Review

The trials and tribulations of membrane protein folding in vitro

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Abstract

Membrane proteins are hard to handle and consequently the purification of functional protein in milligram quantities is a major problem. One reason for this is that once integral membrane proteins are outside their native membrane, they are prone to aggregation, are unstable and are frequently only partially functional. Knowledge of membrane protein folding mechanisms in vitro can help to understand the causes of these problems and work toward strategies to disaggregate and fold proteins correctly. Kinetic and stability studies are emerging on membrane protein folding, mainly on bacterial proteins. Mutagenesis methods have also been used to probe specific structural features or bonds in proteins. In addition, manipulation of lipid properties can be used to improve the efficiency of folding as well as the stability and function of the protein.

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Protein folding refers to the process by which a polypeptide chain folds up to achieve its functional three-dimensional structure. Many years of intensive research into the in vitro folding of water-soluble proteins has brought us to the current status, where knowledge of the in vitro folding mechanism and key forces driving the process can be applied to rational protein design, understanding in vivo folding and contributing to research in protein misfolding and disease [1–4]. As ever, the same cannot be said for membrane proteins, and as ever, the problem lies in the difficulty of working with hydrophobic proteins in aqueous solution. Hydrophobic integral membrane proteins cannot be denatured in vitro by the standard array of denaturants and chaotropes, nor is it easy to find solubilisation conditions that allow dilution of the denaturant and complete refolding of the protein. The folding mechanism of integral membrane proteins has therefore been difficult to study.

Nevertheless, it can be done, and the information that can be gained from such studies is going to prove useful in membrane protein research [5,6]. Understanding membrane protein folding mechanisms in vitro will ultimately contribute to membrane and cell biology research at a number of levels, just as work on water-soluble proteins has benefited. At present, however, its major contribution probably comes from an improved understanding of how to achieve and maintain a functional folded state of an integral membrane protein outside its native membrane environment. This is of course vital during isolation, purification, reconstitution and the subsequent study of the protein.

1. How can integral membrane proteins be folded in vitro?

This review summarises work on α helical membrane proteins. These proteins tend to be hydrophobic and are difficult to unfold and refold in vitro. Most mammalian receptor and transport proteins have structures based on bundles of transmembrane α helices. Proteins dominated by β barrels are found in the outer membranes of bacteria. These proteins are more hydrophilic and amenable to the traditional methods of denaturation by urea or guanidinium hydrochloride and refolding into detergents, or directly into lipids. Recent reviews on the purification, folding and
structure of outer membrane β barrel proteins include Refs. [7,8].

The archaeabacterial protein, bacteriorhodopsin, was the first membrane protein to be completely denatured and refolded in vitro [9,10]. This feat demonstrates that the polypeptide chain can fold spontaneously and efficiently to give a functional structure in vitro, in the absence of any other proteins, protein gradients or native membranes. Bacteriorhodopsin possesses a structure based on seven transmembrane α helices and is a fairly hydrophobic protein with only short loops connecting the helices outside the membrane. Organic acids, such as trifluoroacetic acid, will denature transmembrane α helices, but urea or guanidinium hydrochloride will not. The acid-denatured state can then be refolded by exchange into sodium dodecyl sulfate (SDS), which invariably induces some helical structure. Folding is completed on transfer of this partially folded state to detergents, lipid/detergent mixtures, or lipids. The transfer of denatured bacteriorhodopsin into SDS is significant. Firstly, it shows the importance of an intermediate solvent step on refolding from the denatured state. Secondly, it suggests that a certain core helix content may greatly facilitate membrane protein folding. This implies that as long as a key helix content is kept, it may be possible to allow a certain degree of unfolding (i.e. loss of tertiary structure or partial unfolding of helical sections), but still recover a fully folded protein. This type of approach should be useful during protein purification. Furthermore, it could mean that detergents like SDS will be helpful when folding membrane proteins from aggregates or inclusion bodies. SDS can solubilise the aggregates, but maintain much of the helical structure that could be essential to recovering functional protein when the SDS is exchanged for a milder detergent.

