Review
Folding and stability of integral membrane proteins in amphipols

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Amphipols (APols) are a family of amphipathic polymers designed to keep transmembrane proteins (TMPs) soluble in aqueous solutions in the absence of detergent. APols have proven remarkably efficient at (i) stabilizing TMPs, as compared to detergent solutions, and (ii) folding them from a denatured state to a native, functional one. The underlying physical–chemical mechanisms are discussed.

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Introduction
Folding integral membrane proteins to their functionally active form: still a challenge

Integral membrane proteins – hereafter transmembrane proteins (TMPs) – are key biological players because of their roles in bioenergetics, transmembrane transport, signaling, cell and tissue organization, etc. They are therefore primary targets for pharmaceutical drugs. Structural and pharmacological studies of TMPs are hampered by their low abundance and by difficulties in overexpressing them in a functional form. TMPs can be overexpressed either homologously or heterologously, for example in Escherichia coli, yeast, etc. Plasmid-based overexpression can be designed to target TMPs either to a membrane, which often results in low yields, or to cytoplasmic inclusion bodies, which yields larger amounts of protein, but in a misfolded and aggregated form that has to be folded to a functional state – a difficult achievement. Folding outside the cell environment is also necessary when TMPs are expressed in vitro in a cell-free system. Successful folding in detergent or mixed detergent/lipid micelles has been demonstrated for both known structural classes of TMPs, the α-helix bundle and the β-barrel. Over 50 α-helical TMPs have been folded or refolded in vitro to date [1]. Among the first ones to be studied were bacteriorhodopsin (BR) [2–4], the light harvesting complex LHCl [5,6], several G protein-coupled receptors (GPCRs) such as olfactory receptors [7,8], the BLT1 receptor of leukotriene B4 and the cannabinoid receptor CB1.

Reviews on folding of these proteins have been published [9,10]. The application of lipid bilayers and detergent-free methods for folding the BLT1 receptor has been reported [11], but these methods lack the usual controls for integrity. We will therefore focus on non-lipid-based methods, such as solvents or detergents, to compare them to the foldability of the systems. Detergents are efficient at keeping TMPs soluble in aqueous solutions in the absence of detergent. APols have proven remarkably efficient at stabilizing TMPs, as compared to detergent solutions, and folding them from a denatured state to a functional one. The underlying physical–chemical mechanisms are discussed.

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1 Abbreviations used: A8-35, a poly(sodium acrylate)-based amphipol comprising ~35% free carboxylates, ~25% octyl grafts, ~40% isopropyl groups, whose number-average molar mass is ~4.3 kDa; APol, amphipol; BLT1 and BLT2, two human G protein-coupled receptors of leukotriene LTB4 and BO, respectively; Bacteriorthodopsin from Halobacterium salinarum; CAC, critical association concentration; CB1, human cannabinoid receptor 1; CD, circular dichroism; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; cmc, critical micellar concentration; 2D, 3D, two- and three-dimensional; DAGK, diacylglycerol kinase; dBO, delipidated BO, obtained after solubilizing purple membranes in organic solvents; DDM, n-dodecyl-β-D-maltoside; DHPC, dihexanoylphosphatidylcholine; DS, dodecylsulfate; EM, electron microscopy; ESIMS-MS, electrospray ionization mass spectrometry coupled with ion mobility spectrometry; FomA, major outer membrane protein A from Fusobacterium nucleatum; GHS-R1a, human ghrelin receptor 1a; GPCR, G protein-coupled receptor; His-tag, polyhistidine tag; 5-HT4(a), human serotonin receptor; LDAO, N-lauryl-N,N-dimethylamine-N-oxide; LHCl, light-harvesting complex II; LPS, lipopolysaccharide; MD, molecular dynamics; MOMP, major outer membrane protein from Chlamydia trachomatis; OG, n-octyl-β-D-glucoside; OmpA and OmpT, outer membrane proteins A and T from Escherichia coli; OTG, octylthioglucoside; PAGE, polyacrylamide gel electrophoresis; PagP, lipid A palmytoyltransferase from E. coli; PDS and SDS, potassium and sodium dodecylsulfate, respectively; PM, purple membrane; SEC, size-exclusion chromatography; SUV, small unilamellar vesicles; TMP, transmembrane protein; TOMP, transmembrane domain of OmpK; UV, ultraviolet; Z3-14, 3-[dimethyl(triadeacyl)azaniumyl]propane-1-sulfonate (also called sulfobetaine 3-14, Zwitergent 3-14).

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LTB₄ [9], or the serotonin 5-HT₄(a) receptor [10], channel proteins like the homotetramer KcsA [11], the small multidrug transporter EmrE [12], and the enzyme diacylglycerol kinase (DAGK) [13,14] (for reviews, see e.g. Refs. [1,15,16]). About 40 β-barrel TMPs have been (re)folded in vitro to date [17–20], ranging from simple 8-stranded β-barrels like OmpA [21,22], OmpX [23] or PagP [24–26] to large 22-stranded active transporters like FepA [27,28]. The list also includes eukaryotic β-barrels such as the 19-stranded VDAC human isoforms 1 [29] and 2 [30,31]. It is quite possible that all β-barrel TMPs can be folded in vitro without any cellular folding machinery. An unsuccessful attempt to fold a β-barrel in solution has been reported for the β-barrel domain of AIDA [32]. However even for AIDA, folding was successful when the protein was bound to a nickel-bearing column [33].

β-Helical TMPs like BR or KcsA have been folded from denatured forms in organic solvent like trifluoroacetic acid or mixtures of formic acid and ethanol [2,11]. Folding of BR was initiated by transferring denatured bacterio-opsin (BO, the apoprotein) first into micelles of the denaturing detergent sodium dodecylsulfate (SDS) and then, usually in the presence of retinal, BR’s cofactor, into “mild” detergents, mixtures of detergent and lipids, or pure lipids [2–4]. Rather than diluting it into a large excess of the folding medium [2,3], dodecylsulfate (DS) can be precipitated as its potassium salt, PDS [4]. β-Barrel TMPs such as outer membrane protein A (OmpA, previously called Omp II) from E. coli were first refolded from their SDS-denatured forms by replacing the SDS either with lipopolysaccharide [21] or octylglucoside [22]. Subsequently, most (re)folding protocols for β-barrel TMPs have been based on their solubilization and denaturation in concentrated solutions of urea (8–10 M) [34] or other chaotropic agents (reviewed in Ref. [1]). Denatured β-barrel TMPs are generally folded by transferring them to either detergent micelles or lipid membranes under concurrent strong dilution of the denaturant.

The mechanisms of folding of TMPs of both structural classes have been the subject of extensive research. For β-helical TMPs, BR (see e.g. Refs. [35,36]) or DAGK [37,38] have often served as models. Folding of β-helical membrane proteins is a sequential 3-stage process, in which helices insert independently in a first stage and associate laterally in a second stage, which is followed, in a third stage, by the formation of additional tertiary structure, such as re-entrant loops, and/or by the binding of prosthetic groups (for discussions, see Refs. [35,39]). For β-barrel TMPs, a concerted mechanism has been reported for OmpA, in which folding and insertion are coupled [40,41] (for reviews, see Refs. [20,42–44]).

Using detergent- or lipid-based protocols, folding yields tend to be lower than desired for many pharmacologically and physiologically interesting GPCRs, e.g. GPCRs. For example, the leukotriene B₄ BLT1 receptor has been refolded to a moderate yield of ~30% in mixed micelles of detergent and lipid [9]. To date, this is still one of the highest yields reported using classic methods. A homolog of BLT1, the BLT2 receptor, is an example of a GPCR that cannot be folded with a decent yield (more than a few percents) in any detergent or detergent/lipid mixture that has been tried to date [45]. The development of successful refolding strategies for novel TMPs of interest using classic approaches is extremely time-consuming. It is therefore of great interest to examine new methods.

**Amphipols as new tools for the refolding of integral membrane proteins**

Over the last eight years, a new class of non-detergent surfactants, namely synthetic amphipathic polymers called amphipols (APols) [46], has emerged as very promising tools for folding denatured TMPs to their native state. APols are short polymers comprised of both hydrophilic groups and hydrophobic chains. They can substitute to detergents, providing a milder environment to TMPs while keeping them water-soluble (for reviews, see Refs. [47–51]). All of the three dozen TMPs tested to date form soluble complexes with APols in the absence of detergent, whatever their size, origin, function and secondary, tertiary and quaternary structure (reviewed in Ref. [51]). As compared to preparations in detergent solutions, most TMPs remain active for a much longer time when solubilized in the form of APol/TMP complexes (see e.g. Refs. [45,46,52–55]). APols bind to TMPs by adsorbing specifically onto their hydrophobic transmembrane surface, as demonstrated by NMR spectroscopy [56–60], electron microscopy (EM) [61–69], and molecular dynamics (MD) simulations [70]. Unaltered for other surfactants, APols do not dissociate from the surface of TMPs, even at very high dilution, keeping the protein water-soluble [47,71,72] (reviewed in Ref. [51]).

