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Amphipol-Assisted in Vitro Folding of G Protein-Coupled Receptors[†]

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ABSTRACT: G protein-coupled receptors (GPCRs) regulate numerous physiological functions. The primary difficulty presented by their study in vitro is to obtain them in sufficient amounts under a functional and stable form. *Escherichia coli* is a host of choice for producing recombinant proteins for structural studies. However, the insertion of GPCRs into its plasma membrane usually results in bacterial death. An alternative approach consists of targeting recombinant receptors to inclusion bodies, where they accumulate without affecting bacterial growth, and then folding them in vitro. This approach, however, stumbles over the very low folding yields typically achieved, whether in detergent solutions or in detergent−lipid mixtures. Here, we show that synthetic polymers known as amphipols provide a highly efficient medium for folding GPCRs. Using a generic protocol, we have folded four class A GPCRs to their functional state, as evidenced by the binding of their respective ligands. This strategy thus appears to have the potential to be generalized to a large number of GPCRs. These data are also of interest from a more fundamental point of view: they indicate that the structural information stored in the sequence of these four receptors allows them to reach their correct three-dimensional structure in an environment that bears no similarity, beyond the amphiphilic character, to lipid bilayers.

Folding studies of membrane proteins are important in two respects. From a fundamental perspective, they provide insights into the respective roles of the amino acid sequence, the insertion machinery, and the membrane environment in determining the three-dimensional fold adopted by the polypeptide. From a practical point of view, they may provide a way of producing large amounts of naturally rare proteins in their functional state.

G protein-coupled receptors (GPCRs[1]) are versatile biological sensors that are responsible for a majority of cellular responses to hormones and neurotransmitters as well as for the senses of sight, smell, and taste (*1*). Except for the naturally abundant rhodopsin (*2*), obtaining sufficient amounts of properly folded and stable GPCRs for in vitro structural and functional studies has proven extremely difficult, to the point that decades of efforts have hitherto yielded the crystal structures of only three of them, the β_1- and β_2-adrenergic and the A2A adenosine receptors (*3*−*6*).

Escherichia coli is a host of choice for over-expressing recombinant proteins for structural studies, but integration of GPCRs into its plasma membrane tends to be toxic, usually resulting in poor expression yields (*7*). An alternative strategy is to target recombinant receptors to inclusion bodies, where they can accumulate in milligram amounts without affecting bacterial growth (*8*−*14*), and then to fold them in vitro. However, the use of detergents or detergent−lipid mixtures has led to functional folding of only a handful of GPCRs, usually with limited yields (*8*, *10*, *11*, *14*). Synthetic amphipathic polymers known as amphipols (*15*−*17*) have been introduced as stabilizing environments (*15*, *16*, *18*) in which denatured integral membrane proteins, specifically two bacterial outer membrane β-barrel proteins and the α-helical plasma membrane protein bacteriorhodopsin, have been refolded to their native state (*19*). In the present study, we have examined the ability of amphipol A8−35 (Figure 1A) to promote folding of GPCRs produced as inclusion bodies.

EXPERIMENTAL PROCEDURES

Materials. A8−35 (batch FGH20) was synthesized by F. Giusti (UMR 7099) as previously described (*17*). Leukotriene B_4 (LTB_4) was purchased from Biomol laboratories, U-75302,

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[1]Abbreviations: A8−35, a specific form of amphipol (Figure 1A); CD, circular dichroism; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; KDS and SDS, potassium and sodium dodecylsulfate, respectively; LTB_4, leukotriene B_4; MW, molecular weight; NMR, nuclear magnetic resonance; UV, ultraviolet.

