Can we identify the forces that drive the folding of integral membrane proteins?

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Abstract

Protein folding has been at the forefront of molecular cell biology research for several years. However, integral membrane proteins have eluded detailed molecular level study until recently. One reason is the often apparently insurmountable problem of mimicking the natural membrane bilayer with lipid or detergent mixtures. There is nevertheless a large body of information on lipid properties and in particular on phosphatidylcholine and phosphatidylethanolamine lipids, which are common to many biological membranes. We have exploited this knowledge to design efficient in vitro, lipid-bilayer folding systems for membrane proteins. Bacteriorhodopsin has been used as a model system for our initial studies: we have shown that a rate-limiting apoprotein folding step and the overall folding efficiency seem to be controlled by particular properties of the lipid bilayer. The properties of interest are the stored curvature elastic energy within the bilayer and the lateral pressure that the lipid chains exert on their neighbouring folding protein. These are generic properties of the bilayer that can be achieved with simple mixtures of many types of biological lipid and seem to be important in vivo.

Introduction

Biosynthetic pathways in the cell make use of protein integration machinery to integrate proteins into membranes. Ideally we wish to understand this process in vivo but the complexity of these natural systems means that membrane proteins have to be isolated from their membranes to obtain molecular-level information on their structure, dynamics and folding. Studies of membrane protein folding in vitro [1] are of interest at several levels. Intellectually, folding is an intriguing problem, but folding studies also address a fundamental problem in working with membrane proteins outside their native membrane, namely how can these proteins be kept in a stable, functional form? This problem of maintaining protein solubility and function also impinges directly on the difficulty in obtaining large amounts of the proteins from overexpression systems. Successful overexpression is generally in a heterologous host cell, where the membrane protein inserts poorly into the host membrane and aggregates as inclusion bodies or membrane-associated fragments. The protein is only useful if these aggregates can be solubilized and the protein folded correctly.

To address some of these difficulties, we have been designing a model, lipid-bilayer-based folding system that will allow us to control protein folding and to understand the forces that drive folding. Ultimately we hope that we will be able to predict some of the properties that will aid the solubilization of stable and functional integral membrane proteins.

Hypothesis for the design of a lipid-based folding system for α-helical membrane proteins

A two-stage model has been proposed for the folding of α-helical membrane proteins [2]. Independently stable transmembrane α-helices form in the first stage, because such helices are stable entities in the bilayer. These helices then pack together in the second stage to give the final folded state. Studies in vitro have indicated that certain transmembrane α-helices are indeed stable on their own in lipid bilayers [3-6]. However, it seems that in larger proteins it is not necessary for all helices to be individually stable and that bundles of two, three or more helices can sometimes be needed to help the remaining helices to fold. The two-stage model is based on thermodynamic arguments and therefore does not necessarily represent a mechanism that is followed...
Assembly and Repair of Membrane-Bound Electron Transport Complexes

Either in vitro or in vivo. Nevertheless, it might actually have direct relevance to folding in vivo. For example, the insertion of nascent membrane proteins into the endoplasmic reticulum seems to occur co-translationally at sites termed translocons, which are also responsible for the translocation of secretory proteins [7-11]. These translocons consist of several membrane proteins that form a pore into which nascent polypeptide chains enter. It is not clear how the transmembrane domains of multi-spanning proteins exit from the translocon pore into the bilayer. Recent evidence suggests that transmembrane helices fold within the pore, but exit into the bilayer either individually [10] or, possibly, in pairs. This means that the final transmembrane α-helix packing occurs in the membrane bilayer. The thermodynamic two-stage model indicates that such helices, which emerge individually from the translocon, would be stable in the membrane until the rest of the protein has exited from the translocon and the protein can fold into its functional state.

The ability to manipulate α-helical proteins in the manner suggested by the two-stage model has important implications for work on membrane proteins in vitro, especially when devising systems that maintain protein solubility and function. The folding problem can be broken down; instead of seeking to fold the protein in one attempt, it should be possible to use one solvent mixture first to achieve an intermediate state with some independently stable transmembrane helices. The solvent can then be altered to facilitate the final folding of the protein.