Several types of APols have been synthesized and validated (Fig. 1) (reviewed in Refs. [51,73]). Among these, A8–35 has been most extensively studied. A8–35 (Fig. 1A) is a poly(acrylic acid) partially amidated with octylamine and isopropylamine, leaving ~35% of the carboxylic groups free [46]. In aqueous solution at pH > 7, A8–35 assembles into small micelle-like particles with a mass of ~40 kDa [74–76]. Other APols developed more recently include glycosylated nonionic APols (NAAPols) [77,78] (Fig. 1B and C), sulfonated APols (SAAPols) [79] (Fig. 1D) or phosphorylcholine-based APols (PC-APols) [80,81] (Fig. 1E) (reviewed in Refs. [50,51,73]). SAAPols [79] are derived from a precursor of A8–35 lacking the amidation with isopropylamine. Instead, about ~40% of the carboxyl groups are amidated by tauro-2-aminoethanesulfonic acid (Fig. 1D). The sulfonate groups of taurine do not protonate even at pH 0, which keeps SAAPols water-soluble at pH < 7, whereas under such conditions A8–35 aggregates [74,75]. Homopolymeric NAAPols (Fig. 1B) are synthesized by homotelomerization of a monomer carrying two glycosyl residues [77]. Heteropolymeric NAAPols (Fig. 1C) have been obtained either by cotelomerization of hydrophobic and hydrophilic monomers [82] or by randomly grafting hydrophobic chains onto a glycosylated homotelomer [83]. PC-APols are zwitterionic at neutral and basic pH, cationic at acidic pH. NAAPols and PC-APols, as SAAPols, remain water-soluble under acidic conditions.

APols, originally designed to keep TMPs water-soluble while preserving their activity [46], have been used for a wide range of applications (reviewed in Ref. [51]). Their properties and those of TMP/APol complexes have been reviewed in Refs. [49–51] and detailed protocols for their implementation provided in Ref. [84]. This review focuses on their use for folding TMPs and for stabilizing them against denaturation by heat or chaotropic agents. Since β-helix bundle TMPs and β-barrel TMPs fold according to very different principles (see e.g. Refs. [35,39,41,85]), our discussion is subdivided into separate sections for these two categories of TMPs. Historically, APol-assisted folding of TMPs was first demonstrated for three model TMPs, BR, an β-helical TMP, and two β-barrel TMPs, OmpA and FomA [86]. It was then extended to two more β-barrel TMPs and six GPCRs (Table 1).

**Amphipol-assisted folding of β-helix bundle membrane proteins**

**Folding of BR in A8–35**

BR, a light-driven proton pump [87], has served as a popular model protein in studies on TMP folding since the seminal work of Khorana and coworkers [2] (for reviews, see e.g. Refs. [1,35,88,89]). When overproduced, BR accumulates in the plasma membrane of H. salinarum in the form of 2D protein/lipid crystals, the so-called purple membrane (PM). Its covalently but loessly bound chromatophore, retinal, confers it a characteristic purple
color. When PM is solubilized in SDS, BR denatures to BO, releasing its chromophore, which causes an absorption peak shift from ~555 nm (dark-adapted BR) to ~382 nm (free retinal). The removal of SDS from BO/retinal in the presence of APol A8-35 by precipitating DS as PDS results in the regeneration of the characteristic purple color of BR within minutes, indicating folding of BO and rebinding of the retinal [86]. After a dialysis to remove residual DS, almost quantitative refolding of BR (≥ 90%) is observed for a mass ratio A8-35/BR ≥ 5:1 [86,90]. When refolded at such mass ratios, BR migrates as an A8-35-trapped monomer, as determined by size-exclusion chromatography (SEC). Control experiments carried out in the same way using detergents instead of A8-35 show lower levels of BR refolding. Yields are in fact negligible with octylglucoside (OG), octylthioglucoside (OTG) or C8 E4, but reach ~66% in dodecylmaltoside (DDM).

More stringent experiments were performed in which BO was totally unfolded in formic acid and transferred to SDS after lipids and retinal had been removed by hydrophobic SEC. They established that completely delipidated BO (dBO) refolds in pure A8-35 to 60–80% [86,90]. BR refolded in A8-35 displayed a fully functional photocycle. The light-adapted protein was excited at 640 nm with a 5-ns laser flash. Transient absorption changes, monitored from 10 ns to 100 ms in a spectral range from 370 to 500 nm, were found to be very similar to those observed with lipid-associated native BR trapped in A8-35 [86,90].

A comparison of the folding of BR into A8-35 from BO/retinal or dBO/retinal in SDS confirmed that slightly higher refolding yields are achieved when lipids were present [90]. However, in the presence of lipids, larger amounts of A8-35 were necessary for optimal folding yields. This suggested that lipids may favor BR aggregation at low A8-35 concentrations, in line with observations by SEC that BR aggregates when refolded at 1:2 BO/A8-35 [86]. Interestingly, an attempt at direct refolding of dBO by transfer from trifluoroethanol (TFE) to A8-35, in aqueous buffer in the absence of SDS, resulted in a folding yield of ~40% [90]. This approach, however, has not reached the stage of a protocol yielding reproducible results.

Folding yields were somewhat lower when folding of BR from BO in SDS was initiated by dialysis of SDS instead of precipitating DS as its potassium salt [90,91]. Upon SDS removal by dialysis,
folding yields of BR increased with the A8-35/BO ratio, suggesting that aggregation is a limiting factor [90].

BR was also successfully refolded by diluting a BO/SDS/A8-35 mixture with SDS-free buffer, in which case larger mass ratios of A8-35/BO were required than observed using either SDS precipitation or SDS dialysis: when folding was initiated by dialysis, yields were highest at a mass ratio of 25 [90]. Two mechanisms may contribute to this effect. First, adding more A8-35 reduces the proportion of SDS in the environment of the refolding proteins. Second, the probability that two of them will come into contact and establish intermolecular associations is reduced by diluting them with more APol, making aggregation less likely (cf. Ref. [71]). Folding kinetics were faster at higher concentrations of retinal, consistent with the view that refolded BO can pick up retinal very rapidly if it is present in the BO-associated APol belt, whereas the process is much slower if retinal uptake depends on the BO/APol complex colliding with a retinal-containing free particle of APol (cf. Ref. [71]).

Folding of dBO in A8-35 by PDS precipitation was also successful in the absence of retinal [90]. However, refolded BO is less stable than refolded BR. The addition of retinal within an hour after addition of KCl results in similar yields as observed when folding BO in the presence of retinal, but yields dropped to ~30% when retinal was added after dialyzing refolded BO in A8-35 for three days, indicating that ~1/2 of it had denatured again over this period. This observation is consistent with the view that folding yields of fragile TMPs can be improved in the presence of their ligands.

Transfer from SDS to A8-35 can also be achieved by adsorbing SDS onto Bio-Beads, provided aggregation is limited by immobilizing polyhistidine-tagged BR onto a nickel-bearing column [91].

Table 1
Folding of membrane proteins in amphipols.

<table>
<thead>
<tr>
<th>TMP</th>
<th>Structure</th>
<th>Denaturant</th>
<th>Amphipol</th>
<th>Lipids, cofactors</th>
<th>Method</th>
<th>Yield</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 5%</td>
<td>A8-35 (2–25 g/g)</td>
<td>PM lipids, retinal</td>
<td>DS precipitation with KCl at pH 7, dialysis</td>
<td>Up to 87–92%</td>
<td>[78,86,128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 5%</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>Retinal, no lipids</td>
<td>DS precipitation with KCl at pH 7, dialysis</td>
<td>70–76%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 5%</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>PM lipids, retinal</td>
<td>Dialysis at pH 7, no NaCl</td>
<td>52–72%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 2.5%</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>PM lipids, retinal</td>
<td>Dialysis at pH 7, no NaCl</td>
<td>62–80%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 5%</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>PM lipids, retinal</td>
<td>Dialysis at pH 7, 100 mM NaCl</td>
<td>~76%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 2.5%</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>PM lipids, retinal</td>
<td>Dialysis at pH 7, 100 mM NaCl</td>
<td>74–80%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 0.25%</td>
<td>A8-35 (10 g/g)</td>
<td>PM lipids, retinal</td>
<td>5× dilution at pH 7, no NaCl</td>
<td>40–50%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>SDS 0.25%</td>
<td>A8-35 (10 g/g)</td>
<td>PM lipids, retinal</td>
<td>5× dilution at pH 7, 100 mM NaCl</td>
<td>70–80%</td>
<td>[128]</td>
</tr>
<tr>
<td>BR</td>
<td>7-3</td>
<td>TFE</td>
<td>A8-35 (5 or 10 g/g)</td>
<td>Retinal, no lipids</td>
<td>Dialysis at pH 7</td>
<td>&lt;40%</td>
<td></td>
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<tr>
<td>BLT1</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (1–20 g/g)</td>
<td>None</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>Up to ~50%</td>
<td>[45]</td>
</tr>
<tr>
<td>BLT1</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>Asolectin/A8-35 1:5 w/w</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>65–70%</td>
<td>[45]</td>
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<tr>
<td>BLT2</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>None</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~50%</td>
<td>[45]</td>
</tr>
<tr>
<td>CB1</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>Asolectin/A8-35 1:5 w/w</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~70%</td>
<td>[45,170]</td>
</tr>
<tr>
<td>CB1</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>None</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~30%</td>
<td>[45]</td>
</tr>
<tr>
<td>5-HT3</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>Asolectin/A8-35 1:5 w/w</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~40%</td>
<td>[45]</td>
</tr>
<tr>
<td>5-HT3</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>A8-35 (5 g/g)</td>
<td>None</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~30%</td>
<td>[45]</td>
</tr>
<tr>
<td>GHSR-1a</td>
<td>7-3</td>
<td>SDS 0.8%</td>
<td>NAPol (10 g/g)</td>
<td>Asolectin/NAPol 1:5 w/w, 0.2% (w/v) cholesteryl hemisuccinate</td>
<td>DS precipitation with KCl at pH 8, dialysis</td>
<td>~40%</td>
<td>[78]</td>
</tr>
</tbody>
</table>

FomaA

OmpmA

tOmpmA

OmpT

PagPh

* From Halobacterium salinarum.

