy of AP/Complex I complexes [30, M. Flötenmeyer et al., unpublished data] and the study by SEC, SANS and AUC of BR/A8-35 particles [18] all suggest that in the presence of salt (100 mM NaCl) most of the AP mass lies in a compact, 1–2 nm-thick layer, which is compatible with the volume occupied by hydrated AP (fig. 3). Surface force measurements indicate that as the salt concentration is lowered, the AP layer swells and forms a 'pseudo-brush' [A. Kumpelainen et al., unpublished data]. As a rule, protein/AP complexes feature slightly lower sedimentation coefficients and slightly larger apparent Stokes radii upon SEC than the same protein in detergent solution (see e.g. [17, 18, 27]).

Protein/AP complexes repulse each other electrostatically at high concentration and low ionic strength (fig. 4, *left*). At higher ionic strength, the repulsion disappears, to be replaced by attraction and precipitation if the salt concentration is further increased. At least in the case of BR, the latter does not entail any denaturation of the protein, which can be resuspended by diluting the salt [D. Charvolin and Y. Gohon, unpublished data]. Ideal behavior (fig. 4, *right*) is obtained at lower protein concentrations and/or intermediate ionic strength. Controlling the aggregation state of protein/AP particles is a difficult problem which will be discussed in more detail elsewhere. Basic rules for improving monodispersity are to start from a monodisperse solution of AP particles and a monodisperse solution of protein/detergent complexes – otherwise polydispersity is sure to result – and, at least in some cases, to avoid complete removal of free AP. In the case of the Ca^{2+}-ATPase, extensive removal of the detergent also appeared deleterious, which may be a consequence

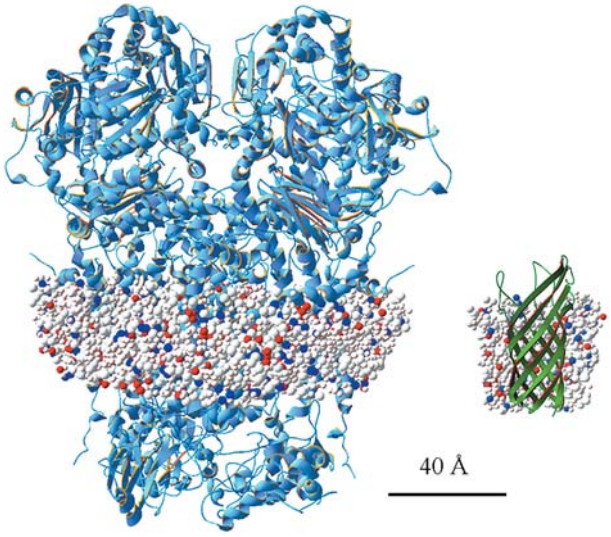


Figure 3. Molecular models of the cytochrome bc_1 complex *(left)* and tOmpA (*right*, in cross-section) in association with amphipol A8-35. Protein models are based on the X-ray structures of the two proteins (PDB accession numbers 1BGY and 1BXW, respectively). The amount of amphipol bound to each protein was experimentally determined either by SANS, using deuterated A8-35, and/or with radio-labelled A8-35 (table 2). The height of the amphipol belt around the transmembrane region of the proteins has been taken to be 4.0 nm. Its volume and thickness were deduced from the mass of amphipol bound per mass of protein [D. Charvolin and M. Zoonens, unpublished data] and the hydration of protein-bound APs as estimated from SEC, AUC and SANS data [18]. Complexes of cytochrome bc_1 and tOmpA with APs represent interesting models for crystallization attempts and for the development of solution-state NMR, respectively. Models built by D. Chazvolin.

of the limited biochemical stability of the protein when associated with pure AP [17]. In the cases of BR, cytochrome bc_1 and tOmpA, it is clear that the detergent (but not all of the AP) can be extensively removed without causing any SEC-detectable aggregation [18, and D. Charvolin and M. Zoonens, unpublished data]. Protein/AP complexes can, at least in the case of BR, be kept frozen for future use: excellent reproducibility was obtained, with no inactivation and no change in dispersity, when a preparation of BR/AP complexes was studied again after being stored for 3 years at –80 °C in the absence of cryoprotectant [18]. Cytochrome b_6f/A8-35 complexes can be frozen (once, but not repeatedly) without inactivating [Y. Gohon, unpublished data].

