Manipulating the folding of membrane proteins: using the bilayer to our advantage

Paula J. Booth*1, A. Rachael Curran*2, Richard H. Templer†, Hui Lu*3 and Wim Meijberg*4

*Department of Biochemistry, Imperial College of Science, Technology & Medicine, London SW7 2AY, U.K., and † Department of Chemistry, Imperial College of Science, Technology & Medicine, London SW7 2AY, U.K.

Abstract

The folding mechanisms of integral membrane proteins have largely eluded detailed study. This is owing to the inherent difficulties in folding these hydrophobic proteins in vitro, which, in turn, reflects the often apparently insurmountable problem of mimicking the natural membrane bilayer with lipid or detergent mixtures. There is, however, 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 develop efficient in vitro lipid-bilayer folding systems for the membrane protein, bacteriorhodopsin. Furthermore, we have shown that a rate-limiting apoprotein folding step and the overall folding efficiency appear 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 the their neighbouring folding proteins. These are generic properties of the bilayer that can be achieved with simple mixtures of biological lipids, and are not specific to the lipids studied here. These bilayer properties also seem to be important in modulating the function of several membrane proteins, as well as the

1To whom correspondence should be addressed. Present address: Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.
2Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520, U.S.A.
3Present address: Department of Medicine, Rayne Institute, University College London, London WC1E 6JJ, U.K.
4Present address: Biomade Technology, Nijjenborgh 4, 9747AG Groningen, The Netherlands.
function of membranes in vivo. Thus, it seems likely that careful manipulations of lipid properties will shed light on the forces that drive membrane protein folding, and will aid the development of bilayer folding systems for other membrane proteins.

Introduction

The folding mechanisms of integral membrane proteins have largely eluded detailed study. Much of this is due to the inherent difficulties in folding these hydrophobic proteins in vitro, which in turn reflects the often apparently insurmountable problem of mimicking the natural membrane bilayer with lipid or detergent mixtures. Membrane proteins can be refolded in vitro from a fully denatured state into functional, native proteins. Bacteriorhodopsin (bR) was the first protein that was shown to refold spontaneously, without the need for any accessory proteins [1,2]. Thus, as for water-soluble proteins, the primary amino acid sequence contains all the information needed to define the tertiary structure. Inevitably, the solvent plays a critical role, and refolding of membrane proteins must be performed in detergents or lipids that mimic biological membranes. Although, on the one hand, this makes measurements more technically demanding than those in aqueous solution, on the other, the ability to alter the lipid bilayer structure and dynamics in a known manner offers the potential to control folding.

The folding of bR has been studied more than that of any other membrane protein. Not only can aspects of its folding from a denatured state be studied in vitro in a variety of detergents and lipids, but several important folding events can also be followed in a native membrane environment [3–7]. Furthermore, nearly every amino acid in the protein has been mutated individually, and the effect on the generation and the function of the folded state investigated. Several crystal structures for bR have been reported, with the highest resolution being 1.55 Å [8–10]. bR is the only protein constituent of the purple membrane of Halobacteria salinaria, where it functions as a light-driven proton pump [11]. The protein consists of seven transmembrane α-helices connected by short extramembrane loops. A retinal chromophore is covalently bound within the helix bundle, via a protonated Schiff-base link, to a lysine residue.

Complete denaturation of bR is possible in organic acids, after removal of the native lipids and the retinal cofactor [1]. SDS is, however, a more suitable denaturant for kinetic studies [12]. The SDS-denatured apoprotein state, bacterio-opsin (bO), is denatured to the extent that it cannot bind retinal, but it retains an α-helical structure equivalent to almost four transmembrane helices. The protein refolds spontaneously on diluting the SDS with renaturing mixed detergent, lipid micelles or lipid vesicles containing retinal. This refolding can be initiated by stopped-flow mixing of equal volumes of the denaturing and renaturing micelles or vesicles, thus giving millisecond time resolution for kinetic studies. Refolding yields of about 95% can be readily obtained, for example, in mixed L-α-1,2-dimyristoyl phosphatidylcholine (DMPC)/CHAPS micelles, DMPC/L-α-1,2-dihexanoyl phosphatidylcholine (DHPC) micelles,
native lipid vesicles, 1-α-1,2-dipalmitoleoyl phosphatidylcholine (DPOPC) vesicles and DMPC vesicles [6,13,14]. Refolding to native protein from a denatured state can be readily assayed by recovery of the characteristic purple absorption band of the bound retinal chromophore, which is indicative of native-like proton pumping ability.

