Biochimica et Biophysica Acta 1818 (2012) 951–956

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Progress in understanding the role of lipids in membrane protein folding ☆

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ARTICLE INFO

Article history:
Received 27 June 2011
Received in revised form 23 December 2011
Accepted 23 December 2011
Available online 4 January 2012

Keywords:
Phospholipid
Phosphatidylethanolamine
Curvature stress

ABSTRACT

Detailed investigations of membrane protein folding present a number of serious technical challenges. Most studies addressing this subject have emphasized aspects of protein amino acid sequence and structure. While it is generally accepted that the interplay between proteins and lipids plays an important role in membrane protein folding, the role(s) played by membrane lipids in this process have only recently been explored in any detail. This review is intended to summarize recent studies in which particular lipids or membrane physical properties have been shown to play a role in the folding of intact, functionally competent integral membrane proteins. This article is part of a Special Issue entitled: Protein Folding in Membranes.

Contents

1. Introduction

Most of the functions associated with biological membranes, e.g. signal transduction, ion movement, energy conversion, osmotic homeostasis etc., are carried out by membrane proteins. Integral membrane proteins regulate a wide range of biological functions and make up about 30% of the open reading frames in the human genome [1]. In spite of this abundance and functional importance the detailed molecular process whereby integral membrane proteins fold in membranes has only recently begun to be explored in earnest. A recent review of membrane protein folding noted that the phrase "membrane protein folding" entered into PubMed returned only 86 publications at the end of 2006 [2]; in the middle of 2011 that number has grown to 143. Among this relatively small number of published studies a distinct minority has been devoted to questions regarding the roles of membrane composition and membrane physical properties in the folding process. Given the substantial technical challenges posed by a detailed examination of membrane protein folding this is not surprising. These challenges arise from the basic protein physical biochemistry of membrane proteins, and flow from the inherent difficulties in isolation and purification of functional proteins under conditions that lend themselves to refolding into membranes. Thus, detailed examination of the various membrane compositional or physical variables that may be involved in membrane protein folding generally means taking a technically demanding procedure and multiplying it by 2 or 3 or 4 or more. However, a small and growing number of investigations have taken up the challenge of examining the lipid membrane side of the membrane folding problem.

Biological membranes consist of a number of lipid types and a wide variety of molecular species within those types. The predominate lipid species in most mammalian cytoplasmic membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM) and cholesterol [3]. In contrast, the inner membrane of Gram-negative bacteria is predominantly PE with a smaller amount of phosphatidylglycerol (PG) and cardiolipin [4]. Within each of these classes there often exists a range of acyl

☆ This article is part of a Special Issue entitled: Protein Folding in Membranes.

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doi:10.1016/j.bbamem.2011.12.029

chain compositions, producing a wide variety of molecular species in most biological membranes. The lipid composition profile of most classes of biological membranes is reasonably well regulated, but bacteria may also alter their membrane lipid composition in response to external factors such as temperature, pressure, pH and salinity [5,6]. In humans the acyl chain composition of many different tissues and membrane types can be altered by diet [7,8]. Thus, organisms have a wide range of lipid molecular building blocks and metabolic machinery at their disposal with which to optimize different membranes for specific biological tasks. A number of investigators have begun to address the many questions related to what aspects of membrane lipid composition are optimized in order to carry out efficient membrane protein folding and insertion.

2. Multi-helical proteins

Among the key contributions to understanding the importance of the membrane properties in membrane protein folding has been a series of studies on the folding of α helical proteins in bilayers with varying composition (reviewed in [9,10]). By systematically varying the phospholipid composition of the target liposomes in a series of integral membrane protein folding studies they have identified several important compositional variables in the protein folding process.

2.1. Bacteriorhodopsin

The folding of the archeal photoreceptor bacteriorhodopsin (bR) is among the most widely studied of any membrane protein (reviewed in [10]). Thirty years ago Khorana and coworkers demonstrated that bR could be refolded from a denatured state in vitro [11,12], paving the way for the use of over expression in *E. coli* to obtain high yields of this important integral membrane protein. BR is clearly not particularly sensitive to its refolding environment as it has been refolded into a functional state in a variety of different detergents and lipids. A common assumption, based on thermodynamics, is that the hydrophobic thickness of the target membrane should be close to the hydrophobic thickness of the protein for efficient folding. However, SDS-denatured bacterio-opsin (bO) refolds with equal regeneration yield (~95%) into bilayers consisting of *di*$C_{14:0}$PC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), *di*$C_{16:0}$PC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) and *di*$C_{18:1}$PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) [13]. The similar regeneration yield for the di-monounsaturated PC and the 2 saturated PCs strongly suggests that acyl chain packing, or membrane fluidity, plays a negligible role in bO refolding.