7-3: bundle of 7 transmembrane α-helices.

* Experiment could not be reproduced.

* From Homo sapiens.

* From Mus musculus.

* From Fusobacterium nucleatum.

* n-β: barrel of n observed or predicted transmembrane β-strands.

* From Escherichia coli.

* Transmembrane domain of OmpA.

A8-35-assisted folding of GPCRs

Successful folding of both α-helical and β-barrel model TMPs in A8-35 [86] suggested that the approach could be general and stimulated attempts at folding GPCRs, for which folding using classical detergent/lipid systems, when successful, is typically limited to yields of 30% or less (reviewed in Refs. [15,92]). The first study bore on BLT1, a GPCR involved in the control of inflammatory processes, using a similar strategy as that established for BR [86]: A8-35 was
added to the denatured GPCR in SDS solution, most of DS precipitated as PDS, and residual DS removed by dialysis. Ligand binding assays indicated that the receptor folded in A8-35 was functional, with a dissociation constant $K_D \approx 9 \text{nM}$, similar to that of native BLT1 expressed in membrane fractions [93]. Based on the number of binding sites, the yield of folding was $\sim50\%$ in the absence of lipids and $65\sim70\%$ in the presence of soybean asolectin (a mixture of about equal amounts of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, with smaller amounts of polar lipids like phosphatic acid, phosphatidylglycerol, phosphatidylserine and sterols) (Table 1). The BLT1/A8-35/lipid mass ratio was 1:5:1. Following folding, BLT1 showed a similar pharmacological profile as the membrane-bound receptor [45], indicating that A8-35 is an efficient medium in which to fold BLT1 obtained from inclusion bodies.

The study was then extended to three other GPCRs: another leukotriene receptor, BLT2, the serotonin receptor 5-HT$_{4a}$, and the cannabinoid receptor CB1 [45]. In the presence of asolectin, A8-35 improved the folding yields of 5-HT$_{4a}$ previously observed by more than a factor of two, namely from 20% to 25% in detergent/asolectin micelles [10] to $\sim30\%$ in pure A8-35 and $\sim60\%$ in A8-35 + asolectin [45]. For BLT2, which shares $\sim45\%$ sequence identity with BLT1, the yield improved from 3–4% in detergent/asolectin micelles to $\sim50\%$ in pure A8-35 and $\sim70\%$ in A8-35 + asolectin [45]. As regards CB1, which had not been folded to any significant extent in detergent/lipid mixtures at the time of these experiments (but has been folded to $\sim30\%$ since [94]), folding yields of $\sim30\%$ were achieved in pure A8-35 and of $\sim40\%$ in A8-35/asolectin mixtures. Altogether, these data, obtained without any extensive search for optimal folding conditions, suggest an interesting potential of A8-35 as a generally useful new tool for the folding of GPCRs. Indeed, more recently, two other GPCRs have been successfully refolded in APols, namely the arginine-vasopressin type 2 and ghrelin GHSR-1a receptors (Table 1) [78,92].

Whereas in no case was a 100% yield reached, separation of active from inactive 5-HT$_{4a}$ could have been achieved using a GR113808 affinity column [10], yielding $\sim96\%$ active receptor [45]. In this context, it is worth noting that, whereas 3D crystallization of APol-trapped TMPs remains a difficult challenge (see Ref. [54]), A8-35-trapped BR has yielded highly organized crystals (diffracting to $<2\text{-Å}$ resolution) following direct transfer to lipidic mesophases [95]. Combining APol-assisted folding of GPCRs with crystallization in mesophases [96–98] might therefore open very interesting perspectives.

**Folding of α-helical TMPs in NAPols**

NAPols (Fig. 1B and C) have been used successfully to fold the ghrelin GHSR-1a GPCR and BR to their native state [78]. Because of their nonionic character, NAPols may provide an even milder environment than polyanionic A8-35 [78], and they present the advantage of being soluble over a broader pH-range [77], covering the mildly acidic regime favorable for NMR work. Folding of BR in homopolymeric NAPols (Fig. 1B) was achieved using the same strategy as previously used for folding in A8-35 [86], i.e. by supplementing SDS-solubilized PM with NAPols, followed by PDS precipitation and removal of residual DS by extensive dialysis. Quantitative analysis by ultraviolet (UV)/visible absorption spectroscopy indicated a yield $\geq 90\%$. NAPol-refolded BR was homogenous, as shown by SEC [78].

Based on folding experiments with BR as a model, NAPols were successfully used for folding the GHSR-1a receptor. Ghrelin, the positively charged ligand of this receptor, binds non-specifically to A8-35 via charge interactions. This causes difficulties in assessing by ligand binding the extent of folding of GHSR-1a in A8-35. More favorable conditions are achieved when the ghrelin receptor is folded in NAPols, where the background is lower. Ligand-binding measurements indicated a folding yield of $\sim40\%$, and a receptor $\sim97\%$ active was obtained after affinity chromatography [78]. The binding properties of the folded GHSR-1a were then further examined to assess the quality of folding. Fluorescence energy transfer from GHSR-1a, labeled with Alexa Fluor 350, to a ghrelin peptide labeled with fluorescein isothiocyanate was recorded in competition experiments with synthetic antagonists. The competition profiles obtained by this method are within the same range as previously inferred from radioactive and TagLite-based measurements of HEK cells transiently expressing GHSR-1a [99]. In addition, GHSR-1a folded in NAPols (i) is able to activate G proteins, (ii) recruits arrestin in an agonist-dependent manner, and (iii) adopts a very similar equilibrium between active and inactive conformations as in the membrane, confirming that it is fully functional [78].

The polyanionic APols A8-35 and SAPols have been found to block in vitro synthesis of TMPs [100], but NAPols do not, as exemplified by successful cell-free expression and folding of BR [78]. This may open an interesting new route to producing hard-to-express TMPs.

**Amphipol-assisted folding of β-barrel membrane proteins**

The list of β-barrel TMPs that have been successfully folded by transfer of their denatured forms in chaotropic denaturants to detergent micelles ranges from small 8-stranded β-barrels like OmpA [21,22,34] to large 22-stranded transmembrane transporters like FepA [28], which contain an additional domain in the barrel lumen. Indeed, most of the known β-barrel TMPs have been successfully (re)folded, the even-stranded TM β-barrels from bacteria as well as the 19-stranded voltage-dependent anion-selective channel VDAC from the mitochondria of eukaryotic cells [29,30,101] (for reviews, see Refs. [1,19]). The mechanism of folding of β-barrel membrane proteins into lipid bilayers has been studied for bacterial outer membrane proteins from E. coli, like OmpA [40,85,102], OmpF [103], OmpG [104], PagP [25,106] and others [26], for FomA from Fusobacterium nucleatum [107], and for VDAC isoform 1 from human mitochondria [29] (for detailed reviews, see e.g. Refs. [20,42,44]). Whereas a folding machinery is required for the folding of β-barrel TMPs in cells, membranes or membrane biomimetics are not an absolute requirement for folding in vitro. OmpA (Omp II [108]) is the first β-barrel TMP for which successful refolding was demonstrated. More than 35 years ago, Schweizer et al. [21] showed that $\sim90\%$ of denatured OmpA regained its native structure in the presence of lipopolysaccharide (LPS) and Triton-X-100 after dilution of the denaturants SDS or urea. Dornmair et al. [22] established that after heat-denaturation in SDS, OmpA, which comprises an 8-stranded transmembrane β-barrel and a periplasmic domain, can refold in micelles of OG in the absence of LPS. Further experiments demonstrated that OmpA can be (re)founded both in lipids and in a wide range of detergents, provided the detergent concentration is above the cmc [109]. OmpA can even fold from fragments in micelles of OG [110], an observation first made in vivo for separately expressed fragments [111].

