Lipid-assisted Protein Folding*

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Although there has been significant progress in our understanding of how water-soluble proteins fold (1, 2), the factors and mechanisms driving correct folding of integral membrane proteins are largely unknown. The folding of membrane proteins, like their soluble counterparts, is dictated by their amino acid sequence and their environment (Fig. 1). Integral membrane proteins can also interact with other proteins within the membrane and with the hydrophobic and hydrophilic components of the lipid bilayer itself during and after attainment of native structure. The role of lipids as an important structure-forming environment was elucidated during the last decade (3). However, the role individual lipids play as part of the protein folding machinery has been largely ignored. Are individual lipids mobilized to protect and guide the nascent polypeptide chain during its membrane assembly? Do lipids act as specific molecular chaperones or transient ligands during the assembly of a membrane protein?

Refolding of Integral Membrane Proteins

The main experimental approach to study soluble protein folding has been to investigate refolding of denatured (unfolded) protein back to the native state (4). The discovery of protein molecular chaperones indicated that in vivo folding of proteins is a more complex process than initially thought (5). Similarly, the folding of membrane proteins during in vitro renaturation may greatly differ from in vivo folding, which involves interaction with the phospholipid bilayer. Therefore, detergent/lipid micelles and lipid vesicles have been included in the renaturation solution used to dilute the denaturant to non-denaturating conditions (6) because they mimic the properties of the lipid bilayer. Using this approach, the α-helical protein bacteriorhodopsin was the first membrane protein refolded from the denatured state in detergent to a folded state in detergent/lipid micelles (7) and lipid vesicles (8, 9). α-Helix formation (the rate-limiting step in folding) is much slower for bacteriorhodopsin than in polymeric protein bacteriorhodopsin relative to that of soluble proteins. Folding, as well as membrane insertion, is slowed even more as the proportion of dimyristoyl-PC and dihexanoyl-PC mixed vesicles or as the non-bilayer-forming lipid phosphatidyethanolamine (PE) is added to PC vesicles. These results indicate that an increase in the radius of curvature of the bilayer (or departure from a flat bilayer) may slow α-helix formation within or inhibit insertion of polypeptide into the membrane bilayer. The kinetics of refolding of the Escherichia coli membrane protein, OmpA, in lipid vesicles has also been studied (10–12). Surprisingly, the yield in renaturation of this β-barrel membrane protein was considerably higher in dimyristoyl-PC/dimyristoyl-PC (80/20) mixed bilayers than in mixtures of 1-palmitoyl 2-oleoyl-PE/1-palmitoyl 2-oleoyl-PE (80/20) mixed bilayers that more closely mimic the lipid composition of the E. coli inner cytoplasmic membrane (13). This protein resides in the outer membrane of E. coli but must pass through the inner membrane where presumably it must maintain a more unfolded state.

In these studies the folding of integral membrane proteins has been investigated as a function of the collective properties of lipid bilayers, i.e. as a function of the hydrophobicity (14), electrostatics (15), rigidity (16), and intrinsic radius of curvature (9). No systematic investigation of the possible role of specific lipids or the chemical properties of membrane lipids on the yield of refolding, formation of refolding intermediates, or alteration of the refolding pathway was included in these studies. However, these reports suggest that membrane protein folding is more complex than interaction with the lipid bilayer approximated as a simple hydrophobic core bounded by water interfaces.

Lipid as a Molecular Chaperone

The role of protein molecular chaperones in directing protein folding, preventing protein misfolding (17), and even unfolding proteins (18) is now well established for the conformational maturation of soluble and membrane proteins (19, 20). The presence of protein molecular chaperones in refolding protocols can significantly increase the yield of native structure. Evidence is now accumulating that specific lipids may also participate as molecular chaperones in the folding and possibly the unfolding of integral membrane proteins.