A significant reduction in folding efficiency was obtained when phosphatidylethanolamine (PE) lipids, with the same acyl chain composition as the host PC lipids, were added to the target liposomes. At a level of 16 mol% PE, regeneration yield were reduced to 85% in *di*$C_{14:0}$PC, 56% in *di*$C_{18:1}$PC and 62% in *di*$C_{16:0}$PC [13]. Interestingly, the transition from a PC head group to a PE headgroup in *di*$C_{14:0}$PC or *di*$C_{16:0}$PC reduces the area elastic bending modulus by ~5%, while in *di*$C_{18:1}$PC this substitution increases the area elastic bending modulus by ~3% [14]. The reduction in refolding efficiency with the substitution of PE for PC is attributed to the increase in curvature elastic energy, or curvature stress, induced in the bilayer by the presence of PE [9,10,13,15,16]. The determinative role of curvature stress in bR folding was tested using target membranes containing variable levels of single acyl chain lysoPC [17]. PE lipids are non-bilayer forming lipids that have a high negative spontaneous curvature, that is, a curvature towards water, and tend to form hexagonal phases rather than bilayers. LysoPC is also a non-bilayer forming lipid, but has a large positive intrinsic curvature, and tends to form micelles rather than bilayers. Incorporating PE into PC bilayers reduced folding efficiency, while incorporating lysoPC into PC bilayers enhanced folding [17]. A simultaneous analysis of both of these substitutions in terms of estimated monolayer curvature, calculated as the sum of the products of mole fraction and spontaneous curvature, shows a positive linear relationship between bR folding yield and monolayer curvature across twelve different bilayer compositions [16]. This linear relationship across a wide range of monolayer curvature values suggests that monolayer curvature plays a major role in controlling bR folding.

2.2. Other multi-helical proteins

The technical challenges posed by quantitative folding of α helical membrane proteins in bilayers are formidable, thus relatively few members of this important class proteins have been examined in terms of lipid involvement in the folding process. A recent comprehensive review of folding proteins into membranes lists only three α helical proteins besides bR where the folding process had been examined in lipid bilayers, and in none of these studies was the question of optimal bilayer composition addressed [2]. Several recent studies by Booth and co-workers have begun to provide important new information about the involvement of specific lipids in the folding of α helical proteins.

Diacylglycerol kinase (DGK) from *Escherichia coli* has three α helices and was first successfully refolded from both urea and guanidine hydrochloride into unilamellar $C_{16:0}C_{18:1}$PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) liposomes by Nagy and coworkers [18]. The role of the bilayer in the folding of DGK was examined in study that used *di*$C_{18:1}$PC as the baseline bilayer composition with *di*$C_{18:1}$PE, *di*$C_{18:1}$PG, *di*$C_{14:0}$PC, *di*$C_{18:1}$PS, lysoOPC and lysoPG added singly over a range of mole fractions [19]. In contrast to the findings for bR folding, the yield of functionally folded DGK was reduced about 3-fold by the addition of *di*$C_{14:0}$PC or lysoPC to a *di*$C_{18:1}$PC bilayer, while the addition of *di*$C_{18:1}$PE caused a modest increase in folding yield. Both *di*$C_{14:0}$PC and lysoPC lower curvature elastic stress of *di*$C_{18:1}$PC, while *di*$C_{18:1}$PE increases it. The biggest increase in folding yield was observed for *di*$C_{18:1}$PG, which increased the yield of functionally active DGK by 3-fold when added to a *di*$C_{18:1}$PC bilayer. The negative charge of *di*$C_{18:1}$PG was ruled out as the dominant factor in increasing refolding yield due to the fact that *di*$C_{18:1}$PS, also anionic, had no effect on folding yield and the enhancement of refolding efficiency by *di*$C_{18:1}$PG was unaffected by increasing ionic strength up to 1 M [19].