There is evidence to support these roles for critical helix content and SDS (detailed examples are given in Table 1 of Ref. [5]). Bacteriorhodopsin can be overexpressed in Escherichia coli in a nonfunctional form, and then folded to a functional state following an organic phase extraction, exchange into SDS and finally into renaturing detergent [11]. E. coli inclusion bodies of the photosynthetic light harvesting protein LHCII can be solubilised in SDS and then reconstituted into lipids [12,13]. Urea has also been used to solubilise LHCII, but the urea is then exchanged into SDS before refolding the protein. Solubilisation of receptor fusion proteins from inclusion bodies overexpressed in E. coli has been reported using SDS, and then changing the SDS for another, nonionic detergent [14], although it is unclear as to how successful this latter method is for achieving fully functional protein. The lack of knowledge of the structure and aggregation of the protein in the inclusion bodies, as well as that of the solubilised SDS state, means it is hard to understand the protein–detergent interactions governing the initial solubilisation. Exchanging detergents on chromatography columns is also successful for membrane proteins [15]. An interesting example here is diacylglycerol kinase from E. coli [16,17]. This protein can be overexpressed with a His-tag, in E. coli. The cell extracts are best solubilised in a detergent such as octylglucoside before loading onto a nickel column. The protein can then be eluted off the column in α-decyl β-D-maltoside (DM) or dodecylphosphocholine (DPC) and subsequently reconstituted into 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) vesicles. Many diacylglycerol kinase mutant proteins containing a single amino acid change were noticeably less active when they were eluted in, and reconstituted from, DM. DPC was found to be a distinctly better choice [18]. This implies two things. It is important to look for optimal conditions for different proteins and even single point mutants of the same protein may require slightly different conditions. It also shows that single chain, lyso lipids such as DPC, which exhibit type I, detergent-like behaviour, can be more useful than detergents such as DM. This may be due to the different head groups of DM and DPC, with the latter being the same as POPC. DPC may stabilise the protein more than DM during elution from the nickel column. Alternatively, there may be a more favourable interaction of DPC with POPC lipid vesicles, which facilitates transfer of the protein into the lipid vesicles.

2. Attaining a functional state

A major problem in membrane protein folding is the final stage of folding to a fully functional state. Inclusion bodies or aggregates can be solubilised in urea or SDS; alternatively, proteins can be extracted from membranes by detergents. In both cases, the proteins can then be exchanged into other detergents or lipids. This gives a state with more or less native-like structure, but it is difficult to ensure that all the protein molecules fold to a native-like functional state with appropriate function or ligand binding constants. This final fine-tuning of tertiary structure formation seems to depend on a number of factors. The folded protein structure is more unstable in detergent solution than in a membrane [19], and this allows more flexibility in the structure and helix packing. A detergent environment is obviously very different to the native membrane and there are different lateral forces holding the helices together. Pure detergent micelles without any lipid present also lack specific protein–lipid interactions that may be important for stability. Lipids and stabilising lipid–protein interactions frequently remain after detergent solubilisation of proteins from membranes; however, this is unlikely to be the case after detergent solubilisation of inclusion bodies. Micelles have to be used in column chromatography during purification of membrane proteins and are frequently amenable to standard spectroscopic methods. However, a final transfer of the protein to lipid bilayers is more likely to result in a fully functional and more stable state. A lipid-bilayer environment is usually more stable than a micelle one, and lipid-containing micelles can be more stable than detergent alone.
This lipid effect can be either specific or a generic property of lipid bilayers. The studies on mutant E. coli diacylglycerol kinase proteins, which were discussed above, provide an example of this. The mutant proteins are more active in lipid bilayers, and importantly, reconstitution into the bilayers is more successful from lipid micelles (i.e. micelles of type I lyso lipids like DPC) than detergent micelles [18]. Alternatives to lipids that may also be worth investigating are amphipols [20], which have a hydrophilic backbone but several long hydrophobic chains that cover the protein surface.

3. How can we begin to understand why particular folding approaches are successful and how can other successful methods be designed?

Several approaches have to be taken to answer these questions. These include hypotheses that could be tested with model peptides or proteins together with the development of experimental methods to probe the kinetics and thermodynamics of folding events in vitro. An understanding of the driving forces and mechanisms involved can be used to help predict folding behaviour. In the absence of such molecular level knowledge, the reasons why some proteins prefer certain detergents, or lipids, over others will remain a mystery and membrane protein reconstitution something of a black art, or luck.