AP-trapped proteins can be adsorbed onto solid supports, e.g. on immobilized metal columns, via a polyhistidine tag [18, and M. Zoonens and F. Zito, unpublished data], on glutathione columns via a glutathione *S*-transferase (GST) tag [H. A. Shuman and M. Zoonens, unpublished data], or on the surface of a BIAcore chip via an avidin/biotin interaction [28] (fig. 2). A8-35/AChR complexes directly adsorb onto ELISA plates between pH 7.4 and 9.6 [J. Humbert and Y. Gohon, unpublished data].

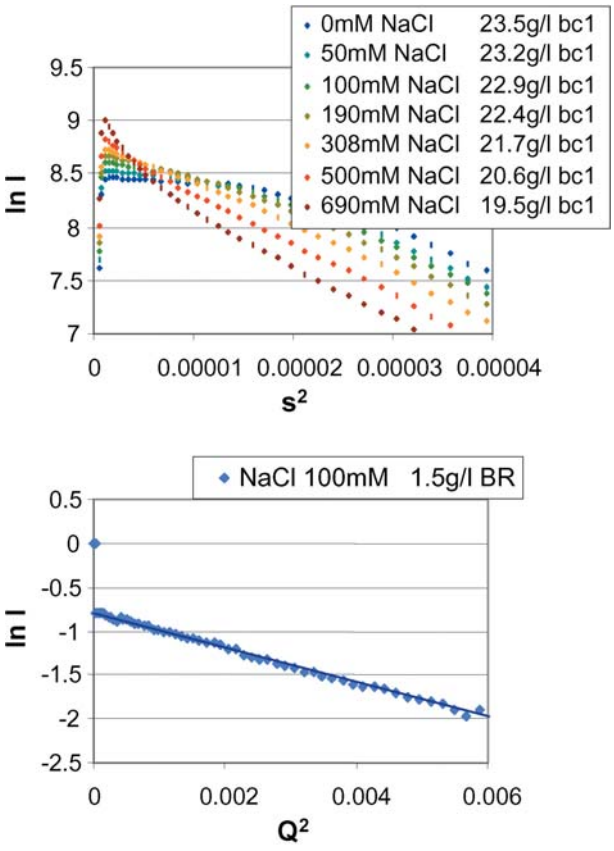


Figure 4. Solution behavior of membrane protein/A8-35 complexes as probed by small-angle scattering. *Top.* Guinier plots of SAXS data collected on a concentrated sample of cytochrome bc_1 trapped in A8-35, at various concentrations of salt and protein. Deviations from linearity in the small-angle region of the plot show the transition from repulsive interactions at low ionic strength (downward deflection) to aggregation at high ionic strength (upward deflection) [D. Charvolin and E. A. Berry, unpublished data]. *Bottom.* Guinier plot of SANS data collected on a sample of BR trapped in deuterated A8-35. The buffer contained 85% D_2O, a concentration at which the deuterated polymer does not contribute to neutron scattering. The linearity of the plot is consistent with the presence of monodisperse BR/AP particles. A more detailed analysis combining SANS and AUC data, however, revealed that the sample actually comprised 75 % monomeric BR, 20% dimers and 5% trimers [18]. *Ordinates*: logarithm of the intensity measured as a function of the scattering angle 2θ. *Abscissae*: $Q^2 = (4\pi\lambda^{-1} \sin\theta)^2$ and $s^2 = (2\lambda^{-1} \sin\theta)^2$, where λ is the wavelength of the radiation (spectively X-rays nd neutrons).