Reduced curvature stress decreased the folding yield of DGK, while the refolding yield of bR is increased when curvature stress is lowered by *di*$C_{14:0}$PC or lysoPC [17]. A potentially important, but unexamined, difference in these two refolding processes is that bR was partially denatured in SDS, while DGK was denatured in urea. Unlike urea, SDS partitions into the bilayer, and could potentially aid the initial insertion process. Thus it is possible that in the presence of SDS the lateral pressure barrier in the acyl chain region is more significant than the lateral pressure barrier in the headgroup/interfacial region. Thus, lysoPC may enhance bR refolding in the presence of SDS by lowering the lateral pressure in the acyl chain region. This line of reasoning suggests that in the absence of SDS the critical barrier for protein α helix insertion is lateral pressure in the headgroup region and the reduction in DGK folding by lysoOPC may be due to the resulting increase in lateral pressure in the headgroup/interfacial region.

The refolding of a small multidrug transporter from *E. coli*, EmrE, has been examined in target bilayers consisting of *di*$C_{18:1}$PC/*di*$C_{18:1}$PE, *di*$C_{18:1}$PC/*di*$C_{18:1}$PG and *di*$C_{18:1}$PG/*di*$C_{18:1}$PE [20]. EmrE monomers consist of 4 transmembrane helices that account for about 75% of the protein [21], and these were partially denatured in a combination of SDS and urea. Increasing the mole fraction of *di*$C_{18:1}$PE in both *di*$C_{18:1}$PC/*di*$C_{18:1}$PE and *di*$C_{18:1}$PG/*di*$C_{18:1}$PE liposomes lowered the folding yield of EmrE. Thus, the folding of EmrE shows the same negative correlation with increased curvature stress

observed for bR. Unlike the case of DGK refolding, where PG lipids enhanced folding about 3-fold, for EmrE refolding there was no significant difference in refolding yield between *di*$C_{18:1}$PC and *di*$C_{18:1}$PG.

Recently the Booth lab extended their studies of the folding of α helical membrane proteins to a large multidomain galactose transporter from *E. coli*, GalP [22]. This member of the major facilitator superfamily is predicted to fold into two 6-helical bundles, and thus presents a significantly more complex folding process than those previously examined. GalP was refolded from 8 M urea into liposomes consisting of varying proportions of *di*$C_{18:1}$PC and *di*$C_{18:1}$PE. Successful refolding of GalP into *di*$C_{18:1}$PC/*di*$C_{18:1}$PE liposomes required a high level of *di*$C_{18:1}$PE of about 60 mol%.

Thus, refolding of GalP is enhanced by increased intrinsic curvature stress in a manner similar to that observed for the *E. coli* diacylglycerol kinase (DGK) [19], which was also refolded from a high concentration of urea. The two α helical proteins where increased intrinsic curvature stress reduced the refolding yield, bR and EmrE, were both refolded out of solutions containing SDS. While there has been a systematic variation of lipid composition, at least with respect to intrinsic curvature, for this small group of α helical proteins, there are also potentially important differences in the refolding conditions. These differences make it to some extent difficult to clearly discern the membrane properties that are essential with respect to the refolding of multi-α helical membrane proteins. Clearly it would be beneficial to determine whether or not the positive effects of reduced curvature stress are related to the presence of SDS. It is also noteworthy that one of the largest effects of bilayer composition on refolding yield for an α helical protein is the 3-fold enhancement of DGK refolding by 32 mol% *di*$C_{18:1}$PG in *di*$C_{18:1}$PC [19]. This enhancement was not related to its anionic character, and the physical mechanism involved in this effect of *di*$C_{18:1}$PG is unclear.

3. β-barrel proteins

At least two factors have increased the number of recently published studies devoted to the refolding of β-barrel proteins in membranes; their abundance in the outer membrane of *E. coli* and the ability to design systems where the folding process is fully reversible, thus facilitating detailed thermodynamic analysis [23]. The folding of β-barrel proteins has been the subject of two excellent recent reviews [2,24], thus this section will be primarily devoted to more recent findings.