There are three basic choices of solvent for membrane proteins: organic solvents, detergents and lipids. Organic solvents can allow membrane proteins to be solubilized but their obvious difference from the natural anisotropic membrane environment, and the fact that many membrane proteins have large extrinsic aqueous domains, make them unsuitable. Lipids provide the best choice for solubilizing a stable protein in a native-like environment, whereas detergents are particularly useful for purification; detergent/lipid mixtures are frequently a good practical solution. Lipids offer the distinct advantage over detergents that there is a wealth of information available on the structure, dynamics and phase behaviour of many common biological lipids [12,13]. Specific lipid properties can therefore be altered in a known manner to control and improve the efficiency of protein folding ([14], and P. J. Booth, R. H. Templer, J. W. Meijberg, S. J. Allen, M. Lorch and A. R. Curran, unpublished work). We have designed a lipid-based system that allows us to do precisely this.

Biological membranes contain a large diversity of lipids, with most membranes containing a mixture of ‘bilayer’- and ‘non-bilayer’-forming lipids. This seems to have a generic, functional importance by enabling the fine tuning of certain bilayer properties that seem to be under homeostatic control in living membranes and vital for the correct function of several of the constituent membrane proteins. The presence of the non-bilayer lipids increases the desire of each monolayer of the bilayer to curve towards the water. However, the monolayers wish to bend in opposite directions, which they cannot do in a bilayer structure. As a result there is a build-up of a curvature elastic stress within the membrane and redistribution of the intermolecular lipid forces, as the monolayers are forced to lie flat, back to back, in the bilayer. This is accompanied by an increase in the lateral pressure in the lipid chain region, as a result of the increase in the number of collisions between the lipid hydrocarbon chains. Our hypothesis is that these lipid forces can be used to control protein folding. In view of the two-stage model discussed above, we seek to focus on the second stage of the folding model, involving helix association within the lipid bilayer.

There is mounting evidence that the lipid intermolecular forces discussed above have key roles both in vitro and in vivo. The recent novel crystallization method reported for bacteriorhodopsin [16] that has led to a 1.55 Å resolution structure [17] is based on the use of a non-bilayer, cubic lipid phase and seems to be dependent on the manipulation of the lipid intermolecular forces. The introduction of non-bilayer lipids into bilayers modulates the function of several membrane-bound proteins, including alamethicin, cytidyl transferase, rhodopsin, Ca²⁺-ATPase and lactose permease [18-23]. There is also increasing evidence that specialized lipid microdomains, or rafts, with particular lipid properties might exist in vitro [24,25]. Lipid rafts are dynamic assemblies containing high concentrations of sphingolipids with saturated hydrocarbon chains, phospholipids with saturated chains, and cholesterol. The high proportion of saturated chains means that the lipid raft phase is more ordered than the surrounding lipids in which unsaturated chains dominate; the saturated raft lipids have a lower lipid-chain lateral pressure and a lower desire for monolayer
curvature. Certain proteins are localized to these rafts, whereas others are excluded; the rafts have therefore been suggested as a means of concentrating particular proteins together, for processes such as signalling.

Choosing the lipid system and the model protein

A simple two-component lipid system allows the lipid properties to be altered in a known manner. We have chosen two common biological lipids, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). Two methods can be used to alter the desire for monolayer curvature in the PtdCho/PtdEtn bilayer: altering the headgroup while keeping the hydrocarbon chains the same, or altering the chain unsaturation while keeping the headgroup the same. As a general rule PtdEtn lipids, or lipids with chain unsaturation, tend to have a greater desire to form non-bilayer phases than PtdCho lipids or lipids with saturated chains. Thus increasing the amount of PtdEtn in a PtdCho/PtdEtn bilayer increases the desire for monolayer curvature and the outward pressure exerted by the lipid chains. Similarly, introducing an unsaturated bond into the lipid chains also increases monolayer curvature and lipid chain pressure.