Whereas the small 8-stranded β-barrel TMPs can usually be folded to nearly 100% using standard methods, this does not necessarily apply to larger barrels. To date, the yield of folding of the 14-stranded fatty acid transporter FADL does not seem to exceed 50%, irrespective of conditions [26]. Folding yields of VDAC (human isoform 1, non-His-tagged) in detergent micelles depend strongly on the pH. They are optimal at pH 3 [29]. The mere introduction of a polyhistidine tag completely alters this pH-dependence of VDAC folding [29,30] (Shanmugavadivelu & Kleinschmidt, unpublished observations).
In spite of all progress, finding the right conditions for folding unfolded β-barrel TMPs obtained from inclusion bodies has remained a time-consuming and, more often than not, frustrating task, which often requires very long incubation times. It is therefore of great interest to develop new methodologies that can be more widely applied, provide higher yields, may be more rapid, and/or facilitate a larger scale production of functionally active β-barrel TMPs.

OmpA [112,113] and FomA [107,114,115] were the first two β-barrel TMPs to be folded using an APol, A8-35, starting from their unfolded forms in 8 M (OmpA) and 10 M urea (FomA) [86]. Folding was very efficient, with yields of >100% for OmpA and >90% for FomA (Table 1), and was achieved within 7 h for OmpA [116] and within 24 h for FomA. Several criteria were used to assess that these 8- and (predicted) 14-stranded β-barrels, respectively, had achieved their native state:

(i) SDS–PAGE, taking advantage of the different electrophoretic mobilities of the folded and unfolded forms.
(ii) Protection of the folded β-barrels against proteolysis.
(iii) Far-UV circular dichroism (CD) spectroscopy.
(iv) Functional studies, that is single-channel conductance recordings after transfer of A8-35-refolded OmpA and FomA to black lipid films (Fig. 2). In these experiments the first three methods did not reveal any differences between the detergent- and A8-35-refolded forms. However, in single-channel recordings of the conductance of the β-barrels in black lipid bilayers, both OmpA and FomA initially displayed smaller conductance when inserted from complexes with A8-35 rather than from detergent-refolded forms. This difference was traced to an asymmetrical distribution of A8-35 in the black lipid films. When A8-35 was present at equal concentrations on both sides of the film, the expected transmembrane conductances were obtained: 75 pS and 290 pS for the small and of large conductance channels of OmpA, respectively, 1.1 nS for FomA channels [86]. The spontaneous transfer of refolded OmpA and FomA from A8-35 to black lipid bilayers is consistent with the transfer, observed earlier, of the α-helical TMP DAGK from another APol, OAPA-20, into lipid bilayers of 1-palmitoyl-2-oleoyl-phosphatidylcholine (multilamellar vesicles) in a functionally active form [117].

Folding of a β-barrel TMP has also been demonstrated using sulfonated APols (SAPols) [79]. After 2 days of incubation, the genetically engineered transmembrane domain of OmpA (tOmpA), isolated after expression into inclusion bodies, folded to yields approaching ~100% (Table 1), as determined by shifts in the electrophoretic mobility from ~16 kDa for the unfolded to ~19 kDa for the folded form [23].

More recently, two other β-barrel TMPs, namely OmpT [118], an integral outer membrane protease with 10 transmembrane β-strands, and PagP [24], an 8-stranded β-barrel TMP that catalyzes the transfer of a palmitate chain from a phospholipid to lipid A, were folded in A8-35 from their unfolded forms in 8 M urea. Using electrospray ionization mass spectrometry coupled with ion mobility spectrometry (ESI-IMS-MS), yields were shown to reach ~100% for OmpT and ~60% for PagP (Table 1) [119]. Folding was confirmed by electrophoretic mobility measurements, by far-UV CD spectroscopy and by functional studies. SEC showed a single narrow peak for OmpT/A8-35 complexes, indicating the presence of a homogeneous single species of OmpT. A broader peak was

![Fig. 2. Single-channel recordings of refolded OmpA and FomA integrated into black lipid films.](image)
observed in SEC after folding of PagP in A8-35, indicating the presence of a mixture of folded and unfolded PagP species, consistent with the ~60% folding yield observed by ESI-IMS-MS. Refolded OmpT trapped in A8-35 was active, as evidenced by the decrease of fluorescence self-quenching of a fluorogenic peptide upon peptide hydrolysis catalyzed by OmpT in the presence of LPS. Interestingly, a small level of activity of A8-35-refolded OmpT was observed even in the absence of LPS [119], which is not observed when OmpT is folded using classical methods [120]. Refolded PagP was active when trapped in A8-35, hydrolyzing p-nitrophenol-palmitate to p-nitrophenol with an activity comparable to that of PagP in detergent solution [25].

Recently, a detailed study [116] examined the kinetics of folding of wild-type OmpA into APol A8-35, initiated by an 18-fold dilution of urea and carried out at various mass ratios, ranging from 0.5 to 16 g of A8-35 per g of OmpA, and at various temperatures [116]. Analyses of the time course of electrophoretic mobility shifts from the unfolded form (Mf ~ 35 kDa) to the folded one (Mf ~ 30 kDa) indicated that folding takes ~6-8 h at pH 10. The minimum mass ratio of A8-35/OmpA required for complete folding was 2 g/g. All time courses followed double exponential kinetics [116], likely caused by the presence of two or more differently protonated states of OmpA. These have been distinguished before by fluorescence spectroscopy and by monitoring the formation of tertiary structure by electrophoresis [43]. Independent of the method used, parallel pathways of OmpA folding via parallel folding pathways both in negatively charged A8-35 particles [116] and in negatively charged lipid bilayers [122]. It appears possible that the parallel pathways are a consequence of charge–charge repulsion, which could be different for the coexisting forms of OmpA. However, parallel pathways have also been observed for the folding of FomA into phosphatidylcholine bilayers, which do not carry a net charge [107]. When OmpA was folded into A8-35, the faster process contributed ~65% to OmpA folding, with a halftime of ~5 min, whereas the slower process had a halftime of ~70 min [116]. Kinetics of folding of OmpA were obtained by fluorescence spectroscopy and by monitoring the formation of tertiary structure by electrophoresis [43]. Independent of the method used, parallel pathways of OmpA folding were observed. SDS–PAGE did not reveal any folding intermediates of OmpA during folding in A8-35 [116]. Such intermediates likely exist, but they do not survive exposure to SDS. Interestingly, the rate constants of the two folding processes did not depend on the concentration of A8-35, indicating that intermolecular interactions between proteins are not involved.

As compared to folding in detergents, an interesting feature of folding in A8-35 is that, because of the APol’s very low critical association concentration (CAC) [123], it can be carried out under very dilute conditions.

The activation energy of the folding of OmpA into A8-35 was determined from the temperature dependence of the folding kinetics. The SDS–PAGE assay yielded an activation energy of ~5.9 ± 4.1 kJ mol⁻¹ (~1.4 kcal mol⁻¹) for the fast process, ~36.5 ± 9.6 kJ mol⁻¹ (~8.7 kcal mol⁻¹) for the slow one. When monitored by fluorescence spectroscopy, these activation energies were ~8.8 ± 2.3 kJ mol⁻¹ for the fast process and 28.9 ± 8.1 kJ mol⁻¹ for the slower process, respectively. Within error margins, these values are comparable. They are smaller than the activation energy of 46 kJ mol⁻¹ reported for the folding of OmpA into bilayers (SUVs) of dioleoylphosphatidylcholine [124].

Stability of membrane proteins in amphipols

A large number of studies have shown that most TMPs of either the α-helical or the β-barrel types inactivate more slowly or at higher temperature when trapped in APols as compared to a detergent environment (see e.g. Refs. [45,46,52–55,78,125,126]; for recent reviews, see Refs. [49–51]). Hereafter, we focus on the stability of TMPs in APols against unfolding induced by either heat or chemical denaturants.

Stability of α-helical TMPs in APols

Heat-denaturation of BR has been studied in various environments by monitoring its UV–visible spectrum as a function of temperature [127,128]. In PM, BR does not denature until ~100 °C [129]. BR solubilized in OTG starts to unfold at 40 °C [128], as indicated by a decrease of the visible absorption peak of the holoprotein and by an increase of the light scattering caused by protein aggregation. Massive aggregation and a shift of the absorption maximum to ~380 nm indicate complete denaturation at higher temperature. In contrast, both A8-35-trapped native BR and BR refolded in A8-35 in the presence of PM lipids do not unfold until a temperature of ~50 °C is reached, indicating a stabilization in A8-35 compared to OTG. A8-35-trapped BR denatures only to ~70% after 20 min at 70 °C [128]. The thermostability of BR refolded in A8-35 in the absence of lipids was also relatively high, but a slightly larger amount of retinal was released at each temperature, indicating that lipids indeed bind to BR in the presence of A8-35 and contribute to the observed stabilization [128].

A8-35 has also been shown to improve the thermostability of BLT1 in comparison to fos-choline-16/asolectin mixed micelles [45]. Dahmane et al. compared the thermostability of BLT1 in preparations that had been folded either in lipid-detergent mixed micelles, in pure A8-35, or in A8-35 supplemented with asolectin. The samples were heated for 30 min at various temperatures, after which the folding state of BLT1 was examined by performing a ligand binding assay [130]. A loss of ligand binding activity was detected above ~27 °C for BLT1 in fos-choline-16/asolectin mixed micelles. In contrast, BLT1 folded in A8-35 was still stable at ~35 °C. An additional improvement of the thermostability up to ~39 °C was observed for BLT1 folded in A8-35 in the presence of lipids.