3.1. The two-stage model

In 1990, Popot and Engelman [21] proposed a simple model for the folding of transmembrane α helical proteins. Transmembrane helices are stable structural elements in the bilayer, largely due to their main chain hydrogen bonds and the hydrophobic effect. Such helices can be considered to be autonomous folding domains and the first stage of the model is proposed to be the formation of independently stable transmembrane helices. The second stage involves the association of the helices to give the tertiary fold within the bilayer. The model is not actually limited to such defined stages. Not all transmembrane helices of the protein have to be independently stable; it may only require a certain core number of stable helices (possibly N terminal) [22]. In addition, helices can extend, tilt and possibly insert or form during the second stage of the model.

The two-stage model is a conceptual, and probably oversimplistic, model based on thermodynamic principles, together with supporting experimental data. It does not address the actual insertion process (for reviews on biophysical studies aimed at understanding the driving forces for insertion, see Refs. [23,24]), nor does it necessarily represent a mechanism in vitro or in vivo. As Popot and Engelman [21] pointed out, the crux of the two-stage model is that much of membrane protein folding can be understood without considering the insertion process, but by focussing on helix association in the bilayer. This notion has led to informative fundamental studies on helix–helix interactions, ranging from the identification of particular motifs such as GxxxG [25–27] to a role for hydrogen bonds between main chain Cα–H and backbone or side chain oxygen atoms [28].

The ideas stemming from this two-stage model also shed some light on the role of detergents such as SDS in membrane protein folding. SDS allows stable, transmembrane helix elements to form, and then it is presumably easier to find detergent and/or lipid combinations that allow these helices to rearrange, extend and pack, rather than trying to find solvent conditions that allow membrane helices to form and pack in one step.

3.2. Folding mechanisms: kinetics, stability and protein engineering

Studies to investigate the folding mechanisms of membrane proteins in vitro have recently been reviewed in detail [5], and only a brief summary will be given here. The bottom line is that many more studies on folding mechanisms are required before major conclusions can be drawn. Studies have been carried out on bacterial proteins, but very little is known about the folding mechanisms of mammalian receptor and transport systems. The main problems hindering such work, especially on mammalian proteins, are the lack of methods to study protein structural changes during folding, irreversible folding/unfolding reactions and lack of appropriate detergent or lipid folding/unfolding systems that give 100% folding.

Kinetic and stability studies have been carried out on bacteriorhodopsin, β barrel outer membrane proteins and E. coli diacylglycerol kinase (see Ref. [5] for a summary of the results of individual studies). The kinetic studies are mainly based on rapid mixing, stopped flow methods. The folding reaction begins when a partially denatured state of the protein (in SDS or urea) is mixed with renaturing detergents or lipids [29]. Folding is then followed by changes in protein fluorescence, secondary structure formation and recovery of function. These studies have shown that proteins insert into different detergents or lipids with different rates and efficiencies, transient intermediates can be detected during folding, a certain amount of transmembrane helical structure is probably necessary for helical proteins to insert into lipids (in accordance with the two-stage model) and aggregation reactions can compete effectively with folding. Stability studies include calorimetric approaches to measure thermodynamic parameters and mutagenesis methods to probe particular helix–helix interactions or helix–lipid interactions. The proteins are more stable in their native membrane than in detergent micelles, although certain synthetic lipid bilayer environments can provide similar levels of stability. Particular helix interaction motifs have been identified (such as GxGGG discussed above) and the stability of E. coli diacylglycerol kinase has been dramati-
cally increased in detergent micelles by mutating amino acids at the bilayer edges [30,31].

3.3. Designing a folding system based on lipid properties

A folding system has been designed for α helical membrane proteins based on phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids [32]. Folding behaviour in this system correlates with a particular property of the lipids, namely their stored curvature elastic stress in a bilayer structure [33]. This shows that this lipid property can be used as a guide in the design of lipid folding systems, and the optimisation of protein folding and function. SDS is used to give a partially denatured state with a degree of helix content. The protein is then folded by diluting the SDS with renaturing lipid vesicles. Thus, for example, SDS-denatured bacteriorhopdopsin can be refolded in L-α-1,2-dioleoylphosphatidylcholine (DOPC) lipid vesicles.