Stability and functionality of amphipol-trapped proteins

As mentioned above, one of the incentives that led to the design of APs was to get rid of the destabilizing effect of detergents. Usually – but not always – AP-trapped proteins indeed appear to be stabilized as compared with their detergent-solubilized counterparts [9, 17]. The difference can be spectacular: in the case of calcium-free Ca^{2+}-ATPase, a particularly fragile protein, the enzyme solubilized in the presence of lipids has a half-life of ~ 1 h after addition of A8-35 and dilution below the CMC of $C_{12}E_8$, compared with ~ 1–2 min in detergent solution [17]. Cytochrome b_6f, on the other hand, is slightly less stable once complexed with either A8-35 or A8-75 than it is in a lipid/detergent mixture [9]. For both proteins, the formation of ternary complexes with lipids (and/or, in the case of the Ca^{2+}-ATPase, detergent) enhances stability [9, 17]. The underlying mechanism is not known. Part of the effect could be due to lipids binding to specific sites, and part to better screening of the transmembrane surface from water. BR, at pH 8 and 4 °C, was found to be much more stable after trapping with A8-35 or A8-75 than it is in C_8-TG solution, but it definitely prefers the first, least-charged AP [9, 18]. DAGK shows roughly the same stability in C_{12}-M and after trapping by PMAL-B-100 (cf. fig. 1, 2) [15]. The biochemical stability of pea photosystem II (PSII) reaction centers supplemented with A8-35 in detergent solution (either CHAPS or C_{12}-M) and then diluted under the CMC of the detergent depends both on the temperature and the nature of the detergent. At 4 °C, the stability is highest for CHAPS-solubilized or AP-trapped centers (independent of the nature of the detergent solution they were trapped from) and lowest for C_{12}-M. With the former three, there is practically no change in activity over 15 h. At 20 °C, all samples are considerably less stable, and the stability order changes to CHAPS > {A8-35 from CHAPS} > {A8-35 from C_{12}-M} > C_{12}-M. The latter observations confirm that even below their CMC, detergents associate with the amphipol layer, which can affect the properties of the trapped protein [A. Zehetner and H. Scheer, personal communication] [31].

Functional studies have yielded contrasting results. In the case of Ca^{2+}-ATPase, association with pure A8-35 has an inhibitory effect, which is partially relieved by lipids and/or detergent [17]. In the case of nAChR, on the other hand, addition of A8-35 and dilution below the CMC of CHAPS relieves the solubilized protein from the perturbing effect of the detergent [27]. Upon illumination, AP-trapped rhodopsin undergoes the transition to the meta-II state (fig. 5), but its interactions with transducin and rhodopsin kinase are affected (see below). DAGK trapped by PMAL-B-100 has been shown to be fully active without addition of either lipids or detergent [15]. After being trapped with A8-35, the maltose transporter, a protein of the ABC-cassette family, features an ATPase activity similar to that observed in proteoliposomes, but its stimulation by the maltose binding protein (MBP) is much reduced, if not absent [M. Zoonens and H. A. Shuman, unpublished data]; C_{12}-M strongly stimulates basal (MBP-independent) activity, which is reminiscent of the observations of Champeil and co-workers on Ca^{2+}-ATPase [17]. *Synechocystis* PCC 6803 PSI reaction centers trapped with A8-35 and deposited on a gold electrode have been shown to be electrochemically active, but no detailed study has been reported [32]. The photochemical activity

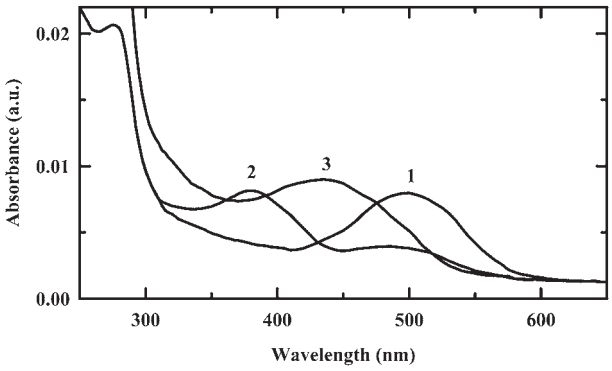


Figure 5. Ultraviolet (UV)-visible spectra of resting-state and light-activated rhodopsin trapped with amphipol A8-35. Rhodopsin was solubilized and purified in 7 $g \cdot l^{-1}$ CHAPS. The preparation was supplemented with 0.2 $g \cdot l^{-1}$ amphipol A8-35 (mass ratio AP/rhodopsin ~14:1) and diluted 20 × below the CMC of CHAPS. If A8-35 was omitted, dilution led to the precipitation of rhodopsin (data not shown). In its presence, rhodopsin remained soluble, stable and exhibited a typical dark-state spectrum (**1**), with maximal absorption of the chromophore at 480 nm. Light-induced activation of AP-trapped rhodopsin (30 s illumination at $\lambda > 495$ nm) led to the formation of the meta-II intermediate, with an absorption maximum at 380 nm, as typically observed in detergent solutions (**2**). Acidification of the bleached sample to pH 1.9 (in the presence of 2 $g \cdot l^{-1}$ SDS in order to prevent precipitation of A8-35) led to the formation of the protonated retinyl Schiff base, which absorbs maximally at 440 nm (**3**). Similar results were obtained starting from rhodopsin purified in C_{12}-M. Spectra were recorded on a Perkin Elmer 7 UV/visible spectrophotometer. Cell path 1 cm [C. Creuzenet and H. G. Khorana, unpublished data].

of A8-35-trapped pea PSII reaction centers, measured at room temperature by the accumulation of the pheophytin free radical upon illumination, is intermediate between that in CHAPS and that in C_{12}-M solutions [A. Zehetner and H. Scheer, personal communication] [31]. The oxygen-evolving activity of cyanobacterial PSII reaction centers isolated from *Thermosynechococcus elongatus* (as determined by dynamic luminescence quenching) increases by ~15% after trapping with A8-35 as compared with PSII particles solubilized by the best detergent, C_{12}-M [M. Nowaczyk and M. Rögner, personal communication].