In summary, all observations available to date indicate that APols increase the thermal stability of BLT1 and BR as compared to that in a detergent or detergent/lipid environment. The higher thermal stability of α-helical TMPs in APols translates into an improved stability upon long-term storage, as observed for BR [46,78,126,128], the sarcoplasmic calcium pump SERCA1a [52,125], BLT1 [45], or cytochrome bc [54].

Less direct observations also suggest stabilization of α-helical TMPs by APols as compared to detergents. A case in point is the ion channel TRPV1, which has been studied by EM after trapping in A8-35. Galleries of images of negatively-stained particles indicate that their overall shape is much more reproducible in A8-35 than it is in DDM, suggesting stabilization [62,64]. Similarly, the resolution of cryo-EM images of the human γ-secretase complex is higher for A8-35-trapped than for digitonin-solubilized preparations, suggesting improved conformational homogeneity [69].

Stability of β-barrel TMPs in APols

The heat stability of the major outer membrane protein from the pathogenic bacterium *Chlamydia trachomatis* (MOMP) in APol A8-35 vs. 0.05% of the detergent n-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (zwitgentte Z3-14) has been compared using CD spectroscopy [53,55]. MOMP represents ~60% of the expressed protein in the cell and is therefore much more abundant in the cell than BR or BLT1.

Note added in proofs: a study has recently been published of the stability of the dimer of the transmembrane α-helix of glycophorin A in A8-35 and A8-35/SDS mixtures [171].
the protein content of the outer membrane of \textit{C. trachomatis}. No high-resolution structure has been published to date. However, MOMP seems to share many structural features with the 16-stranded \(\beta\)-barrel porins from other bacteria [131]. At pH 7.4, the midpoint of the transition of heat-induced unfolding of the MOMP trimer in Z3-14 is \(\sim 52^\circ\text{C}\), whereas A8-35–trapped MOMP does not unfold at all up to 78 \(^\circ\text{C}\), indicating an exceptionally strong stabilization [53], which translates into a remarkable stability upon extended storage [55].

Unless aggregation is prevented by APols [132,133] or an excess of detergent [22], heat denaturation of soluble or membrane proteins is usually not reversible, because the denatured protein precipitates. To determine free energies of unfolding/folding, conditions must be found where unfolding/folding is fully reversible and where unfolded and folded forms are at equilibrium. The classic approach is to use chaotropic reagents like urea or guanidinium chloride to unfold and solubilize proteins. Both are strong denaturants that also prevent protein aggregation. When these denaturants are diluted, soluble proteins, as well as most \(\beta\)-barrel TMPs, refold if provided with a suitable folding environment: for TMPs, detergent micelles [21,22,109], lipid bilayers [34,85], or APols such as A8–35 [88] or SAPols [79]. Under equilibrium conditions, a free energy for folding can be calculated from titrations with urea [116,134] or similar denaturants. Reaching equilibrium, however, can be extremely lengthy (weeks).

Equilibrium folding/unfolding has been compared for OmpA in A8–35 vs. the detergent LDAO [116], allowing the calculation and comparison of the free energies of unfolding (Fig. 3). Unfolding and folding titration curves superimposed only after very long incubation times: 30–40 days for OmpA/A8–35 complexes, 18–25 days for OmpA/LDAO ones. As shown in Fig. 3, it is the unfolding reaction that imposes these long equilibration times. The activation free energy for unfolding OmpA in urea, pH 10, is much higher in A8–35 than in LDAO, as indicated by the slower kinetics.

Titrations for folding indicate that equilibrium is reached much faster when the reaction is started with unfolded OmpA. This indicates that the activation energy for folding is lower than that for unfolding. Reversible folding/unfolding conditions were only achieved at pH 10, in line with an earlier study of the stability of OmpA in sonicated lipid vesicles [134]. At pH 10, OmpA (\(pI = 5.5\)) is strongly negatively charged, because of the deprotonation of some of the 17 lysine (\(pK_a = 9.5–10.5\)) and 17 tyrosine (\(pK_a = 9.5–10\)) residues. The resulting increased negative charge of OmpA leads to intermolecular repulsion and a stronger side-chain hydration, which prevents aggregation and ensures a better solubility. However, the increased net negative charge of OmpA may also lead to less stable complexes with the negatively charged A8–35, because of charge–charge repulsion. This might be the reason for the reduced thermodynamic stability of folded OmpA observed in A8–35 as compared to LDAO (Fig. 3). Neutral APols can be expected to prevent aggregation.

![Fig. 3. Unfolding (solid symbols) and refolding (open symbols) titrations of OmpA in LDAO (A) and in A8–35 (B) in the presence of various concentrations of urea, determined by fluorescence spectroscopy. Samples of OmpA in LDAO were incubated for 3 (\(\bullet\)), 7 (\(\bigcirc\)), 10 (\(\bigtriangleup\)), 17 (\(\bigtriangledown\)), and 25 (solid and open bowties) days (panel A) and samples of OmpA in A8–35 for 3 (\(\bullet\)), 10 (\(\bigcirc\)), 24 (\(\bigtriangleup\)), 38 (\(\bigtriangledown\)), 52 (solid and open bowties) days (panel B) at 40 \(^\circ\text{C}\). (C) and (D) Energies of unfolding calculated from the folding and unfolding titrations shown in panels (A) and (B). Equilibrium was reached after 25 days for LDAO (C) and after 52 days for A8–35 (D), respectively. At equilibrium, free energies of unfolding were \(\sim 60\) kJ/mol for OmpA in LDAO solution and \(\sim 8\) kJ/mol for A8–35–trapped OmpA. Adapted from Ref. [116].](http://example.com/figure3.png)
to provide greater thermodynamic stabilization under such extreme conditions as pH 10. Despite the thermodynamic destabilization of OmpA, the activation energy of unfolding of OmpA from complexes with A8-35 is much higher, so that it takes longer for OmpA to unfold in A8-35 than in LDAO [116]. This is consistent with the view that part of the stabilizing properties of APols result from damping the conformational excursions of OmpA, which slows down denaturation (see below).

In lipid bilayers (large unilamellar vesicles), an equilibrium unfolding titration of OmpA could not be observed even after 12 days of incubation [135]. The folding titration had a midpoint around 2 M urea, similar to that observed for folding of OmpA into A8-35 [116]. This midpoint is much lower than the midpoint observed for unfolding in LDAO. Since the presence of a surfactant is necessary for equilibrium unfolding studies of membrane proteins, it cannot be entirely excluded that the higher thermodynamic stability of OmpA seen in LDAO (or in octylmaltoside [136]) vs. A8-35 be due to surfactant-urea interactions. The activation energy for unfolding of OmpA from lipid bilayers is so high that it prevented the estimation of the free energy from unfolding titrations [135].

As a conclusion, these observations indicate that OmpA, at pH 10, is greatly stabilized by A8-35, as compared to LDAO, against denaturation by urea, not as a result of an increased thermodynamic stability, but because of a large increase of the activation energy of unfolding.

What is going on?

From the data summarized in the previous sections, it seems reasonable to conclude that (i) as a rule, TMPs, whether of the \( \alpha \)-helical or the \( \beta \)-barrel type, tend to be more stable after trapping with APols than they are in detergent solution, and (ii) APols appear as an efficient medium in which to fold TMPs to their native state. In the present section, we will discuss first various mechanisms that seem to be involved in stabilization, and try to relate them to the physical–chemical properties of APols. We will next examine the issue of folding or refolding in APols.

Stabilization of membrane proteins by amphipols

Three mechanisms have been invoked as possible sources of TMP stabilization by APols: (i) reduction of the hydrophobic sink; (ii) weak competition with protein/protein and protein/lipid interactions; (iii) damping of TMP dynamics (reviewed in Refs. [49–51]). Let us examine them in turn, provide some evidence in their favor, and discuss which properties of APols underlie them.