So far only two proteins have been refolded from a completely unfolded state: bacteriorhodopsin [26] and OmpA [27]. Bacteriorhodopsin is the only protein constituent of the purple membrane of Halobacteria salinaria. Its structure is now known to 1.55 Å and is a seven-transmembrane α-helical bundle with a covalently bound retinal cofactor. OmpA is an outer-membrane protein of Escherichia coli forming a β-barrel in the membrane. Several other proteins have also been partly unfolded and refolded (see Table 1) but bacteriorhodopsin provides a good model system for testing hypotheses on the folding of α-helical membrane proteins [28–30]. Another advantage of bacteriorhodopsin is that it is possible to study its folding kinetics, which allows mechanistic information to be gained. Furthermore, there is evidence that lipid forces affect the folding kinetics. Bacteriorhodopsin can be folded from a partly denatured SDS state into mixed-lipid 1-α-1,2-dimyristoylphosphatidylcholine/1-α-1,2-dihexanoylphosphatidylcholine (DMPC/DHPC) micelles [31]. A rate-limiting apoprotein folding step has been identified that leads to the formation of an intermediate state with seven transmembrane helices, which then goes on to bind retinal (see Scheme 1). The rate of this folding step is dependent on the amount of DMPC in the mixed DMPC/DHPC micelles. Increasing the DMPC content slows the folding rate. A possible explanation is that the increase in DMPC, which has a longer chain (C14) than the C12 DHPC, increases the collisions between the lipid chains and thus the lateral pressure imposed on the protein. This increase in lipid pressure seems to slow protein folding. However, it is not easy to obtain a direct correlation of this effect in the DMPC/DHPC mixed-micelle system because altering the lipid composition also alters the micelle structure.

We have shown that bacteriorhodopsin can be folded from its SDS-denatured state directly into PtdCho lipid vesicles, to give an almost 100% yield of folded functional protein [14]. This has been achieved in several different PtdCho lipid-bilayer vesicles, including DMPC (C14 saturated chain), 1-α-1,2-dimyristoylphosphatidylcholine (DMoPC) (C14,1, with one unsaturated bond), 1-α-1,2-dipalmitoylphosphatidylcholine (DPOPC) (C16,1, with one unsaturated bond) and 1-α-1,2-dioleoylphosphatidylcholine (DOPC) (C18,1, with one unsaturated bond). On the basis of our hypothesis we have found that the introduction of the corresponding PtdEtn lipid, which increases the chain lateral pressure, has two effects on protein folding: first it lowers the folding yield; secondly it slows down folding. This is true for the following refolding systems: DMPC/DMPE, DPOPC/1-α-1,2-dipalmitoylphosphatidylethanolamine and DOPC/1-α-1,2-dioleoylphosphatidylethanolamine.

Other work in our laboratory has shown that the rate-limiting folding step involves transmembrane α-helix formation [32] and probably some helix packing [33]. The removal of structure in the loops connecting helices C and D, by replacing the loop with a structureless Gly-Gly-Ser repeating linker, also makes the protein more susceptible to the lipid lateral pressure effect [34,35]. A similar effect was observed when the EF loop was replaced by a Gly-Gly-Ser repeating linker. The stability of the seven-transmembrane apoprotein state is lower than that of the wild type for both the CD and FG loop mutant proteins. The folding yield of these loop mutants is also decreased from approx. 100% to as low as 25% as the lipid lateral pressure is decreased (whereas there is only an approx. 5–10% loss of yield for wild-type protein). This also correlates with a
slower folding of both mutant proteins to the seven-transmembrane apoprotein state.

The results outlined above are in line with our hypothesis for designing membrane protein folding systems. The use of two different solvent systems, SDS micelles followed by lipid vesicles, corresponds to the earlier discussion on using more than one solvent system to fold membrane proteins, as deduced from the two-stage model. The SDS-denatured state of bacteriorhodopsin retains almost half of the protein's native helix content; thus both helix formation and packing