The development of mutants of E. coli (21, 22) in which the membrane phospholipid composition can be readily altered has made it possible to separate phospholipid-assisted and -unassisted folding events and study the role of individual phospholipids in protein folding. The absence of the major phospholipid PE in one such mutant results in the misfolding of the polytopic membrane protein lactose permease (LacY) without affecting its insertion into the membrane. Refolding dependent on phospholipid of unfolded LacY was monitored using the Eastern-Western blotting procedure (23, 24). This procedure involves the transfer of proteins from a SDS-polyacrylamide gel by standard Western blotting methodology to a solid support coated with phospholipids that interact with partially denatured proteins as they refold. These refolding experiments have uncovered a role for PE as a non-protein molecular chaperone.

LacY initially assembled in the presence of PE but not in its absence has full biological function (25) and displays a continuous epitope (4B1) recognized by the conformation-dependent monoclonal antibody 4B1 (23, 24). This epitope is in a periplasmic loop near the middle of LacY between transmembrane helices VII and VIII. The proper conformation of this epitope is required for full function of LacY as a transporter (26) and depends on exposure to PE during assembly (23, 24). Sufficient conformational information was retained to detect epitope 4B1 after partial unfolding of LacY by SDS, complete removal of PE, and refolding in the absence of PE. Therefore, once information is imparted by PE during LacY folding in vivo, the presence of PE is no longer required to maintain proper conformation of this epitope consistent with the primary definition of a molecular chaperone (27). LacY lacking recognition by monoclonal antibody 4B1, because of initial assembly in the absence of PE, can be induced to form this epitope if partially denatured and then refocused in the presence of PE or phosphatidylserine (PS) specifically; other phospholipids such as PC, PG, or cardiolipin (CL) do not support the regain of epitope 4B1 during refolding. Thus, the misfolded conformation of LacY can be corrected by specific lipids (absent during in vivo assembly) by interaction with non-native LacY, also consistent with an important criterion in the definition of a molecular chaperone (27).

Refolding of denatured full-length protein is not an ideal model because the folding of the nascent peptide in vivo begins during translation (28). Moreover, molecular chaperones were shown to interact differently with denatured proteins than with their newly translated counterparts (17). Molecular chaperones can bind transiently to nascent proteins and prevent premature folding (28) or can assist in folding by binding post-translationally at a later stage of assembly (29). The liposome-induced conformational transition of colicin E1 occurs concomitantly with insertion into membranes.
also regained both full transport function and epitope 4B1 after in situ synthesis of PE in isolated membranes originally lacking PE. LacY synthesized and assembled in the absence of PE is partially functional as a facilitating transporter and is consistent with the observation that LacY in the absence of PE is correct, there should exist molecular species of lipids other than PE that integrate into, pass through, or come in contact with a membrane. For glycerophosphate-based lipids chemical diversity in the fatty acid chains combined with the major headgroup classes define individual molecular species ranging in the hundreds to thousands of non-protein molecular chaperone (tentatively named a “lipochaperone”). The transformation of chemical information from a one-dimensional amino acid sequence into a three-dimensional protein structure is dictated by primary sequence, is governed by folding “rules,” and is affected by the environment (solvent composition, temperature, etc.). Soluble proteins fold primarily in an aqueous environment whereas membrane proteins fold primarily in a hydrophobic environment but also interact with ionic components in the membrane and in the extramembrane space. Protein molecular chaperones have been established to prevent misfolding and/or aggregation of both soluble and membrane proteins by interaction with stress-denatured proteins and folding intermediates of newly synthesized proteins. Specific lipids (termed “lipochaperones”) are postulated to act in a similar manner as protein molecular chaperones in the folding of proteins that integrate into, pass through, or come in contact with a membrane.

The rate of unfolding of acetylcholine esterase from <i>Pedoto californica</i> was greatly enhanced in the presence of PC vesicles with concomitant insertion of the protein into the lipid bilayer (32). For β-barrel membrane proteins, folding and membrane insertion are coupled processes that involve kinetically distinguishable steps (10, 33). However, by using mutants lacking PE coupled with the ability to add PE post-translationally to LacY already assembled in the membrane, the process of membrane insertion was shown to be separate from a late step of conformational maturation requiring specific PE assistance (24).