The hydrophobic sink effect. It is well known to membrane biochemists that the stability of a given TMP in a given detergent very often depends on the concentration of micelles it is exposed to: the closer one is to the cmc of the detergent, the more stable the

![Fig. 4.](image_url)
protein. This is illustrated in Fig. 4A in the case of the photosynthetic complex cytochrome b$_{56}$f, a superdimer whose stability is exquisitely dependent on the detergent used and its concentration. Upon migration in a sucrose gradient containing 0.2 mM DDM, that is just above the cmc of ~0.17 mM, the complex migrates as a dimer, retains all of its subunits, and it is active in electron transfer. At 3 mM DDM, a large fraction of the preparation disaggregates into inactive monomers, with the loss of cofactors and subunits [137]. Because the chemical potential of DDM monomers remains essentially constant under these two conditions, buffered as it is by the micelles, there is no reason to think that the physical–chemical properties of the DDM belt surrounding the complex vary significantly upon increasing the DDM concentration from 0.2 to 3 mM. What does vary enormously is the concentration of micelles. Taking 0.17 mM to be the approximate cmc of DDM under these conditions, the 0.2-mM gradient contains ~0.03 mM of micellar DDM, the 3-mM one ~2.87 mM, that is ~0.015 and ~1.5 g L$^{-1}$, respectively. This corresponds to a ~100× increase in the volume of micellar detergent. This can obviously favor monomerization, either directly, by displacing a dimer/monomer equilibrium, or indirectly, by diluting hydrophobic or amphipathic molecules – lipids, cofactors, subunits – that are associated to the complex and may stabilize its dimeric state. In the case of cytochrome b$_{56}$f, delipidation has been identified as a major factor of destabilization. This is illustrated in Fig. 4B, where the electron transfer activity of the complex has followed as a function of time in the presence of a high concentration of Hecameg (a detergent similar to OTG), supplemented or not with egg lecithin in a molar ratio to micellar detergent of 1:10 or 1:5 [137].

That the hydrophobic sink effect also exists in preparations of TMPs trapped in APols is suggested by Fig. 5, in which the stability of BR, solubilized from purple membrane (PM) along with PM lipids, has been compared at two temperatures and in the presence of a variable excess of A8-35 or OTG (data from Ref. [138], figure from Ref. [50]). BR is known to bind ~2 g A8-35 per g protein [126]. The excess of A8-35 over that actually bound to the protein is therefore, respectively, ~2.5×, ~5×, ~10× and ~25× for overall mass ratios of 1:5, 1:10, 1:20 and 1:50. It is also known that, in the first of these conditions –which is the one in which BR is usually handled in A8-35–, PM lipids remain quantitatively associated to it, and that BR is mostly monomeric [126]. Experiments in which BR has been refolded in A8-35 either in the presence or absence of lipids have shown that lipids improve its resistance to thermal denaturation [128]. Finally, because the CAC of A8-35 is very low, ~0.002 g L$^{-1}$ [123], and the concentration of free APol in the experiments of Fig. 5 is much higher (~0.7–10 g L$^{-1}$), nearly all APOL that is not bound to BR is present as particles, into which lipids can partition. At room temperature, there is no clear effect of the concentration of APol on the stability of BR, the protein remaining perfectly stable over a period of a week (Fig. 5, left). At 40 °C, however, there is a tendency for BR to inactivate more rapidly at the highest concentrations of APOL (Fig. 5, right). The most likely interpretation of this data is that there is some redistribution of PM lipids between the surface of the protein and free APol particles and that, as a result, inactivation sets in somewhat more rapidly in the presence of a large excess of APOL.

In solution, it is recommended to keep a small excess of free APol in equilibrium with TMP/APOL complexes, so as to avoid the formation of small oligomers (see Refs. [54,71,126,139,140]). This excess is, however, of the same order of magnitude as the amount of TMP-bound APol, in our example ~0.44 g L$^{-1}$. When TMP/APOL complexes cannot aggregate, as occurs upon immobilization onto chips or beads, free APOL can be dispensed with entirely, because APOLs will not spontaneously desorb unless they are displaced by other surfactants [47,141,142]. When working with detergent solutions, on the contrary, not only must one keep the concentration of the detergent above its cmc, but it is advisable to keep a margin of safety above it, e.g. 0.2 × cmc if the cmc is relatively well known, because the cmc is not a universal constant and it can be affected by such factors as the presence of salt, pH, or the temperature. With a detergent like OTG, whose cmc is ~10 mM, this means working with ~2 mM of micellar detergent, i.e. ~0.6 g L$^{-1}$. At such a high concentration of protein as that used in Fig. 5 (~8 μM BR), the difference between the concentration of free, self-associated APOL or detergent is not large, meaning that the volume of the hydrophobic sink is comparable (note that BR is nevertheless more stable in A8-35 than in OTG, indicating that, as discussed below, other factors must come into play). In many other experiments, however, e.g. spectroscopic or functional measurements, where the protein concentration is often a couple of orders of magnitude lower, the concentration of free APOL particles can be lowered by as much, because what matters is the TMP/APOL mass ratio, not the absolute concentration of APOL. This does not apply to free detergent micelles, which must be kept at a safe level above the cmc whatever the concentration of protein. Everything else being equal, the distribution of lipids will therefore be strongly

Fig. 5. Time stability of bacteriorhodopsin (BR) in amphipol A8-35 versus octylthioglucoside (OTG). BR was extracted with OTG from Halobacterium salinarum purple membrane (PM), along with PM lipids, trapped in A8-35 at various BR/A8-35 mass ratios, and stored in the dark either at room temperature (A) or at 40 °C (B) in a buffer containing 100 mM NaCl and 20 mM sodium phosphate, pH 7.0 ([BR] = 0.22 g L$^{-1}$). 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 aggregated detergent as that of A8-35 particles in the samples trapped respectively at 1:10 and 1:20 BR/A8-35 mass ratios. The absence of data points past 2 h in OTG at 40 °C is due to the aggregation of the protein, accompanied by complete bleaching. From Ref. [50], original data from Ref. [138].
displaced toward binding to the protein in the APol-trapped vs. the detergent-solubilized preparation, which is a stabilizing factor. Needless to say, when APol-trapped TMPs are immobilized onto solid supports and flushed with surfactant-free solutions, the hydrophobic sink effect disappears altogether.

**The poorly dissociating character of APols.** In the experiments of Fig. 5, the volume of aggregated surfactant is roughly the same in the presence of 18 mM OTG (resp. 25 mM) and at 1:10 (resp. 1:20) BR/A8-35 mass ratios. Yet, BR inactivates much faster in OTG than in A8-35. Under such conditions, the hydrophobic sink effect cannot account for the stabilization of BR by A8-35 as compared to OTG. Several lines of observations suggest that, as compared to detergents, APols are poor competitors for protein/protein and protein/lipid interactions. This is reflected in their inability to solubilize biological membranes [47], and even, in most cases, pure lipid vesicles [143]; according to this criterion, APols are not, or only extremely weak detergents. The issue is somewhat complex, in the sense that dispersion of lipids can occasionally be observed, given the right combination of APol chemical structure, lipid composition, ionic strength, temperature and, typically, long period of incubation (days) (see Ref. [143], and Refs. therein). Nevertheless, APols can be applied to lipid vesicles [117] or black lipid membranes [86] without breaking them, and to live cells without lysing them [47,50].

Another line of evidence is provided by studies of the functional cycles of BR [128] and, possibly, the nicotinic acetylcholine receptor [144]. Upon excitation of its chromophore, retinal, by green light, BR undergoes a complex series of transconformations, the photocycle, which results in building up a transmembrane proton gradient [145,146]. Solubilization by detergents affects the kinetics of the photocycle: the early steps are accelerated, an effect that largely persists after transfer to A8-35 and may reflect the loss of the 2D lattice, whereas late phases are slowed down (see Ref. [128], and therein). When detergent-solubilized BR is supplemented with A8-35 and the detergent removed, the late kinetics becomes similar to that in PM. If PM is solubilized in SDS, the mixture supplemented with A8-35, and dodecylsulfate precipitated as PDS, renatured BR exhibits a photocycle identical to that of BR trapped with A8-35 without having been denatured. If, however, BR is renatured in A8-35 in the absence of lipids, its photocycle resembles that in OTG. The most likely interpretation of these observations is that interactions with lipids control the kinetics of the late steps of BR photocycle, that these interactions are absent or perturbed in OTG solution, and that they reform when OTG is replaced with A8-35. The implication is that OTG efficiently competes with lipids for binding to the surface of BR, whereas A8-35 does not [128].

The evidence regarding the nicotinic acetylcholine receptor (nAChR) is less detailed, but points in the same direction. The nAChR is a chemically controlled cation-specific channel that lies in the postsynaptic memhrane of the neuromuscular junction and other synapses. Upon binding the neuromediator acetylcholine, its conformation shifts from a non-conducting, low-affinity state to one in which the channel is open, and then to non-conducting, high-affinity, desensitized states [147]. In native receptor-rich membrane fragments from the electric organ of the electric ray *Torpedo marmorata*, the nAChR, in the absence of ligands, pre-exists in an allosteric equilibrium where a majority of receptors is in the low-affinity resting state, ready to be activated, and a minority of them pre-exists in one of the high-affinity desensitized states. Upon solubilization with CHAPS, the equilibrium shifts in favor of the desensitized states. Upon addition of A8-35 and dilution under the cmc of CHAPS, the equilibrium comes back to that in the original membranes [144] (Fig. 6). The most straightforward interpretation of this observation is that CHAPS displaces lipids from the surface of the nAChR. Upon dilution in the presence of A8-35, it desorbs. Recovery of the original allosteric equilibrium could be due either to the desorption of CHAPS and its replacement with A8-35 or, as in the case of BR, to the rebinding of lipids.