Among the β-barrel that have been successfully refolded into lipid bilayers perhaps none have been more thoroughly examined with respect to bilayer composition than OmpA from the outer membrane of *E. coli*. This eight-stranded β-barrel refolds upon denaturant dilution with kinetics of refolding that depend on membrane thickness and are enhanced by a high radius of curvature [25]. In a comprehensive study of the equilibrium thermodynamics of the fully reversible refolding of OmpA, Hong and Tamm showed that elastic bilayer forces such as curvature stress and hydrophobic mismatch govern OmpA refolding [23,26]. Using a host-guest system composed of $C_{16:0}C_{18:1}$PG/$C_{16:0}C_{18:1}$PC, with $C_{16:0}C_{18:1}$PG held constant at 7.5 mol%, as the host they examined the free energy of unfolding as a function of lipid composition for a series of *di*-saturated, saturated-monounsaturated and *di*-monounsaturated PCs, varying in carbon number from ten to twenty. OmpA refolded efficiently into 30 nm SUVs composed of lipids ranging in length from *di*$C_{10:0}$PC (1,2-didecanoyl-*sn*-glycero-3-phosphocholine) to *di*$C_{20:1}$PC (1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine), which covers a range of hydrophobic thickness from 15 Å to 30 Å [23]. In an earlier study using LUVs OmpA refolded successfully into short chain PCs, but was unable to fold in LUVs composed of *di*$C_{18:1}$PC or *di*$C_{14:0}$PC [25]. The folding free energy of OmpA in each lipid was determined by extrapolating the experimental free energy values obtained with a range of mole fractions in the host–guest bilayer to 100% guest lipid. The resulting values of stabilization free energy were examined as a function of hydrophobic thickness. For the series *di*$C_{n:0}$PC (n = 10, 12, 14), $C_{16:0}C_{18:1}$PC, $C_{18:0}$, $C_{18:1}$PC there was a positive linear correlation between stabilization free energy and hydrophobic thickness. For the di-monounsaturated series *di*$C_{n:1}$PC (n = 14, 16, 18, 20) there was a negative linear correlation between stabilization free energy and hydrophobic thickness. These two linear trends with opposite slopes are consistent with the trends in lateral pressure in the bilayer interior for the two different classes of lipid. For *di*-saturated PCs this lateral pressure increases with chain length [27], while for *di*-monounsaturated PCs it decreases as the chains are lengthened [28]. In addition, the entire *di*-monounsaturated series had higher free energy values than the entire *di*-saturated/saturated-monounsaturated series. For example both *di*$C_{14:0}$PC and *di*$C_{16:1}$PC have a hydrophobic thickness of ~23 Å [29], but the stabilization free energy in *di*$C_{16:1}$PC is three times higher than in *di*$C_{14:0}$PC (6 kcal/mol compared to 2 kcal/mol). This comparison suggests that while membrane hydrophobic thickness is clearly an important determinant of folding efficiency and membrane protein stabilization, the lateral forces, or bilayer curvature stress, also play a significant role, and may make a larger contribution to the overall free energy of stabilization. The importance of increased internal lateral pressure due to intrinsic curvature stress was further examined by adding 30 mol% $C_{16:0}C_{18:1}$PE to the reference bilayer. This produced an increase in free energy of 60%, which was the largest stabilizing effect observed for any lipid studied. The strong correlation between increased lateral pressure at the center of the bilayer and increased free energy of stabilization was interpreted in terms of the three dimensional structure of OmpA which is essentially hourglass-shaped [30]. Hong and Tamm proposed that insertion of OmpA relieves intrinsic curvature stress and the stored curvature stress energy stabilizes the folded OmpA structure in the membrane [23]. For OmpA increased lateral pressure at the center of the bilayer enhanced folding, while for bR and DGK it inhibited folding and this difference is rationalized in terms of differences in the structures of the proteins.

A recent study examined the effects of target liposome composition on the spontaneous, 2-step refolding of two other eight-stranded β-barrel proteins, $Opa_{50}$and Opa_{60} from the outer membrane of *Neisseria gonorrhea* [31]. In contrast to the findings with OmpA, these two β-barrel proteins were unable to successfully fold into SUVs composed of *di*$C_{18:1}$PC, $C_{16:0}C_{18:1}$PC or 92.5/7.5 $C_{16:0}C_{18:1}$PC/$C_{16:0}C_{18:1}$PG. For the *di*-saturated series $C_{n:0}$PC (n = 10, 12, 14, 16, 18) the greatest total fraction folded was found in *di*$C_{14:0}$PC where folding efficiency was ~60%, while for all other chain lengths folding efficiency was 30% or less. In order to examine a possible effect of acyl chain order, or membrane fluidity, *di*$C_{16:1}$PC was combined with *di*$C_{14:0}$PC at mole fractions from 25% to 100%. *di*$C_{16:1}$PC was selected because it has a similar hydrophobic thickness to *di*$C_{14:0}$PC. This modification of the target liposome composition drastically reduced the total fraction folded, with even 25 mol% *di*$C_{16:1}$PC causing a greater than 3-fold reduction in folding efficiency for both Opa_{50} and Opa_{60} [31]. Comparison of this reduction in refolding with the results obtained for OmpA where *di*$C_{16:1}$PC provided a 3-fold increase in stabilization free energy compared to *di*$C_{14:0}$PC [23] indicates the difficulty in identifying bilayer compositional variables and bilayer physical properties that facilitate β-barrel folding and insertion in a general way.