In <i>in situ</i> synthesis of LacY in the presence of membranes originally lacking PE either with co- or post-translational expression of PE resulted in the appearance of the PE-dependent epitope 4B1. In <i>in situ</i> synthesis of PE in isolated membranes originally lacking PE and containing <i>in vivo</i> synthesized LacY also resulted in the appearance of epitope 4B1. Thus PE appears to be required in a late step of conformational maturation occurring after membrane insertion and involving final adjustments for the transition from near native to the native state. This late stage involvement is similar to the temporal point of action of most protein molecular chaperones (34) and is consistent with the observation that LacY in the absence of PE is partially functional as a facilitating transporter in <i>vivo</i> and in <i>in situ</i> (25). LacY synthesized and assembled in the absence of PE also regained both full transport function and epitope 4B1 after initiation of PE synthesis in <i>vivo</i>. Therefore PE can act as a non-protein molecular chaperone (tentatively named a “lipochaperone”) that specifically mediates the late stage folding of LacY.

How Widely Might Lipid Assistance Be Required for Protein Folding?

If the general argument for phospholipids as molecular chaperones is correct, there should exist molecular species of lipids other than PE that are involved transiently in the folding of integral membrane proteins. Several reports on the unfolding, refolding, and assembly of outer membrane porins of <i>E. coli</i> strongly suggest a molecular chaperone role for lipopolysaccharide (LPS). Refolding of denatured OmpA occurred exclusively in the presence of LPS and not with other lipids (35). An immature form of OmpA was detected in the inner membrane that undergoes a conformational change upon interaction with LPS making the protein “outer membrane compatible” (36). No lipid was found tightly associated with either conformational variant of OmpA. Similarly, the glycolipid part of LPS appears to be required for folding outer membrane PhoE porin monomers of <i>E. coli</i> as they pass through the periplasmic space. LPS molecules are also in transit to the outer membrane. LPS inhibits the assembly of PhoE into its functional trimeric units while in the inner membrane. Release of LPS very likely is required for efficient trimerization because no interaction was detected between LPS and the mature trimer that forms upon assembly of monomers in the outer membrane. The transient interaction of LPS with the PhoE monomer hydrophobic interfaces could prevent aggregation during passage through the inner membrane consistent with this glycosphospholipid acting as a molecular chaperone (37).

DegP, a periplasmic protein of <i>E. coli</i>, undergoes PG-dependent conformational changes that may protect the unfolded protein from self-aggregation at relatively low temperatures keeping critical interactive sites apart, thus preventing misfolding (38). This phenomenon was not observed in the presence of PE or CL, demonstrating again specificity of folding of DegP with the aid of the putative lipochaperone PG.

A sulfated glycosphingolipid (sulfatide) appears to act as a molecular chaperone by transiently interacting in a functional manner with non-native reversibly aggregated crystals of insulin (39). This interaction prevents the irreversible aggregation of insulin into an amorphous form while either promoting or facilitating monomerization into the biologically active form. This is the first example of molecular chaperone action to simultaneously preserve a reversibly aggregated form and to convert protein aggregates into a biologically active form.

These observations add to a growing body of evidence that lipids may bind to certain areas of unfolded proteins, thus reducing the likelihood of misfolding or aggregation. Therefore, lipids act in an analogous manner to protein molecular chaperones, thereby adding them to the growing list of non-protein chaperones (27), which includes RNA and oligosaccharides (40, 41).

What Structural Features of Lipids Account for Their Molecular Chaperone Ability?

For glycerophosphate-based lipids chemical diversity in the fatty acid chains combined with the major headgroup classes define individual molecular species ranging in the hundreds to thousands (42). In addition lipids assume various physical organizations such as the bilayer, micellar, inverted hexagonal, or cubic phase (43). The collective physical properties of an overall bilayer organization of lipids will have different physical properties depending on the proportion of non-bilayer- and bilayer-forming lipids present.