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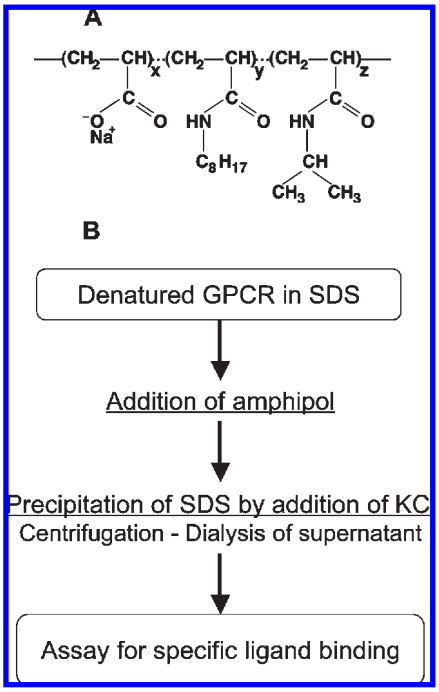

FIGURE 1: BLT1 folding in A8−35. (A) Chemical structure of A8−35, with $\langle x \rangle \approx 35\%$, $\langle y \rangle \approx 25\%$, $\langle z \rangle \approx 40\%$, and ⟨molecular weight⟩ = 9−10 kDa (from ref (*14*)). (B) Schematic representation of the protocol used for receptor folding.

CAY10508, and LY255283 from Cayman Chemicals, [^{3}H]GR 113808 from GE Healthcare, and asolectin from Fluka. All detergents were from Anatrace with the exception of sodium dodecylsulfate (SDS), which was purchased from Euromedex.

Receptor Purification. Human BLT1 and mouse 5-HT$_{4(a)}$ receptors were prepared as KSI fusions as previously described (*11*, *20*). His-tagged mouse BLT2 was expressed using the method described in ref (*11*). Mouse CB1 was expressed in *E. coli* inclusion bodies as a fusion with GST as described for a series of GPCRs (*21*). Both receptors were purified under denaturing conditions as described for BLT1 (*20*). In all cases, the receptors were solubilized in SDS buffer (50 mM Tris-HCl, 0.8% SDS, pH 8) before folding.

Receptor Folding. Receptor folding in detergent and lipid−detergent mixed micelles was carried out as previously described (*11*, *20*). For amphipol-mediated folding, A8−35 was added at ratios of 1, 5, 10, or 20 g of A8−35 per gram of SDS-unfolded receptor in the absence or presence of asolectin at a 0.2:1 asolectin/A8−35 weight ratio. After 30 min of incubation at room temperature, receptor folding was initiated by precipitating dodecylsulfate as its potassium salt (*19*). After 30 min, the KDS precipitate was removed by two 10 min centrifugations at 13 000 rpm (Heraeus tabletop centrifuge). The supernatant was then dialyzed against KP buffer (150 mM KCl, 30 mM potassium phosphate, pH 7). The amount of active receptor after folding was determined by ligand-binding experiments using equilibrium dialysis. Measurements were carried out at stoichiometric receptor/ligand ratios and high concentration (in the 10-μM range), to ensure saturation of the receptor ligand-binding sites. Receptor concentrations were calculated from UV absorbance (*10*, *11*). LTB$_4$, serotonin, and anandamide were used as ligands for BLT1 and BLT2, 5-HT$_{4(a)}$, and CB1, respectively. The free and total concentrations of ligands were calculated from their UV absorbance (*10*, *11*, *22*). Nonspecific binding in the equilibrium dialysis experiments was estimated by substituting under the same conditions similar amounts of pure A8−35 to the A8−35-folded receptor preparation. The magnitude of this correction varied from less than 1% of the specific binding for LTB$_4$ to ~6% for GR 113808.

Ligand Titration. Ligand titration experiments with the leukotriene and serotonin receptors were carried out as previously described (*10*, *11*). CAY10508 binding to the CB1 receptor was assessed by monitoring the changes in the fluorescence emission of the purified receptor ($\lambda_{exc} = 295$ nm; $\lambda_{em} = 340$ nm) as a function of the ligand concentration, as previously described for the binding of LTB$_4$ to BLT1 (*10*). The binding profiles are presented as the percentage of maximal ligand binding as a function of ligand concentration. Titration data were analyzed using the PRISM software version 4.0 (Graphpad Inc.).