Issues regarding acyl chain length, acyl chain unsaturation, vesicle size, PE content, PG content and cholesterol were addressed by Burgess et al. in a comprehensive study of nine different outer membrane proteins from *E. coli* [32]. OmpX, OmpW, OmpA, PagP, OmpT, OmpLa, FadL, Omp85 and OmpF, varying in size from eight to sixteen β-strands, were refolded from concentrated urea into target liposomes composed of a wide range of acyl chain and headgroup compositions. A noteworthy contribution of this study is an extensive

comparison of refolding in SUVs vs. LUVs. In SUVs composed of native-like synthetic lipids (75% $C_{16:0}C_{18:1}$PE, 25% $C_{16:0}C_{18:1}$PG) five of the nine outer membrane proteins were capable of refolding, with the smaller proteins generally showing higher fractions folded than the larger proteins. However in LUVs with the same lipid composition only OmpT was capable of refolding, and only at a very low level [32]. For the *di*-saturated series, *di*-$C_{n:0}$PC (n = 10, 11, 12, 14), and *di*-monounsaturated series *di*-$C_{n:1}$PC (n = 14, 16, 18; 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine) the differences in refolding yield were not as dramatic between SUVs and LUVs. Folding yields were generally higher in SUVs in all bilayer compositions, but the increase in folding yield was somewhat incremental in the *di*-saturated series and in *di*-$C_{14:1}$PC. However, for LUVs composed of *di*-$C_{16:1}$PC and *di*-$C_{16:1}$PC only five and one of the outer membrane proteins, respectively, were capable of folding, while this was increased to seven and six, respectively, in SUVs. This difference in folding efficiency in SUVs and LUVs does not appear to be strictly related to hydrophobic thickness as both *di*-$C_{16:1}$PC and *di*-$C_{14:0}$PC have the same hydrophobic thickness [29]. A complicating factor in this comparison of folding in SUV s and LUVs is the fact that SUVs are not thermodynamically stable, and thus not well suited for the measurement of equilibrium processes [33]. The potential importance of lipid to protein ratio is suggested by comparing the results for OmpA folding of Burgess et al. with those of Marsh et al. [34]. Burgess reported greater than 80% folding efficiency of OmpA into the entire series *di*-$C_{n:0}$PCs (n = 10, 11, 12, 14) in 100 nm extruded LUVs at a lipid to protein ratio of 800:1, while Marsh et al. found no insertion or folding of OmpA in *di*-$C_{14:0}$PC in similar LUVs at lipid to protein ratios of 100:1, 200:1 and 400:1 [34].

Burgess et al. found that in SUVs all nine outer membrane proteins were capable of folding with some degree of success in the three shortest PCs; *di*-$C_{10:0}$PC, *di*-$C_{11:0}$PC (1,2-diundecanoyl-*sn*-glycero-3-phosphocholine) and *di*-$C_{12:0}$PC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine). However, this trend did not serve as a good predictor of folding efficiency for a given outer membrane protein. Three of the smaller outer membrane proteins, each with eight β-strands, folded with high efficiency in all seven PC bilayers examined, but the same pattern was observed for Pmp85 which is predicted have either 12 or 16 strands. This suggests that the requirement for matching hydrophobic thickness between bilayer and protein is unlikely to be strictly related to barrel size [32].