**bR folding kinetics**

The simplest reaction scheme that accounts for the kinetic data in lipid-based micelles is [6,13]:

\[
\begin{align*}
& bO \quad \leftrightarrow \quad l_1 \quad \leftrightarrow \quad l_2 \quad \leftrightarrow \quad l_R \quad \rightarrow \quad bR \\
& \quad \downarrow \quad R \quad \downarrow \quad l_1 \quad \downarrow \quad l_2 \quad \downarrow \quad l_R \quad \rightarrow \quad bR
\end{align*}
\]

where R is retinal, and I₁ and I₂ are intermediates that form before retinal binding. Retinal binds in at least two steps: first, non-covalently to give Iᵣ and, secondly, via its covalent link to Lys-216 to give bR. The scheme does not include branches or parallel pathways that may well exist. Iᵣ, for example, seems to consist of at least two states (see below), one where the retinal absorption band is similar to that of free retinal at about 380 nm (Iᵣ380) and another where the retinal band is red-shifted to 440 nm (Iᵣ440).

The intermediate I₁ could be an apoprotein folding intermediate, but also seems to reflect a change in the micelle-vesicle structure as a result of stopped-flow mixing. The apoprotein intermediate I₂ is a key component of the folding process. Formation of I₂ is rate-limiting in apoprotein folding and must occur before retinal can bind. As a result, the formation of I₂ gives rise to an observed lag phase in the formation of folded purple bR [12]. The rate of I₂ formation can also be controlled by manipulating particular characteristics of the refolding lipid environment, with the time constant ranging from seconds to minutes (see later). The changes in protein secondary structure have been time-resolved during this stage of folding by far-UV CD [15]. The SDS-denatured bO state has an α-helical content of about four transmembrane helices, whereas the remaining equivalent of three transmembrane helices are disordered. The secondary structure of I₂ is native-like and corresponds to seven transmembrane helices. About half of the SDS-disordered structure folds to form helices during the 20 s dead-time of these particular far-UV CD experiments, whereas the remaining 30 or so amino acids form helices with a time constant equivalent to that of I₂ formation (i.e. seconds to minutes).

Only one retinal-binding step has been observed in the folding of bR in mixed micelle systems, where retinal binds non-covalently to I₂, probably within some sort of loosely formed binding pocket [16,17]. The exact mechanism by which retinal gains access is unknown. It is also unknown what correct tertiary contacts between the helices are present in the I₂ state, and what controls the specific packing of the helices. There are probably some specific helix–helix contact sites present in the partially folded I₂ state. Retinal binding to I₂ would then allow the helices to pack round the bound retinal to form the folded state. The aqueous loops that connect the helices could also help to provide some specificity and close packing.
Studies of retinal binding to I\textsubscript{2} are complicated by the preceding formation of I\textsubscript{2}. However, the binding reaction can be more readily investigated by allowing bO to prefold to a state equivalent to I\textsubscript{2} and then adding retinal [12,16]. There appear to be at least two non-covalent, retinal–protein I\textsubscript{R} states, both of which form with the same observed rate of approx. 1.1 s\textsuperscript{-1}. One retinal–protein intermediate (I\textsubscript{R440}) is observed in transient absorption measurements because the retinal absorption band red-shifts from 380 to 440 nm. However, the formation of I\textsubscript{R440} cannot by itself account for the observed retinal–protein concentration dependence of the kinetics [17]. It is suggested that another intermediate, I\textsubscript{R380}, forms in parallel with I\textsubscript{R440}, with the same observed kinetics, thus giving a reaction scheme where the two parallel paths from I\textsubscript{2} to bR are kinetically indistinguishable:

![Reaction scheme](image)

Which of the two routes from I\textsubscript{2} to bR is followed is determined by pH [17]. Optimum refolding, in terms of a maximum refolding yield and overall rate, is observed at pH 6, and the maximum observed concentration of I\textsubscript{R440} also occurs at pH 6. At pH 8, the refolding yield drops to approx. 80% of that at pH 6, whereas the concentration of I\textsubscript{R440} drops to 30% of that at pH 6. The pH dependence of the two paths from I\textsubscript{2} to bR could result from a distribution of protein conformers in I\textsubscript{2} that have slightly different protonation equilibria of their side-chains and can interconvert due to thermal energy. Binding of retinal to these I\textsubscript{2} conformers would then occur via the same transition state (or distribution of interconverting transition states on a microscopic scale) to form I\textsubscript{R}. I\textsubscript{R} also contains a similar distribution of protein conformers as I\textsubscript{2}; however, the different protonation equilibria of the protein conformations affect the retinal absorption band, and thus show up in this retinal-bound state as I\textsubscript{R380} and I\textsubscript{R440}. The retinal absorption band is sensitive to its electrostatic environment. Therefore, an alternative model is possible where the I\textsubscript{2} and I\textsubscript{R} states have altered protonation states of individual residues (or bound solvent ions) near retinal, but with no difference in polypeptide conformation. I\textsubscript{R440} and I\textsubscript{R380} both decay, with the same observed time constant of a few minutes, to form refolded bR with a 560 nm absorption band in which the Schiff base bond has formed and retinal is covalently bound to Lys-216. This bR state contains all-trans retinal within its binding pocket. 

The retinal then isomerizes at a much slower rate (time constant about 1 h) to a state equivalent to the dark-adapted state of bR that contains a mixture of all-trans- and 13-cis-retinal [17].
Lipid folding systems

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 allowing fine tuning of certain bilayer properties that seem to be under homeostatic control in living membranes, and is vital for the correct function of several of the constituent membrane proteins [18,19]. The presence of the non-bilayer lipids increases the desire of each monolayer of the bilayer to move towards water. However, the monolayers tend 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 a 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 at the centre of the bilayer, as a result of the increase in the number of collisions between the lipid hydrocarbon chains. There is mounting evidence that these intermolecular forces within lipid bilayers play key roles, both in vitro and in vivo. The novel crystallization method reported for bR [20], which has led to near-atomic resolution [10,21], seems to be dependent on the manipulation of the lipid intermolecular forces. The introduction of non-bilayer lipids into bilayers has also been shown to modulate the function of several membrane-bound proteins, including alamethicin, cytidyl transferase, rhodopsin, Ca$^{2+}$ ATPase and lactose permease [22–26].

We are currently investigating how these changes in lipid-chain lateral pressures affect the folding of bR. Our aims are two-fold: first, we wish to investigate the molecular origin of forces that control folding in vitro, and possibly also in vivo; secondly, we aim to develop efficient folding systems for membrane proteins. We have shown that an increase in the lateral pressure within the bilayer may be responsible for the slowing of a rate-limiting folding step for bR [13]. This study used a two-component lipid mixture consisting of lipids with phosphatidylcholine (PC) headgroups but different chain lengths: six-carbon DHPC and 14-carbon DMPC. Increasing the amount of the longer-chain 14-carbon lipid increases the lateral pressure in the chain region and slows the rate-limiting folding step of bR (i.e. formation of I$_2$). Thus it appears possible to control the rate of protein folding. These DMPC/DHPC mixtures form micelle structures in solution and are thus amenable to the optical methods (fluorescence, absorption and CD) used to study the folding kinetics. However, the exact nature of these mixed micelles at different lipid compositions is unknown and there are additional, unknown constraints on the lipid dynamics and pressures in the micelles, as opposed to a bilayer system.