The advantages of this PC/PE system are that lipid properties can be used to control the protein folding, and the lipids offer an advantage over detergents as the liquid crystalline phase properties of lipids and lipid mixtures are much better understood. Biological membranes consist of several different lipid types, with different head groups and chains. The individual lipids have different properties, with some forming fluid lamellar or bilayer phases and others preferring cubic or hexagonal, nonbilayer phases. Including nonbilayer lipids in membranes imparts a desire for each monolayer to curve toward water. Because the monolayers are constrained to lie back to back in a bilayer, this inclusion of nonbilayer lipids results in an increase in curvature elastic energy in the bilayer (see Fig. 1). There is also a redistribution of the lateral forces within the bilayer, which can be simplistically described as a decrease in outward lateral pressure in the lipid head group region and an increase in outward lateral pressure in the lipid chain region. The curvature energy and chain lateral pressure of a PC bilayer can be increased either by adding a lipid with unsaturated chains or with a different head group such as PE.

Kinetic studies of peptide insertion and protein folding in membranes have shown that this increase in curvature stress has several consequences for protein folding. Firstly, the efficiency of folding is reduced. The yield of bacteriorhopdopsin that folds to a functional state decreases as the PE content of PC/PE bilayer is increased [32]. Secondly, the stability of the helix bundle of bacteriorhodopsin appears to increase as the PE content increases [5]. Thirdly, the activation energy for insertion of a transmembrane helix increases with increasing PE content [34]. These results suggest that increasing the lipid chain lateral pressure and curvature elastic energy of a bilayer hinders the insertion of transmembrane helices (from an aqueous phase) and the insertion of the whole protein (from SDS). Conversely, the increase in curvature energy increases the protein stability due to an increased pressure on the helical bundle.

The optimal curvature stress seems to be different for different aspects of the reconstitution process. Alamethicin is a water-soluble peptide that binds and inserts into lipid bilayers, forming a transmembrane helix that oligomerises and acts as an ion channel. Increasing the PE content of PC/PE bilayers lowers the binding constant for monomers to the bilayers, but favours the multimeric channel state in the bilayer and stabilises this helical-bundle channel [35,36]. Nonbilayer-type lipids, with either PE head groups or unsaturated chains, also affect the function of several membrane proteins. Rhodopsin, for example, exhibits optimal function when reconstituted into membranes with unsaturated lipids or PE lipids [37]. The bilayer should not be too stressed for maximal incorporation of a protein; however, a greater stress and lateral chain pressure may be needed to improve the protein stability, and the optimal stress for function may be different again. During protein purification, similar effects can be achieved with mixed lipid micelles or detergent/lipid micelles. For example, a mixture...
of two PC lipids, with short and long chains that can form micelles, could be used for protein purification. Altering the relative amounts of the two lipids would change properties in the micelle similar to the lateral chain pressures in lipid bilayers and may result in enhanced protein stability during purification.

The detergent that is used to solubilise the protein before insertion into lipid bilayers is important. Firstly, it affects how the membrane/aggregates are solubilised and the degree of structure in the state before refolding. Secondly, the detergent will aid the insertion of the protein into the bilayer. Detergents relax the bilayer, in terms of reducing the bilayer curvature stress. Indeed there may be situations where a stressed bilayer favours detergent-assisted insertion, because the detergent will release the bilayer stress. This rather contrasts with the increase in activation energy for insertion of a transmembrane helix as the bilayer becomes more stressed. Here, the increased bilayer stress and pressure near the bilayer centre makes it more difficult for the helix to cross the bilayer when there is no detergent present. There will be a balance between the structure the detergent induces in the protein, the dilution or removal of the detergent to allow the protein to insert into the lipids and the role of the detergent relaxing the bilayer to allow protein insertion.