Another significant contribution made by Burgess et al. [32] is a systematic comparison of the effects of PE, PG and cholesterol in LUVs. The background, or host, bilayer was *di*-$C_{12:0}$PC and 5 mol% or 20 mol% *di*-$C_{12:0}$PE, *di*-$C_{12:0}$PG or cholesterol was added. For outer membrane proteins with high folding efficiency in the host *di*-$C_{12:0}$PC membrane; OmpX, OmpA and Omp85; the presence of PE and PG had no effect on folding efficiency. For three of the four largest outer membrane proteins; FadL, OmpLa and OmpF; folding efficiency was reduced by both PE and PG, while for PmpT and PagP folding efficiency was enhanced by both *di*-$C_{12:0}$PE and *di*-$C_{12:0}$PG. These results suggest that it may be difficult to predict the effects of PG and PE on β-barrel refolding in LUVs. While cholesterol is not a constituent of the bacterial membranes where outer membrane proteins reside, the authors examined the effects of cholesterol on folding efficiency to address whether or not folding efficiency of theses β-barrels might be altered by reduced membrane fluidity. For all nine bacterial outer membrane proteins cholesterol reduced folding efficiency in an essentially dose-dependent manner. The consistency of this finding is somewhat remarkable considering the non-uniformity of the responses of the various proteins in this study to changes in PE, PG, acyl chain length, acyl chain unsaturation and vesicle size. This uniformity strongly suggests that reduced membrane fluidity, or more constrained acyl chain packing, is generally disadvantageous to β-barrel folding and insertion.

4. Membrane protein topogenesis

A significant body of evidence has accumulated that membrane lipids are an important factor in determining the topological organization of some membrane proteins in a co-translational and post insertion manner. Considering that the topology of membrane proteins is determined by their amino acid sequence it is a noteworthy example of the multifunctional roles of membrane lipids that the topogenesis of lactose permease (LacY) [35,36], phenylalanine permease (PheP) [37]and γ-aminobutyrate permease (GabP) [38] of *E. coli* are directed by the lipid composition of the membrane.

The membranes of *E. coli* normally contain about 70% PE, 20% PG and 5% cardiolipin (CL) [39]. Insight into the roles of membrane lipids in membrane protein topogenesis was made possible by the development of viable *E. coli* strains in which the membrane phospholipid composition could be varied. LacY is a proton/lactose symporter in *E. coli* that consists of 12 transmembrane α helices. Early studies of LacY reconstituted into phospholipid vesicles suggested that PE is required for full function [40]. Analysis of LacY function and topology in cells lacking the ability to express PE confirmed the important role of PE in LacY function, organization and insertion [41]. A series of additional studies established that PE acts as a 'non protein molecular chaperone' in the membrane insertion and folding of LacY [35,36,42,43]. LacY initially folded in vivo in the absence of PE, lacks the native-like structure of the periplasmic P7 loop that defines epitope 4B1. Refolding of this LacY from SDS in the presence of PE restores epitope 4B1, but refolding in the presence of PG, PC or cardiolipin does not restore this epitope [35,43]. The PE structural requirements for this in vitro correction of a folding defect resulting from in vivo LacY folding and assembly in the absence of PE have been examined in detail [42]. The ability of phospholipids to correct the loop P7 folding defect in LacY expressed in PE-deficient cells was analyzed with an Eastern-Western blotting technique that exposes phospholipids on a solid support during re-naturation of LacY from SDS. As expected, PE species extracted from *E. coli*, primarily $C_{16:0}C_{18:1}$PE, were fully successful in restoring recognition by mAb 4B1. Two di-saturated PE species, *di*-$C_{16:0}$PE and *di*-$C_{18:0}$PE were as effective as *E. coli* derived lipids.