The activity of AP-trapped proteins may conceivably be affected indirectly, e.g., in the case of polyacrylate-based APs, because the optimal pH, if acidic, cannot be reached or because the concentration of divalent cations must be kept low. Champeil and co-workers measured the enzymatic activity of the Ca^{2+}-ATPase under the standard conditions used for the detergent-solubilized protein (5 mM Mg-ATP, 0.1 mM Ca^{2+}, pH 7.5). From an analysis of the competition between A8-35 and murexide for calcium, they concluded that complexation of Ca^{2+} by the AP did not account for the observed inhibition of the enzymatic activity [17] and estimated the K_D for calcium binding by A8-35 to be 0.15–0.2 mM [unpublished data]. The pH dependence was similar in the presence and absence of amphipols, suggesting that changes in local pH were not responsible for the drop in activity either [17]. Sanders and co-workers observed that DAGK was much more active in zwitterionic than in anionic APs. They reported precipitation of Mg^{2+} (which is used at 10 mM in their assay) by the anionic APs PMAL-B-0 and PMAL-B-50 [15].

It is clear from the above that the effects of APs on membrane protein function vary from protein to protein, from one AP to the next and that they can be modulated by the presence of lipids and/or detergent. It would be premature to try and generalize on the basis of such scattered observations. One may, however, offer the following remarks:

- First, detergents often perturb the function of membrane proteins, either in situ or in solution. In most cases, this effect is likely to involve competition between the detergent and lipids for the transmembrane surface of the protein. APs, being poor detergents, may be expected to displace lipids less efficiently, which could account for some of their functional effects. The relief by APs of a perturbing effect such as that of CHAPS on the nAChR may therefore result either (i) from lipids rebinding to critical sites because APs do not as efficiently displace them as CHAPS does, and/or (ii) from the polymer replacing the detergent, but without its perturbing effect on allosteric equilibria [27].
- Second, we have noted above that APs may not shield the transmembrane surface of the solubilized protein from water as efficiently as a layer of detergent does. If this hypothesis is correct, one may expect the hydrophobic effect to unbalance the equilibrium between conformational states if those expose different amounts of hydrophobic surface. A protein that undergoes important transmembrane conformational changes, as may be the case of the Ca^{2+}-ATPase or the maltose transporter, may then find itself pushed towards that state that exposes the minimum hydrophobic surface. Lipids and detergents may moderate this effect by diminishing the access of water to the transmembrane surface.
- Finally, and on an even more speculative note, it would be interesting to compare the molecular dynamics of detergent-solubilized and AP-trapped proteins. Protein transconformations typically occur on a millisecond time scale, while wriggling and shifting movements of APs at the transmembrane surface, one can speculate, are likely to take place mostly in the sub-microsecond one. Despite these widely different time scales, one may wonder whether the dense crisscrossing of the protein surface by APs does slows down or limits the amplitude of large-scale vibrational movements, which could affect the rate of conformational transitions ('Gulliver' effect).