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**Table 1**

Examples of membrane proteins that have been partly denatured and refolded *in vitro*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure and function</th>
<th>Protein denaturation</th>
<th>Formation of native-like protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halobacterial bacteriorhodopsin, bR</td>
<td>● 7 TM α-helices and retinal cofactor&lt;br&gt;● Light-driven protein pump</td>
<td>● Apoprotein can be fully denatured in trifluoroacetic acid [36]&lt;br&gt;● Apoprotein partly denatured in SDS, leaving ~55% of native helix content [32,36]</td>
<td>● Initially transferred from organic acid to SDS and then folded by mixing with DMPC/CHAPSO or DMPC/DHPC micelles, lipid vesicles e.g. DMPC, DOPC, containing retinal [14,26,30]</td>
</tr>
<tr>
<td>Major light-harvesting complex of higher plants, LHCII</td>
<td>● 3 TM α-helices and 1 short amphipathic helix at membrane surface. Several bound pigment molecules&lt;br&gt;● Photosynthetic light-harvesting protein</td>
<td>● Apoprotein partly denatured in SDS, leaving about 30% of native helix content [37]</td>
<td>● Reconstituted with thylakoid membrane extracts by freeze-thaw [38]&lt;br&gt;● Reconstituted in OG micelles containing pigments, or mixed OG/lipid micelles (e.g. PG, DGDG) and precipitation of LDS [39,40]&lt;br&gt;● Refolded in DM micelles.</td>
</tr>
<tr>
<td>E. coli diacylglycerol kinase, DGK</td>
<td>● Postulated structure: 3 TM α-helices and 2 cytoplasmic α-helices&lt;br&gt;● Phosphorylates diacylglycerol to give phosphatic acid</td>
<td>● Apoprotein slightly denatured by SDS, leaving ~85% of native helix content [41]</td>
<td></td>
</tr>
<tr>
<td>E. coli outer membrane protein, OmpA</td>
<td>● Membrane domain of 170 amino acids in 8-stranded β-barrel&lt;br&gt;● Exact function unknown, possibly gives cell structural stability, might form a channel</td>
<td>● Protein completely denatured in urea [27]</td>
<td>● Folding in lipid vesicles (e.g. DMPC) by mixing the urea-denatured state with the vesicles [27]</td>
</tr>
<tr>
<td>E. coli outer membrane protein, OmpF (porin)</td>
<td>● 16-stranded β-barrel&lt;br&gt;● Trimer forms pore in outer membrane</td>
<td>● Protein completely denatured in urea or GdmCl [42]</td>
<td>● Poor folding in lipid vesicles on mixing the urea-denatured state with the vesicles. Folding yields increased if detergent used (e.g. DM/DMPC mixed micelles) [43]</td>
</tr>
</tbody>
</table>
**Scheme I**

Proposed reaction scheme for the folding of bacteriorhodopsin (bR)

<table>
<thead>
<tr>
<th>bO</th>
<th>SDS-denatured apoprotein</th>
<th>I₁</th>
<th>Possible transient intermediate</th>
<th>I₂</th>
<th>Key apoprotein intermediate with native, 7TM α helix content</th>
<th>I₃</th>
<th>Transient intermediates probably formed in parallel, with retinal non covalently bound and absorption maxima of 380 nm or 440 nm</th>
<th>bR</th>
<th>Folded, functional bR, with all trans retinal covalently bound</th>
</tr>
</thead>
</table>

**I₁ formation**  
- Time constant of hundreds of milliseconds.

**I₂ formation**  
- Rate-limiting apoprotein folding step (time constant of about 10-100s).

**I₃ formation**  
- Bimolecular protein/retinal reaction, apparent time constant about 1s.

**bR formation**  
- Time constant about 2 min.

Conclusions

Lipid bilayer systems can be designed that allow the folding of an α-helical membrane protein to be controlled. Further study on both the protein and the lipid system should give insight into the molecular origin of the forces that control the folding rate and efficiency. A lipid system of two common biological lipids, PtdCho and PtdEtn, shows that increasing the proportion of the PtdEtn lipid, which increases the desire for monolayer curvature and the lateral pressure exerted by the lipid chains, slows protein folding and decreases the yield of correctly folded protein. Developments are now required in two main areas. The first is the nature of the lipid forces in the protein/lipid system: nearly all the results on PtdCho/PtdEtn systems come from purely lipid mixtures, but it is likely that the protein affects the lipid forces. Secondly, methods are needed that probe the protein structural changes during folding. Finally, we hope that in future it will be possible to extend these ideas to other membrane proteins.

References


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Assembly and Repair of Membrane-Bound Electron Transport Complexes

Possible role for molecular chaperones in assembly and repair of photosystem II
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Abstract
Genes of the HSP70 chaperone family are induced by light. In Chlamydomonas reinhardtii, the induction of HSP70 (70 kDa heat shock protein) chaperones by light results in a partial protection of photosystem II against damage by photo-inhibitory conditions. Underexpression of a chloroplast-localized HSP70 protein caused an increased sensitivity of photosystem II to light. Overexpression of this protein had a protective effect. Fluorescence measurements and studies of the turnover of photosystem II core components suggest that this HSP70 might function in both the protection and the regeneration of photosystem II. This concept is supported by fractionation studies in which the plastid HSP70 was found associated with chloroplast membranes. Because the light-induced elevation of HSP70 levels provides protection for photosystem II, we examined whether the chloroplast is involved in