Altogether, and even though more systematic studies would be desirable, it seems therefore likely that part of the stabilizing character of APols could be due to their inability to efficiently compete with the protein/protein and protein/lipid interactions that keep biological membranes together and stabilize the native structure of TMPs. This may be due to the fact that the relatively short hydrophobic chains of APols are bound to a polymeric backbone, making it more difficult for them to intrude into protein/lipid assemblies and disrupt their structure than it is for detergent molecules. The underlying mechanism may be enthalpic or, perhaps more likely, entropic.

**Amphipols damp the dynamics of membrane proteins: the “Gulliver effect”.** APols as a rule do not perturb the function of TMPs (reviewed in Refs. [49–51]). There is however one remarkable exception, the sarcoplasmic calcium pump from twitch muscle (SERCA1a) [148], whose enzymatic cycle is reversibly inhibited by APols with respect to what it is both in permeabilized sarcoplasmic vesicles and in detergent solution [52]. The inhibition is not due to a trivial mechanism such as interference with calcium or ATP binding. It is accompanied by a strong stabilization against the irreversible denaturation that is observed upon calcium removal in detergent solution. Later studies revealed another interesting feature: when different APols are compared, or when mixtures of A8-35 and detergent are used, a correlation appears between the level of inhibition and the degree of stabilization: the greater the stabilization, the stronger the inhibition [125].

This gave a first hint that the same mechanism might underlie both effects. Indeed, i) the enzymatic cycle of SERCA1a involves large (nanometric) rearrangements of its transmembrane helices [149], which is not the case for the other TMPs whose function has been studied in the APol-trapped state to date; and ii) calcium is known to bridge SERCA1a transmembrane helices, which leads to the suggestion that the rapid inactivation of the enzyme upon calcium removal in the presence of detergent [150] may result from the opening of the transmembrane helix bundle, a first step on the way to irreversible denaturation.

These observations led to a hypothesis, dubbed the “Gulliver effect”, according to which APols, because they attach to the transmembrane surface of TMPs at multiple points, may damp large-scale (nanometric) rearrangements of the surface of transmembrane protein domains [47,50,51,125]. A possible mechanism would be that conformational transitions of the protein that entail a rearrangement of the backbone of the polymer are affected by a free energy penalty that makes them less probable than they are in detergent solution. This could account both for the slowing down of the enzymatic cycle of SERCA1a and for lowering the frequency and amplitude of transient openings of the transmembrane helix bundle, purported to be the first step towards denaturation, potentially explaining the observed correlation between the two effects.

Long highly speculative, this hypothesis has been progressively buttressed by a number of observations:

(i) MD [76,151] and inelastic neutron scattering [151] data indicate that A8–35 particles have a hydrophobic core whose viscosity is comparable to that of the hydrophobic region of biological membranes, whereas their interface with the
aqueous solution, where most of the APol backbone lies (Fig. 7, top), is more viscous than that of detergent micelles, and even that of lipid bilayers.

(ii) According to MD data, conformational excursions of A8-35-trapped OmpX (Fig. 7, middle) are damped as compared to those in a detergent or a lipid environment [70] (Fig. 7, bottom).

(iii) As described above, the stabilization of OmpA by A8-35 against urea-induced denaturation at pH 10 is not due to a thermodynamic stabilization, but to a higher energy barrier on the way to unfolding [116].

Taken together, these observations suggest that there is probably some truth in the view that one of the mechanisms of TMP stabilization by APols is a relative freezing of their movements as compared to those in detergent solution.

Stabilization of membrane proteins by amphipols has not a unique origin

It will be apparent from the above that stabilization of TMPs by APols has a multifactorial origin. Depending on the protein considered, on the APol, and on experimental conditions, one mechanism may be more important than another. For instance, it is generally observed that trapping TMPs with APols in the presence of lipids adds to their stability as compared to APol alone (see e.g. Refs. [45,128]). It is likely that, in the presence of lipids, stabilization due to the reduction of the hydrophobic sink will lose some of its importance, as illustrated in Fig. 4B for the b_{6}f complex in detergent solution.

There are suggestions that, as is generally the case for detergents, the more highly charged an APol is, the less stabilizing. Thus, SERCA1a is more stable in A8-35 (which carries 35% of charged units) than in SAPols (75% of charged units) [125], and cytochrome b_{6}f is stabilized by NAPols (no charges), but not by A8-35 [46,78]. This can be understood if electrostatic repulsion favors extended or dissociated states of TMP/APol complexes. Yet, the ion channel TRPA1 is reported to be stabilized by SAPols as compared to A8-35 [67]. Clearly, more systematic studies are needed before a general view can emerge.

Amphipol-assisted folding of membrane proteins

As described above, folding TMPs in APols has been, to date, remarkably successful. This statement must be qualified by remarking that only a limited range of structural types has been
Organization and dynamics of TMP/APol complexes. (Top) Radial distribution of the various moieties of A8-35 in a particle of pure APol, and of water molecules and Na+ ions around it. Data extracted from all-atom MD trajectories after coarse-grained and reverse coarse-grained simulations (from Ref. [76]). The density for the A8-35 moieties is plotted for six distinct molecular groups, corresponding to backbone segments, either ungrafted (BB), grafted with isopropylamine (Iso BB), or grafted with octylamine (Oct BB), and to side-chain segments, there being one side-chain group per isopropyl graft (Iso SC) and two per octylamine graft (Oct SC1, Oct SC2), the latter corresponding respectively to the four methylenes closer to the backbone and to the three distal methylenes and the methyl group. Figure from Ref. [151]. (Middle) Snapshot illustrating the configuration of OmpX/A8-35 complexes in a particle of pure APol, and of water molecules and Na+ ions around it. Data extracted from all-atom MD trajectories after coarse-grained and reverse coarse-grained simulations (from Ref. [76]). The density for the A8-35 moieties is plotted for six distinct molecular groups, corresponding to backbone segments, either ungrafted (BB), grafted with isopropylamine (Iso BB), or grafted with octylamine (Oct BB), and to side-chain segments, there being one side-chain group per isopropyl graft (Iso SC) and two per octylamine graft (Oct SC1, Oct SC2), the latter corresponding respectively to the four methylenes closer to the backbone and to the three distal methylenes and the methyl group. Figure from Ref. [151]. (Bottom) Eigenvalues for the principal components of OmpX dynamics in different environments. Projections onto the first and second principal components for OmpX trapped in A8-35 (green) or solubilized in dihexanoylphosphatidylcholine (DHPC; blue). From Ref. [70]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Organization and dynamics of TMP/APol complexes. (Top) Radial distribution the various moieties of A8-35 in a particle of pure APol, and of water molecules and Na+ ions around it. Data extracted from all-atom MD trajectories after coarse-grained and reverse coarse-grained simulations (from Ref. [76]). The density for the A8-35 moieties is plotted for six distinct molecular groups, corresponding to backbone segments, either ungrafted (BB), grafted with isopropylamine (Iso BB), or grafted with octylamine (Oct BB), and to side-chain segments, there being one side-chain group per isopropyl graft (Iso SC) and two per octylamine graft (Oct SC1, Oct SC2), the latter corresponding respectively to the four methylenes closer to the backbone and to the three distal methylenes and the methyl group. Figure from Ref. [151]. (Middle) Snapshot illustrating the configuration of OmpX/A8-35 complexes in a particle of pure APol, and of water molecules and Na+ ions around it. Data extracted from all-atom MD trajectories after coarse-grained and reverse coarse-grained simulations (from Ref. [76]). The density for the A8-35 moieties is plotted for six distinct molecular groups, corresponding to backbone segments, either ungrafted (BB), grafted with isopropylamine (Iso BB), or grafted with octylamine (Oct BB), and to side-chain segments, there being one side-chain group per isopropyl graft (Iso SC) and two per octylamine graft (Oct SC1, Oct SC2), the latter corresponding respectively to the four methylenes closer to the backbone and to the three distal methylenes and the methyl group. Figure from Ref. [151]. (Bottom) Eigenvalues for the principal components of OmpX dynamics in different environments. Projections onto the first and second principal components for OmpX trapped in A8-35 (green) or solubilized in dihexanoylphosphatidylcholine (DHPC; blue). From Ref. [70]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

explored yet: 7-α-helix bundles and single β-barrel outer membrane proteins (Table 1). Yet, in all cases that have been tested to date, folding yields were at least as good as and usually better than those obtained, using generally more complex procedures, in the presence of detergent or lipid/detergent mixed micelles. Furthermore, no lengthy search for optimization was required, as is usually needed when endeavoring to fold a new TMP in a detergent environment. Indeed, a more or less universal protocol of urea dilution (β-barrel TMPs) or DS precipitation (α-helical ones) in the presence of APols gives, as a rule, satisfying results without departing very much from the conditions initially established for model proteins, OmpA and FomA on the one hand, BR on the other [86].