Stability Assays. Thermostability was assessed by incubating different receptor samples at the specified temperature for 30 min. The samples were then placed on ice and LTB$_4$ binding assessed by circular dichroism (*23*). Time-dependent stability was assessed by measuring the amount of ligand-competent receptor after storage at 4 °C for the duration indicated.

Size Exclusion Chromatography. Gel filtration was carried out on a Superdex S200 column (10 × 300 mm; GE Healthcare) equilibrated with KP buffer, connected to an Akta purifier system (GE Healthcare). The sample was loaded on the column directly after dialysis against KP buffer. Elution profiles were typically followed at 220 and 280 nm. The oligomerization state of the eluted protein was assessed by chemical cross-linking as described in ref (*24*).

RESULTS

A8−35-Mediated Folding of the BLT1 Receptor. BLT1, one of the two receptors of LTB$_4$ (*25*), belongs to class A GPCRs. It is involved in the control of inflammatory processes (*26*). In vitro folding of BLT1 has been previously achieved in detergent−lipid mixed micelles (*20*, *24*). Although moderate, the yield reached under these conditions (~30%) is among the highest reported to date for a GPCR. In the present work, we have examined whether higher folding yields could be achieved by replacing the mixed micelles with amphipols. BLT1 was purified from *E. coli* inclusion bodies in SDS solution as previously described (*20*). According to CD evidence, SDS-solubilized BLT1 features significant helicity; however, it does not bind LTB$_4$, and therefore, it is not in its native state (data not shown). The strategy we applied to fold BLT1 from its SDS-solubilized state is schematically presented in Figure 1B. A8−35 was added at different protein/A8−35 ratios and SDS removed by KCl precipitation followed by dialysis (*19*). In contrast to what occurs in detergent and detergent−lipid solutions, no significant protein loss through precipitation occurred during amphipol-mediated folding. Folding efficiency was then assessed by measuring the fraction of ligand competent receptor. A high folding yield was observed at all protein/amphipol ratios tested (Figure 2). The ~50% folding yield achieved at 1:5 protein/A8−35 weight ratio is remarkable, considering that the maximal yield of ~30% observed in fos-choline-16/asolectin mixed micelles is the highest ever reached for this receptor after testing more than 30 detergents and lipid−detergent combinations[2].

The denatured BLT1 receptor, being extracted from inclusion bodies and purified in the presence of SDS, is unlikely to contain

[2]J.-L.B., unpublished data.

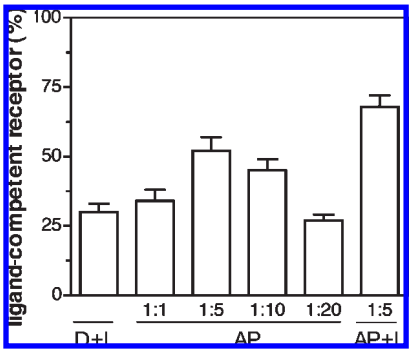

FIGURE 2: Folding of BLT1 in detergents and A8−35. Fraction of ligand-competent receptor obtained after folding SDS-solubilized BLT1 in fos-choline-16/asolectin (D + L), in A8−35 at increasing amphipol/protein ratios (AP), and in A8−35 in the presence of asolectin (1:5:1 protein/amphipol/lipid weight ratio; AP + L). Protein/amphipol mass ratios are indicated under each bar. The fraction of ligand-competent receptor was calculated as the amount of receptor able to bind LTB_4 divided by the amount of unfolded receptor used in the folding assay. Error bars span the range of values observed in duplicate experiments.

any significant amount of lipids. Earlier studies showed that its folding efficiency in detergent micelles increases upon addition of lipids (*24*). The same holds true in A8−35: as shown in Figure 2, the folding yield increased to 65−70% in the presence of even limited amounts of asolectin. Increasing the amount of lipids did not significantly increase the folding yield (not shown), which represents over a two-fold increase over that obtained in detergent−lipid mixed micelles.