Lipid-bilayer vesicle systems have been developed for the refolding of bR [14]. Two-component lipid mixtures are used where the desire for monolayer curvature and the lateral pressure in the lipid-chain region can be increased by changing either the lipid headgroup or the lipid chain. Changing the headgroup from PC to phosphatidylethanolamine (PE) increases the desire for monolayer curvature towards water, as does incorporating an
unsaturated bond into the lipid chains. Several systems have proved successful for bR refolding: L-\(\alpha\)-1,2-dioleoyl phosphatidylcholine (DOPC)/L-\(\alpha\)-1,2-dioleoyl phosphatidylethanolamine (DOPE) (18-carbon unsaturated chains with a \textit{cis} double bond at position C-9:C-10), DPOPC/L-\(\alpha\)-1,2-dipalmitoleoyl phosphatidylethanolamine (DPOPE) (16-carbon unsaturated chains with a \textit{cis} double bond at position C-9:C-10), DMPC/L-\(\alpha\)-1,2-dimyristoyl phosphatidylethanolamine (14-carbon saturated chains) and DMPC/DOPC [14]. Increasing the amount of PE in the PC/PE mixtures, or the chain unsaturation in the DMPC/DOPC mixture, increases the lipid-chain lateral pressure. Furthermore, the different chain lengths in the PC/PE mixtures give rise to different lateral pressures, with for example the longer, 18-carbon dioleoyl chains having a greater chain pressure than the 16-carbon dipalmitoleoyl. It is possible to control the overall refolding yield of bR in these lipid systems. All 100% PC systems allow bR to fold to approx. 100% yield. The yield then decreases as the PE content increases, with a greater decrease being observed in the DOPC/DOPE system than in the DPOPC/DPOPE system [14]. This reflects either an inability of the denatured protein to insert into the stressed bilayer, or an effect of the lipids on protein folding within the bilayer and the accumulation of an intermediate involved in the protein’s assembly. We are currently investigating how the slowing of the rate-limiting folding step to I\(_2\), which occurs with lipid-chain pressure, correlates with the decrease in folding yield. Time-resolved optical measurements are more difficult in the lipid vesicle systems than in the DMPC/DHPC micelles, because the lipid vesicles scatter light and can change with time during the course of a folding reaction.

Conclusions

Studies of membrane-protein folding mechanisms lag severely behind those of water-soluble proteins and remain a major challenge in protein research. A major difficulty in the study of membrane proteins lies in finding appropriate detergent or lipid systems that maintain the structural and functional integrity of the protein. This is highlighted in refolding studies where appropriate solubilization conditions must be found both to unfold and refold the protein and, at the same time, to prevent aggregation of the often highly hydrophobic proteins. There is, however, a large body of information on lipid properties and, in particular, on PC and PE lipids, which are common to many biological membranes. We have shown how this can be exploited to develop efficient \textit{in vitro} lipid-bilayer folding systems. Furthermore, we have shown that a rate-limiting apoprotein-folding step and the overall folding efficiency appear 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 proteins. These are generic properties of the bilayer that can be achieved with simple mixtures of many types of biological lipid and are not specific to the lipids studied here. These bilayer properties also seem to be
important in modulating the function of several membrane proteins as well as membrane function \textit{in vivo}.

It is also possible to perform detailed biophysical studies on membrane protein folding and to identify intermediate states and folding mechanisms \textit{in vitro}. At least two intermediates are involved in the folding of bR \textit{in vitro}. It has also been possible to identify two parallel folding pathways during the final stages of folding. These paths are kinetically indistinguishable and are determined by pH, indicating that they arise from different protonation states of the protein.

References