Binding of ligands to amphipol-trapped proteins

One could expect, a priori, that the binding of ligands to sites located away from the transmembrane region should not be directly affected by the presence of the AP layer. There may be cases, however, where the hydrophobicity of the site would make it energetically favorable for an AP alkyl chain carried by a loop or tail to intrude into it, leading to competitive inhibition. A somewhat fuzzy AP layer could sterically prevent interactions at sites close to the transmembrane region. In early experiments with cytochrome b_6f, we observed that the AP-trapped protein was functional after injection into a C_{12}-M-based reaction medium [9]. This means that it was interacting with both plastoquinol and plastocyanin, whose binding sites are located respectively within and outside of the transmembrane region. The exact composition of the surfactant layer under the conditions where electron transfer was being measured is not known, but it is likely to have been a ternary mixture of lipids, detergent and APs. AP-trapped nAChR was found to bind a fluorescent analog of its soluble ligand, acetylcholine, whose binding sites are carried by extramembrane domains, with kinetics indistinguishable from those recorded with the membrane-bound protein [27]. The same receptor has recently been observed to bind monoclonal antibodies directed towards various regions of its extramembrane surface with the same affinity as in detergent solution [J. Humbert, personal communication]. Similarly, immobilized OmpF/A8-35 complexes bind both anti-OmpF antibodies and the water-soluble R domain of colicin N [28]. In the latter case, the affinity was improved by more than one order of magnitude as compared with OmpF/detergent complexes. Preliminary studies with rhodopsin have yielded less satisfactory results: phosphorylation by rhodopsin kinase was observed, although at a reduced rate, but no activation of transducin [C. Creuzenet and H. G. Khorana, unpublished data]. Since these attempts were carried out in the presence of free AP, it is not clear whether AP binding to the hydrophobic moiety of the G protein may not have created particle-particle electrostatic repulsion, which could account for this inhibition. As noted above, the AP-trapped maltose transporter is functional, but the regulation of its ATPase activity by MBP is impeded. This could be due either to direct interference of APs with MBP binding, or, perhaps more likely, to perturbation of allosteric equilibria [M. Zoonens and H. A. Shuman, unpublished data].

As for functional measurements, it is clearly too early to generalize from these few observations. Let us simply say that, while the formation of water-soluble membrane protein/AP complexes is universal and biochemical stabilization of the protein is frequent, the effects of complexation by APs on the protein's functionality and on its interactions with ligands cannot be safely predicted and must be ascertained case by case.

Applications

Even though data are still preliminary and many conclusions remain tentative, the information given above suggests that APs provide an interesting new way of handling membrane proteins in vitro. Mainly because methodological development is best carried out on already well-known proteins, it is fair to say, however, that up till now few novel biological insights have been obtained specifically thanks to APs and that applications are still in the process of being worked out.

Most of the methodological advantages of APs derive from the possibility of working in the absence of detergent. As indicated in the introduction, many of the problems encountered by membrane biochemists when handling membrane proteins in solution can be traced to the dissociating effects of detergents. The fact that AP-trapped proteins tend to be more stable than their detergent-associated counterparts is probably largely due to the fact that APs are very weak detergents, which do not, by themselves, disperse membrane components very efficiently and are expected to be poorly delipidating. This is compounded by the fact that no excess of free surfactant need be present: free APs can be eliminated from the preparations more or less completely, driving protein/lipid equilibria towards association. As a result, APs make it easier to handle fragile proteins, and they probably provide an extremely interesting novel way of exploring protein/lipid associations and membrane protein supercomplexes, which are known to exist in situ (see e.g. [33]) but do not resist purification in detergent solution. The potential usefulness of APs in various analytical procedures is certainly worth investigating. In cell biology, APs can probably be used, among other things, to introduce membrane proteins and other compounds into the membrane of living cells without killing them. In immunology, it seems that APs can be used to stabilize immunogenic preparations as well as to detect, in detergent-free systems, antibodies directed against membrane proteins.

Most AP-trapped membrane proteins being stable in the absence of detergent, it becomes possible to study their interactions with ligands and with other macromolecules in the absence of this complicating factor. The first biologically relevant new information on a membrane protein brought by the use of APs was the distinction between molecular and physical control of the allosteric properties of the nAChR by its environment: APs made it possible to separate physical effects due to the disappearance of the membrane upon solubilization from equilibrium displacement due to detergent binding [27]. A promising area of development is the study of the interaction of soluble factors with immobilized AP-trapped membrane proteins, where the solution that is flown over the chip or resin does not need to contain any free surfac-

tant. One such example is the recent analysis of the interaction between OmpF and the R fragment of colicin N, which cannot be studied in the presence of detergent [Q. H., J.-L. P. & J. L., unpublished data].