Ligand-Binding Properties of the BLT1 Receptor Folded in A8−35. To probe the quality of folding, the ability of BLT1 folded in A8−35 to bind its specific ligands with the proper pharmacology (*10*) was examined. The binding isotherm of LTB_4 to A8−35-folded BLT1 (Figure 3A) yielded a K_D value of 8.9 ± 0.3 nM ($n = 3$), which is in the same range as that observed both with membrane fractions from cell lines expressing BLT1 under its native form and with BLT1 folded in detergent−lipid mixed micelles (~13 nM) (*20*). Competition experiments were carried out with two BLT antagonists, U-75302 and LY255283. U-75302 is a specific BLT1 orthosteric antagonist, whereas LY255283 is an antagonist of the closely related BLT2 receptor, with no high-affinity binding to BLT1. As shown in Figure 3C, U-75302 readily displaced LTB_4 from its binding site on BLT1, with an EC_{50} value (139 ± 29 nM; $n = 3$) comparable to that observed with BLT1 folded in detergent−lipid mixtures[2] (168 nM). In contrast, no significant displacement was observed with the BLT2-specific antagonist LY255283 (Figure 3C). The fact that BLT1 folded in A8−35 displays a similar pharmacological profile as the membrane-bound receptor is highly indicative of its having adopted its native fold.

Stability of the BLT1 Receptor Folded in A8−35. Stability is essential to studying membrane proteins in vitro. In detergent solutions, this is often problematic (*27*, *28*), as is the case for BLT1 folded in detergents (*23*). Amphipols generally stabilize membrane proteins as compared to detergent solutions (*16*, *18*). We therefore examined whether this holds true for the BLT1 receptor folded in A8−35. Thermostability was assayed by heating the receptor folded either in lipid−detergent mixed micelles or in amphipol at various temperatures for 30 min before performing an LTB_4 binding assay (*23*). As shown in Figure 4A, loss of ligand binding occurred above 27 °C with BLT1 folded in fos-choline-16/asolectin mixed micelles. A8−35-folded receptor, on the other hand, was stable up to ~35 °C. Further thermostability, up to ~39 °C, was achieved after folding BLT1 in the presence of A8−35 and lipids, indicating that lipids increase both the folding efficiency and the thermal stability of the protein (Figure 4A). Stability over time followed the same trend: after folding in fos-choline-16/asolectin, high-affinity LTB_4 binding dropped to ~50% of its initial value after ~20 days at 4 °C, while after folding in A8−35 no significant loss of activity was observed for up to 25 days, whether lipids were present or not (Figure 4B). Taken together, these data indicate that trapping BLT1 in A8−35 significantly increases its stability as compared to that in detergent solutions.

A8−35-Mediated Folding of the BLT2, 5-$HT_{4(a)}$, and CB1 Receptors. To probe the generality of this new folding strategy, we tested three other GPCRs: the human serotonin receptor 5-$HT_{4(a)}$, which folds to 20−25% in detergent−lipid mixtures (*11*), the cannabinoid CB1 receptor, whose folding has never been achieved whether in pure detergents or in detergent−lipid mixtures, and the other LTB_4 receptor, BLT2. Although BLT2 features 45% sequence identity with BLT1 (*25*), its folding yield in mixed micelles has never exceeded 3−4%[2]. The three receptors were purified from *E. coli* inclusion bodies solubilized in SDS. We then folded 5-$HT_{4(a)}$, BLT2, and CB1 BLT1 from their SDS-solubilized states as described above for BLT1. All three receptors were found to fold efficiently both in pure A8−35 and in the presence of A8−35 and lipids (Figure 5). In the latter case, functional folding yields of ~60, 70, and 40% were achieved for the 5-$HT_{4(a)}$, BLT2, and CB1 receptors, respectively (Figure 5).