In a couple of cases where GPCRs did not fold efficiently in A8-35, the preparation in SDS appeared heterogeneous upon SDS-PAGE (unpublished data). It is unsurprising that preparations in which a large fraction of the material is either aggregated or misfolded do not fold in good yield. Indeed, in early experiments in which DS precipitation was used to refold and reassociate two BR fragments in a lipid environment, satisfactory yields were not achieved before a way was devised to transfer the fragments from organic solution to SDS without inducing any aggregation [4]. Every effort should therefore be spent to obtain starting material that yields a single band upon SDS–PAGE before spending too much time on refolding attempts. In difficult cases, one could consider resorting to organic solvents to achieve complete unfolding, followed by transfer to SDS [2,4,128]. Replacing SDS with tetracetylcysulfitc, which is a stronger denaturant [152], is perhaps another option, which has not been tested yet. It is worth noting that, in all experiments published to date, no special precaution was taken to control the refolding of disulfide bridges, if any. It is to be expected that control of the redox potential, e.g. by folding first in a reducing medium, followed by oxidation, or by folding in the presence of a mixture of reduced and oxidized glutathione, will turn out to be necessary in specific cases.

In all cases where it has been tested, the presence of lipids (i) was not necessary to obtaining good folding yields, but (ii) improved the yield as compared to that observed in their absence. As lipids were used in small amount as compared to APols (typically in a 1:5 mass ratio), an effect on the physical properties of the APol belt surrounding the refolding protein seems rather unlikely. More probably, molecular interactions are at work. Crystallographic structures of TMPs have revealed well-defined binding sites for lipids, often at the protein/lipid interface, sometimes within protein transmembrane domains. Lipids in biological membranes, therefore, should not be considered as a mere two-dimensional solvent: they also play the role of cofactors (for discussions, see e.g. Refs. [88,153–157]). One way to understand their favorable effect on folding yields in APols is to assume that, in the course of the conformational excursions experienced by a (re)folding protein, transient, partially folded states appear, some of which may exhibit lipid-binding sites. Binding of lipids to such an intermediate state will stabilize it, lengthening the period of time during which folding has a chance to proceed to completion. In other words, binding of lipids to partially folded states may steer folding towards a native-like conformation [51,128]. Once the native structure is achieved, the binding of lipids will stabilize it, diminishing the frequency of conformational excursions that could lead to denaturation, misfolding and/or aggregation.

Why is it that folding in APols is, apparently, so efficient, even though this medium is so unlike a lipid bilayer, both in its chemistry and in its organization? Among the various mechanisms of stabilization that have been discussed above, the most relevant one...
may be their poorly dissociating character. When folding is carried out in detergent, or in lipid/detergent mixtures, the detergent—a molecule that was initially selected for its dissociating properties—competes with reforming protein/protein and protein/lipid interactions. In the scheme of Fig. 8A, this means that folding to the native structure has to compete with partial folding, in which protein/surfactant interactions replace some protein/protein ones, misfolding, in which non-native-like interactions form intramolecularly, and aggregation, induced by intermolecular protein/protein interactions. APols, because of their low detergency, can be expected to favor the productive path over, in particular, pathway, because of their lesser detergency. See text. Adapted from Ref. [86].
to the “denatured” state, from which it can fold again. In a “non-denaturing” detergent, which is not so dissociating, it is at risk of reaching a misfolded $\zeta$ and/or aggregated $\gamma$ state, from which it may not recover (Fig. 8A). In an APol environment, chances that a protein that has reached a partially folded state $\beta$ will move to the native state $\alpha$ can be expected to be higher, because of the lesser competition of protein/surfactant interactions with native-like protein/protein and protein/lipid ones. This, in turn, should diminish the risks of moving to the irreversible states $\zeta$ and $\gamma$ (Fig. 8B).

According to this view, APols could provide a good folding medium because, on the one hand, they adsorb onto hydrophobic surfaces, keeping unfolded TMPs from aggregating (or slowing down their aggregation), while, on the other hand, they do not compete efficiently with the protein/protein and protein/lipid interactions that define the native structure. Thus, they would substitute for SDS or urea at the surface of the unfolded protein, keeping it soluble, but be progressively displaced from those protein surfaces that can form stronger interactions either with other proteic elements or with lipids. Although the term of “molecular chaperones” has been overused and misused, it may be to some extent appropriate here, in the sense that APols may slow down the formation of non-specific, unproductive interactions between hydrophobic segments, which would lead to misfolding and/or aggregation, while moving out of the way when specific interactions establish themselves.

Three types of APols have led to successful (re)folding of TMPs to date: A8-35, SAPols and NAPols (Table 1). Except for BR, for which comparable folding yields are achieved in A8-35 and in NAPols [78,107,128], no comparative studies of the folding yields achieved for one given TMP using one or the other APol have been carried out yet. On the basis of our early experiments, we had proposed that A8-35 formed around refolding TMPs a sort of protective “bubble” that would allow folding to proceed while slowing down the formation of non-productive intermolecular interactions [86] (Fig. 8). One possible mechanism providing a relative isolation of refolding proteins one from another could be the electrostatic repulsion between complexes incorporating either A8-35 or SAPols: both of them are polyanions, whose interactions indeed strongly depend on the ionic strength [71]. The fact that the totally uncharged NAPols also allow folding in good yield [78] seems to indicate that such an electrostatic mechanism, if present, is not essential.

Detailed protocols for folding TMPs in APols are given in Ref. [84].

Perspectives

Once trapped or folded in APols, TMPs can be purified, handled and studied using most of the approaches classically used in detergent solutions, plus some extra ones made possible, in particular, by the use of labeled or functionalized APols (reviewed in Refs. [49–51]). The stability conferred to them by the APol environment facilitates experiments that are lengthy and/or must be carried out under destabilizing conditions, such as high concentrations of detergent and/or high temperature (for a general discussion, see Ref. [51]). A case in point is solution NMR, whose conditions tend to be harsh (reviewed in Refs. [60,91,158,159]). In EM single-particle image reconstruction, the use of APols facilitates the study of fragile TMPs and TMP complexes (see e.g. Refs. [61,62,64,67–69,160], and Refs. therein). As noted above, an interesting observation is that, in the case of the ion channel TRPV1, more homogeneous images are obtained in A8-35 than in detergent [64], which may be related to stabilization. In vaccination, better protection against a bacterial disease is obtained following immunization against an APol-trapped than a detergent-solubilized outer TMP used as immunogen, which may be related either to its stabilization and/or to a better presentation to the immune system [53,55,161]. Also worth noting is the developing use of tagged APols for immobilizing TMPs onto solid surfaces [141,142,162–164]. In such experiments, several advantages of APols are exploited simultaneously: they stabilize the TMP during extended screening sessions, they mediate its immobilization, and, because of the stability of TMP/APol interactions, they make it possible to work in surfactant-free media (reviewed in Ref. [165]).

As noted above, trapping TMPs in APols in the presence of lipids generally increases their stability. This can of course be combined with other stabilizing strategies, such as adding ligands, a case in point being BR [128]. It is interesting that, in this particular case, the ligand, retinal, is extremely hydrophobic. When added to the refolded apoprotein in the presence of APol, it must distribute between protein-bound APol belts and free APol particles. It is likely delivered to retinal-free BO/APol complexes when they collide with retinal-containing particles (for a discussion, see Refs. [71,128]).

An interesting question is whether TMPs that have been made detergent-resistant by accumulating stabilizing point mutations (see e.g. Refs. [166–169], and therein) will show improved stability in APols as compared to wild-type proteins. This may not be the case if the stabilization mechanism involves the selection of detergent-binding sites at the surface of the protein. Note also that detergent-resistant GPCRs have been engineered primarily with the view of forming 3D crystals in detergent solution, whereas crystallizing in aqueous solution is not the easiest application of APols (see Refs. [51,54,95], and therein).

One of the many open questions is which types of TMPs will be amenable to APol-assisted folding. A particularly difficult case is that of oligomeric TMPs. There are however reasons to be cautiously optimistic:

(i) Many (currently close to 20) oligomeric TMPs have been folded or expressed in vitro using classic surfactants (reviewed in Ref. [1]).

(ii) As illustrated by the delivery of retinal after BO has folded, being trapped with APols does not prevent a TMP to interact with a molecule carried by another APol particle [128] (see also Ref. [171]).

(iii) By increasing the TMP/APol ratio, APol-trapped TMPs can be induced to interact one with another [71,126,139].

(iv) There are indirect indications that GPCRs folded in A8-35 can dimerize. Whereas no strong, direct demonstration has been provided yet, this is suggested by the fact that there is an optimum protein/A8-35 ratio for the BLT1 leukotriene receptor to fold [45]. In the absence of lipids or any other cofactor that could become diluted, the most straightforward interpretation of this observation is that newly folded receptors become stabilized by dimerization, which occurs less efficiently in the presence of too large an excess of APol.

One of the main challenges in folding oligomeric TMPs is that, in most cases, unassembled monomers can be expected to be only marginally stable. Conditions must be found that favor their folding and assembly while discouraging the formation of improper intermolecular interactions. Given that it can be done in detergent or mixed lipid/detergent micelles (see Ref. [1]), there is every reason to hope that APols will make it easier. It is likely that, in most cases, simultaneous (re)folding of all subunits will turn out to be the best strategy. In cases where unassembled monomers have very different stabilities, however, alternative routes could be experimented with, such as folding or expressing the most unstable subunit in the presence of its already folded partner(s).