Applications to membrane structural biology form a fascinating field, but definitely not the easiest one to break into. In principle, it is extremely attractive to be able to handle an AP-trapped membrane protein as though it were a soluble protein – and this was a major incentive at the inception of this work – but difficulties are many. Single-particle electron microscopy is probably a field where the use of APs ought to be easiest to implement[2] [26, 30] (fig. 6). In solution-state NMR, where accelerating the tumbling rate of particles is one of the keys to obtaining well-resolved spectra, the lower mass of AP bound to proteins as compared with detergents may be a deceptive advantage because of the high hydration of the polymer and, possibly, a less compact structure. HSQC spectra of the transmembrane regions of glycophorin A [Y. Gohon, K. MacKenzie, D. M. Engelman and D. E. Warschawski, unpublished data] and of OmpA [M. Zoonens, D. E. Warschawski, F. Ferrage and G. Bodenhausen, unpublished data] trapped with APs have been obtained, but much work is still needed to optimize them to the point where they could be used to establish a structure. As noted above, a significant improvement could come from the use of APs that remain soluble at acidic pH. It may be difficult – or, in any case, lengthy – to engineer molecules and to devise conditions that will yield spectra of better quality than those obtained using the best detergents. However, one should recall that the NMR conditions used currently tend to be extremely drastic (high temperature, often high concentrations of detergent), and that the few transmembrane regions whose three-dimensional (3D) structures have been hitherto explored (those of glycophorin A, OmpA, OmpX and PagP) are unusually resistant to detergents (they are not denatured by SDS at room temperature). Being able to do away with the detergent may open to investigation proteins that would not stand such harsh conditions. Finally, 3D and 2D crystallization of membrane protein/AP complexes is being vigorously pursued, with little success so far. Crystallization attempts are critically dependent on the homogeneity of the particles, which, as we have seen, is not easily achieved, and on striking the right balance between reducing the repulsion between AP layers and preventing aggregation (fig. 4, *top*). Given the general difficulty of crystallizing membrane proteins, however, there is a strong need for innovative approaches, and this one certainly deserves to be thoroughly investigated.

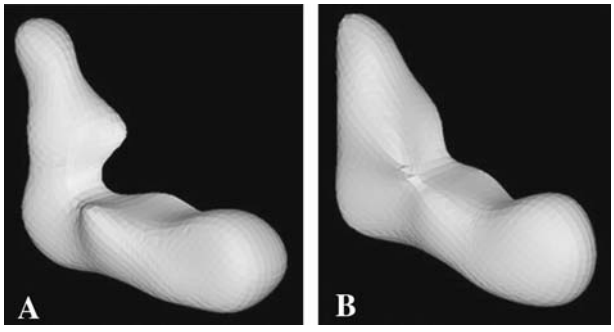


Figure 6. 3-D reconstruction of single particles of mitochondrial Complex I solubilized in detergent (*A*) or trapped by amphipol A8-35 (*B*). (*A*) A 3D reconstruction of Complex I made by the conical tilt method, from images of single particles solubilized in detergent and stained negatively with uranyl acetate. The horizontal arm is the membrane domain and includes detergent density. The vertical arm is the cytoplasmic domain. Each arm is ~20 nm in length [35]. (*B*) A preliminary 3D reconstruction made by the multi-reference alignment method for single particles stabilized (in the absence of detergent) by amphipol A8-35 [30, M. Flötenmeyer et al.]. In this case, samples were unstained and imaged in the frozen-hydrated state. Both reconstructions were filtered to a cut-off of 3 nm and, at this resolution, show comparable features.

Conclusion

In this first survey of work done on or with APs, we have tried to offer a reasonably balanced view of the assets and problems of this novel approach, and to point out some directions that seem to deserve investigation. It should be kept in mind that our understanding of the physical chemistry of APs and protein/AP complexes remains extremely patchy. The development of improved molecules and procedures, which will be necessary for many applications, will be a lengthy process, whereby physical and biochemical studies will guide the design of better or more specialized APs. Close interdisciplinary collaborations involving chemists, physical chemists and biologists will remain essential to success. One difficulty, as pointed out above, will be to properly balance the efforts invested into developing new molecules and gaining a detailed understanding of their behavior and those targeting their exploitation for biological investigations. As has been the case up till now, there will necessarily be a trial-and-error process in which some applied projects will be launched prematurely and will lead to disappointing results, followed by backtracking. All the same, the poten-

[2] The use of detergent for solubilizing membrane proteins is a well-established technique for electron microscopic studies of negatively stained single particles. However, the presence of free detergent, which is needed to prevent the protein from aggregating, makes cryoelectron microscopy of unstained specimens difficult if not impossible: the free detergent lowers the surface tension of the protein solution, causing uneven distribution of the protein molecules in the ice film, with the result that most of the proteins tend to aggregate or to cluster around the edges of the ice-filled holes in the supporting carbon film. If amphipols are substituted for detergent and excess amphipol removed, there is very little reduction in surface tension and rapidly frozen suspensions have an excellent distribution of protein particles.

tial of this new methodology appears rich enough to warrant taking risks and sustaining long-term development efforts.

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