After folding in A8−35, 5-$HT_{4(a)}$ displayed a comparable affinity for its GR113808 antagonist ($K_D = 0.60$ nM) as it does after folding in dimyristoylphosphatidylcholine/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) bicelles ($K_D = 0.5$ nM) (*11*). Similarly, the K_D for LTB_4 of BLT2 folded in A8−35 (169 nM) is indistinguishable from that in mixed micelles[2] (177 nM). Finally, the K_D of the purified CB1 receptor for its inverse agonist CAY10508, that is, 293 nM, is close to that reported for the receptor in membrane fractions (243 nM; ref (*29*)). The fact that all four receptors folded in A8−35 display similar pharmacological profiles as their membrane-bound forms again indicates that the amphipol stabilizes them in their native conformation and that it does not interfere with ligand binding.

We have observed before that bacterio-opsin renatures in A8−35 to a higher yield in the presence of purple membrane lipids (>90%) than in their absence (~75%) (*19*). A similar effect is encountered for the four GPCRs tested in the present work. Therefore, it seems that, while a pure amphipol environment suffices for these five proteins to reach a native-like conformation, the presence of lipids favors folding and stabilizes the final fold.

Solution Properties of Receptor/A8−35 Complexes. Size exclusion chromatography analysis of the BLT1/amphipol complexes obtained after folding in pure A8−35 did not reveal the presence of any aggregates (Figure 6). The elution profile observed (Figure 6) and cross-linking experiments (not shown) are compatible with the preparation comprising a mixture of monomeric and dimeric receptors, as found after folding in detergent−lipid mixed micelles (*22*). Similar profiles were obtained for all of the other receptors tested (Figure 6). With the preparation of folded CB1, where the folding yield was lowest, a minor peak was observed close to the exclusion volume. This probably reflects the presence, in this case, of a small fraction of

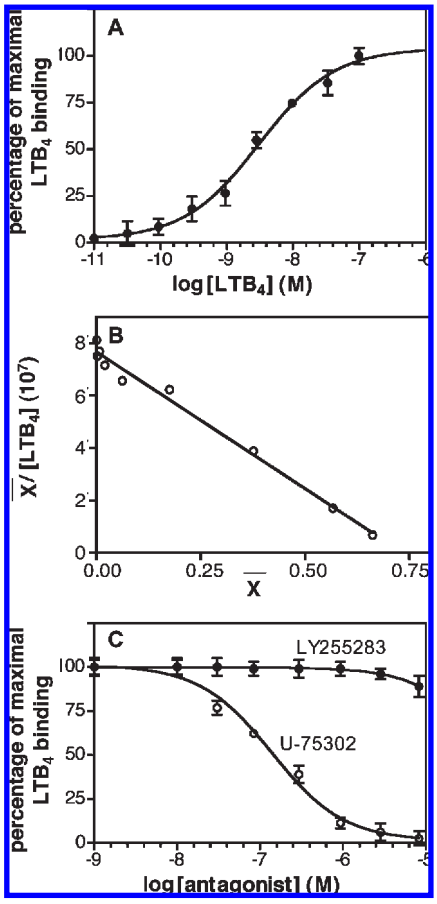

FIGURE 3: Ligand-binding properties of A8−35-folded BLT1. (A) [^{3}H]LTB$_4$ binding isotherm, (B) corresponding Scatchard plot where $\overline{X}$ is the molar ratio of bound LTB$_4$ per BLT1, and (C) competition between the [^{3}H]LTB$_4$ and the U-75302 (○) and LY255283 (●) antagonists. BLT1 had been folded in A8−35 in the presence of asolectin (1:5:1 protein/amphipol/lipid weight ratio). Error bars span the range of values observed in duplicate experiments.