One of the strengths of the PC/PE folding system described here is that it is based on curvature elastic stress, which is a general property of lipid bilayers. This stress is a nonspecific property, in the sense that it is not a specific chemical interaction between a particular lipid and protein. It is a property of the bilayer that can be achieved with several lipids with different chemical structures. Thus, for example, the curvature stress of a PC bilayer can be increased either by changing the lipid head group or by increasing chain unsaturation. Furthermore, this suggests that it is not always necessary to use native lipids for efficient protein folding and reconstitution. Nevertheless, although curvature stress can give a guide as to successful folding conditions, there are also likely to be specific lipid–protein interactions for some proteins, and head group charge interactions. In these cases, it may be necessary to add a specific lipid to the folding system to stabilise the folded state for optimal function. In some cases, specific lipids can be seen in the protein crystal structure. Examples include cytochrome \( c \) oxidase and a bacterial photosynthetic reaction centre (for a recent review, see Ref. [38]). Cardiolipin can be resolved in the cytochrome \( c \) oxidase crystal structure and this lipid cannot be removed from the protein without a loss of activity.

### 3.4. Membrane protein topology

A fascinating role for PE has been found in the folding of the \( E. \ coli \) transport protein, lactose permease (LacY) [39]. This work shows that lipid composition can affect membrane protein topology [40]. PE is required for correct folding of lactose permease, but is then redundant in the folded state when the PE can then be removed without loss of protein function. Folding was assessed in these studies by formation of an antibody-binding site on a periplasmic loop between helices VII and VIII of this 12 transmembrane-spanning protein. \( E. \ coli \) mutant cells lacking PE produce lactose permease that is not recognised by this antibody, because the N-terminal helices I–VI adopt an inverted topology. However, the subsequent synthesis of PE causes the protein to switch to its correct topology in the membrane with correct folding of loop VII–VIII. These experiments are carried out in PE-deficient cells where the translocon is present, suggesting that lipid composition is important for membrane protein assembly in vivo. The authors raise the intriguing possibility that there may be lipid-dependent, post-assembly transmembrane helix reorganisation, or that there is a lipid post-assembly proofreading process.

Strategies have been developed that optimise detergent-mediated reconstitution of membrane proteins into lipid vesicles. These are based on a stepwise solubilisation of lipid vesicles by detergent, with incorporation of the protein at different stages of the solubilisation [41]. Three stages have been observed during detergent solubilisation of lipid vesicles. Firstly, the detergent incorporates into the lipid bilayers. Secondly, the lipid vesicles start to collapse and mixed micelles begin to form. Finally, the vesicles are completely solubilised as micelles. Differences in protein reconstitution have been observed for different detergents. Reconstitution of the lactose transport protein, LacS from Triton X-100 leads to the protein being incorporated into the lipid vesicles in only one direction, while reconstitution from DM gives a random distribution [42]. The structures of the lipid/detergent mixtures have been followed by cryoelectron microscopy as Triton X-100 or DM were added to mixed \( E. \ coli \) and egg yolk PC vesicles. This showed that the detergents have different effects on the second, mixed micelle formation, stage, of the reconstitution process and this could explain the differences in directionality of protein incorporation. An equilibrium exists between Triton X-100-saturated, intact vesicles and mixed micelles; removal of Triton X-100 by Bio-beads results in small vesicles and favours protein insertion. However, this insertion is unidirectional, because one surface of lacS is more hydrophilic than the other and does not want to cross the bilayer. In contrast, vesicles immediately start to disintegrate after incorporation of DM and are mainly present as membrane sheets. LacS can insert from either side into such sheet structures, thus giving a random topology.

### 4. Conclusions

Much more fundamental research is required on the folding of integral membrane proteins. We need to know how to handle these proteins outside their native environments, specifically how to purify them and how to maintain
their function and stability. This is important in obtaining protein for crystallisation trials and to understand crystallisation methods. Solvents and sample preparation methods will also need to be optimised for NMR methods, which are starting to come of age for membrane proteins. Protein dynamics often get overlooked; static structures of open and closed states can give an indication of how transport proteins work, but the mechanism can only be found from kinetic and thermodynamic studies of the pathways, transition states and transient intermediates involved. These are crucially dependent on an understanding of the solvent. Detergents are very useful in membrane protein studies, but lipids must also be considered either because there may be specific lipid–protein interactions that stabilise the protein or because they can affect the protein topology. General properties of the bilayer such as curvature stress can also help in the final reconstitution of membrane proteins to attain stable, functional proteins.

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