But not all molecular forms of PE were capable of supporting proper refolding with respect to recognition by mAb 4B1. The di-monounsaturated species *di*-$C_{16:1}$PE and *di*-$C_{18:1}$PE were completely incapable of supporting proper refolding, but when combined in a binary mixture with 50 mol% *di*-$C_{16:0}$PG full refolding was achieved. Similar complete lack of folding and 'rescue' by 50 mol% *di*-$C_{16:0}$PG was also observed for plasmalogen-PE [42]. The similar property of these three molecular forms of PE; *di*-$C_{16:1}$PE, *di*-$C_{18:1}$PE and plasmalogen-PE; is that they form a non-bilayer inverted hexagonal II phase (H_{II}) under the experimental conditions employed [44]. Lamellar-preferring lipids can stabilize H_{II}-preferring lipids in bilayer structures in mixed binary systems when the mole fraction of the lamellar-preferring lipid is greater than about 30% [45]. Thus, the three forms of molecular PE incapable of supporting proper LacY refolding were able to facilitate proper refolding in mixtures that would normally adopt a lamellar structure. A similar conversion with respect to lamellar vs non-lamellar phase was also observed for lysoPE, which prefers to be in micellar structure under the experimental conditions employed. LacY showed no functionally correct refolding in neat lysoPE, but was capable of complete refolding in 85 mol% *di*-$C_{16:0}$PG, 15 mol% lysoPE. The possibility that competent LacY refolding was enabled by high levels of *di*-$C_{16:0}$PG rather than the presence of lamellar PE in the mixtures cited above is ruled out by the results obtained for PC/PG mixtures. No LacY refolding was obtained in *di*-$C_{18:0}$PC, and the addition of 50 mol% or even 85 mol% *di*-$C_{16:0}$PG had no effect on the refolding yield [42].

Remarkably, this in vitro correction of a folding and assembly error is also observed in vivo. When synthesis of PE is initiated

post-assembly the resulting change in membrane lipid composition triggers recovery of normal conformation and topology of at least one LacY subdomain and restores active transport [36]. A PE-induced, post-assembly restructuring has also been observed for the phenylalanine permease PheP [37]. Expression of PheP in PE-deficient cells produced complete inversion in topological orientation of the N terminus and adjoining transmembrane hairpin loop. Introduction of PE, following assembly of PheP, leads to reorientation of the N terminus and adjacent hairpin and restoration of wild-type function [37]. The *E. coli* γ-aminobutyric acid (GABA) transporter (GabP) is also topologically misfolded when expressed in PE-deficient cells [38]. Like PheP, GabP is a member of the amino acid/polyamine/organocation superfamily, and like PheP the absence of PE causes the N-terminal hairpin to be inverted, with the hinge point of the topological inversion in transmembrane domain III. Thus, the absence of PE has a very different effect on the topology of these two transporters than it does on LacY, which is a member of the major facilitator superfamily. In LacY the transition point between native and aberrant topology, in the absence of PE, occurs in the middle of the protein, between the two six-TM helical bundles [46,47], which is also the location of the substrate binding site [48].

5. Summary

The breadth and depth of the studies discussed here make it clear that in recent years significant progress has been made with respect to indentifying aspects of membrane composition and membrane physical properties that contribute to the folding of functional integral membrane proteins. However, the difficulty in identifying membrane properties that universally contributes to integral membrane protein folding highlights the relative infancy of the field. One aspect of membrane structure that appears to be perhaps not as crucial as might have been expected is membrane hydrophobic thickness. Studies on both multi-helical proteins and β-barrels proteins indicate that successful refolding can occur over a range of membrane thicknesses. The presence of phosphatidylethanolamine (PE) in the membrane inhibits folding of some multi-helical proteins, such as bR and EmrE, while for proteins such as GalP a high level of PE is required for successful refolding. The effects of PE on the refolding of β-barrel proteins are similarly varied, with PE enhancing refolding for some proteins, inhibiting or having no effect on others. Studies on both classes of proteins suggest there is often an important interplay between the membrane lateral pressure profile and the transmembrane shape of the protein.

This brief survey suggests that progress toward a general understanding of the role of the membrane in membrane protein folding could be enhanced by a certain amount of standardization of experimental protocols. The experimental details of studies in this field are often necessarily dictated by the chemical and physical requirements for successful isolation and refolding of the particular protein under examination. However, the increased use of non-detergent chaotropic agents such as urea would contribute greatly to meaningful comparisons between different proteins of membrane properties involved in the folding process. Refolding studies involving detergents will continue to be important; however the possible role of detergent monomers in the refolding process may make it more difficult to isolate the key membrane properties that contribute to the process. A second area of potentially useful standardization lies entirely on the lipid side of the process, the use of large unilamellar vesicles (LUVs) instead of SUVs. The variation in refolding yields between these two vesicle types for the same lipid composition highlights the effects of intrinsic membrane curvature for some proteins. More widespread use of LUVs would make it easier to compare the refolding yields for different proteins and different membrane compositions. As the field progresses to address more complex issues on the lipid side, such as the role of membrane phospholipid asymmetry, it will be helpful to have a significant body of results obtained in a uniform physical system.

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