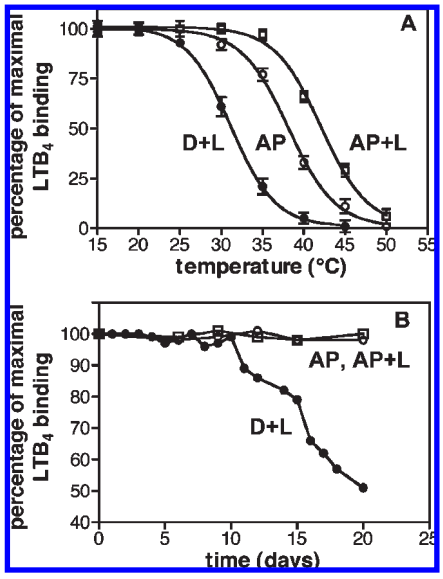

FIGURE 4: Stability of A8−35-folded BLT1. (A) Temperature- and (B) time-dependent stability of BLT1 folded in amphipols (1:5 protein/amphipol weight ratio) in the absence (AP; ○) or presence (AP + L; □) of asolectin (1:0.2 amphipol:lipid weight ratio), compared to that of BLT1 folded in fos-choline-16/asolectin mixed micelles (2:1, w/w) (D + L; ●). Error bars span the range of values observed in duplicate experiments.

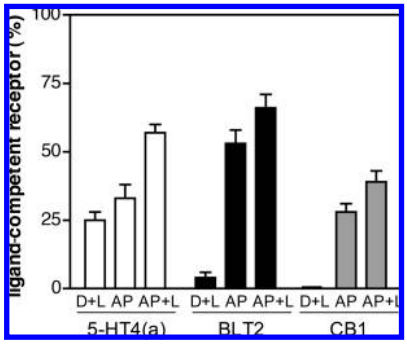

FIGURE 5: Folding of 5-HT$_{4(a)}$, BLT2, and CB1 receptors. Amount of ligand-competent receptor obtained after folding the SDS-unfolded receptors in fos-choline-16/asolectin (D + L), in A8−35 (1:5 protein/amphipol weight ratio; AP), and in A8−35 plus asolectin (1:5:1 protein/amphipol/lipid weight ratio; AP + L). The fraction of ligand-competent receptor was calculated as the amount of ligand-binding receptor over the amount of unfolded receptor used in the folding assays. Error bars span the range of values observed in duplicate experiments.

the aggregated receptor. Other than that, however, the similarity of the size exclusion chromatography (SEC) profiles suggests that, for all receptors tested, folding in A8−35 did not lead to any significant aggregation of the protein.

Separation of Functional from Nonfunctional 5-HT$_{4(a)}$ Receptor. The refolding efficiency achieved here using amphipols, that is, in the 40−70% range depending on the receptor considered, implies that an additional step will be required to obtain the functional receptor in pure form. To assess whether SEC could afford such purification, we tested the different fractions eluted from the Superdex S200 column for ligand binding. The specific activity of all receptors folded in A8−35 did not vary significantly across the main elution peak, indicating that all fractions contain a similar mixture of folded and misfolded receptor. SEC therefore is not adapted to separating the active from the inactive receptor. An alternative possibility would be to use affinity columns with an immobilized ligand, as previously reported for the β_2-adrenergic and the neurotensin receptors (*30*, *31*). The 5-HT$_{4(a)}$ receptor folded in dimyristoylphosphatidylcholine/CHAPS bicelles can be purified on a GR 113808 affinity column (*11*). The same procedure was applied to the receptor folded in an A8−35/asolectin mixture. The fraction recovered after chromatography on the immobilized antagonist was ~96% active, to be compared to ~60% before purification (not shown). Affinity chromatography therefore can readily be used to separate amphipol-trapped active and misfolded receptors for further characterization.

DISCUSSION

The present observations are of interest from both practical and fundamental points of view. From a practical perspective, it is worth noting that, even for those few GPCRs that have been previously folded using conventional surfactants, folding yields are usually low. Furthermore, identifying efficient folding conditions has always been extremely time-consuming. In the present study, conditions initially established to refold bacteriorhodopsin (*19*) have been applied without much change to folding four distinct GPCRs, with yields between 40 and 70%. Should this approach turn out to be as general and easy to implement as

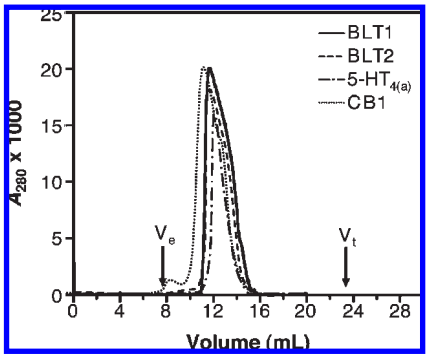

FIGURE 6: Size exclusion chromatography profiles of BLT1, BLT2, 5-$HT_{4(a)}$, and CB1 folded in A8−35 (1:5 protein/A8−35 mass ratio). The molar masses of BLT1, BLT2, 5-$HT_{4(a)}$, and CB1 are 37.5, 37.8, 43.9, and 52.8 kDa, respectively.

the present data suggest, it would represent an important breakthrough for in vitro studies aimed at understanding the molecular bases of the function of class A GPCRs and, possibly, of other membrane proteins.

It is to be noted that, while all of the protein subjected to the folding protocol was kept soluble by A8−35, as indicated by the absence of precipitate or aggregates, only 40−70% thereof was functional, depending on the receptor considered. This means that some of the receptors trapped by the amphipol as monomers or dimers do not bind their ligands. This is not necessarily a hindrance for pharmacological or functional measurements, as long as the misfolded protein keeps silent. For structural studies, on the other hand, it implies that additional separation of the misfolded protein from the functional one will be required. Separating out functional receptors in amphipol by SEC is not effective, since all fractions in the main elution peak display a similar specific activity. An alternative possibility is to use affinity purification, which allowed the separation of the active from the misfolded forms of 5-$HT_{4(a)}$. Other approaches are conceivable, such as transferring the folded receptor from amphipol to detergent (*32*) where the misfolded BLT1 receptor precipitates (*10*).

Depending on the experiments to be performed next, receptors folded using amphipols can be either handled as protein−amphipol complexes, with the benefits inherent to amphipol-induced stabilization, or transferred to other environments. Amphipol-trapped proteins are directly amenable to many functional and structural studies, including ligand-binding measurements (refs (*33* and *34*) and present work) and solution NMR investigations (*35*, *36*). In particular, functionalization of A8−35 with an appropriate group, such as a biotin, makes it possible to use it as an intermediate to immobilize membrane proteins onto solid supports (*34*). Biotinylation does not impede the ability of A8−35 to assist membrane protein folding (unpublished data). The use of such functionalized amphipols therefore opens the way to attaching amphipol-folded GPCRs onto chips or beads for the purpose of binding studies. For other applications, such as crystallization and some forms of reconstitution, it may be advantageous instead to transfer the folded protein to a detergent environment (*32*). Integration into lipid bilayers can be achieved, in some cases, by directly exposing protein−amphipol complexes to preformed membranes, such as black films (*19*) or vesicles (*37*). A milder and more general approach would be to perform a conventional reconstitution procedure after transferring the protein to a detergent solution.

From a more fundamental point of view, we note that seven integral membrane proteins have now been successfully folded in a synthetic polymer, A8−35, whose chemical structure and supramolecular organization (*17*) bear no similarity whatsoever, beyond the amphiphilic character, to lipid bilayers. This is consistent with all of the chemical information needed for these proteins to correctly fold being stored in their sequences. It also implies that, while an amphipathic shield protecting the folding protein from the aqueous solution is essential, and lipids, if present, help in the folding process or stabilize the resulting structure, the presence of a lipid bilayer is not needed to decode this information. This may bear some relevance to the process of membrane protein folding in vivo, which, in the case of most α-helical membrane proteins, starts inside the translocon, in a protein rather than in a lipid environment. The lipid bilayer seems to intervene in a second stage, by selecting among prefolded helices those that will exit laterally the translocon channel and integrate into the membrane (*38*). Much evidence supports the view that the folding of α-helical integral membrane proteins involves a stage in which preformed transmembrane helices assemble into a bundle (*39*−*41*). In keeping with the results of folding experiments carried out previously in detergent solutions (*10*, *11*), the present observations indicate that neither an environment mimicking the highly complex and anisotropic lipid bilayer nor even the presence of lipids is required by any of the four GPCRs tested here for their transmembrane helices to form and correctly orient and pack one with another, and for the receptor to adopt its functional three-dimensional structure.

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