Using LacY synthesized and assembled <i>in vivo</i> in the absence of PE, the physical and chemical properties of a lipid required to assist proper refolding of epitope 4B1 of LacY <i>in vitro</i> were investigated using the Eastern-Western technique (44). A primary anionic (either PE or PS) was most effective. PE derivatives of increasing degrees of methylation were progressively less effective in supporting refolding with PC being totally ineffective. Monoacyl phospholipids were not functional, and the diacyl phospholipids had to contain at least one saturated fatty acid with a preference for chain lengths above 14 carbons. The requirements within the hydrophobic domain correlated closely with an apparent requirement for phospholipid mixtures that assume a collective bilayer rather than non-bilayer organization under experimental conditions. Therefore, as with bacteriorhodopsin (8), insertion of LacY into a lipid domain and subsequent folding may require a bilayer structure, and these may precede the requirement of PE as a lipochaperone. Unnatural diastereoisomers of PS (at either chiral center) were ineffective unless in binary mixtures with natural isomers of PG that were unable in themselves to support proper refolding of LacY. Therefore, the molecular chaperone effect is highly selective with respect to lipid chemical composition, chirality, and physical properties supporting a role for lipid that goes well beyond simply providing a nonspecific detergent-like or two-phase environment for refolding of LacY. PC, which is not found in <i>E. coli</i>, promoted the unfolding of native epitope 4B1 (44), suggesting that lipochaperones could function as do protein chaperones to maintain or induce an unfolded state.

Zardeneta and Horowitz (45) investigated the interaction of the mitochondrial phospholipid CL with unfolded and native rhodanese, a nuclear-encoded mitochondrial matrix enzyme. They demonstrated that unfolded non-native rhodanese, but not native rhodanese, associates transiently with CL-containing micelles, and this interaction increases the amount of enzyme reactivation by preventing aggregation. The anionic lipid PS was not as effective as

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2 M. Bogdanov, P. Heacock, and W. Dowhan, unpublished data.
CL, indicating a structural preference in addition to simple charge. Formation of secondary and tertiary structure during refolding of the outer membrane protein OmpA of E. coli correlated with the micelle-forming properties of short chain phospholipids and detergents and did not depend on the polar headgroup or hydrophobic chain length of phospholipids and detergents that were examined (46). Monomeric detergent solutions were not active. Finally, negatively charged LPS derivatives that tend to organize bilayer structures were better facilitators of folding of PhoE in situ than their bilayer-forming counterparts (47). All these data taken together demonstrate the importance of both individual chemical makeup and supramolecular organization in the function of lipochaperones.

**How Do Lipids Facilitate Membrane Protein Folding?**

The binding of partially folded proteins to a molecular chaperone prevents their misfolding and the aggregation of folding intermediates. Similarly, lipochaperones interact with non-native conformations of proteins and have a low affinity for native proteins (23, 24, 37). Interaction of protein chaperones with substrate proteins also displays an ionic component (48). Both the properties of the ionic headgroup and the organization of the hydrophobic domain of PE (44) and LPS (47) also mimic critical components of protein chaperones. Several examples support the view that some proteins require molecular chaperones to fold correctly not to avoid a potential aggregation problem but to reverse stable misfolded states (18, 49). Lipochaperones could participate at a late stage of protein folding in facilitating such interactions and conformational changes.

PE appears to interact with late folding intermediates to assist in attaining native structure of LacY. In situ folding studies on LacY (24) and the fact that LacY in the absence of PE is partially functional (25) as a facilitating transporter indicate that considerable conformational maturation must occur prior to interaction with PE followed by the imprint of conformational information by exposure to PE. More extensive unfolding of LacY using urea-SDS eliminates epitope 4B1 in LacY and prevents its proper refolding in the presence of PE (23), also suggesting a requirement for existing structural organization prior to the involvement of PE. A similar concept of “protein memory” was used to describe the action of intramolecular chaperones represented by the propetidies of some proteases (50). Subtilisin and α-lytic protease purified and denatured in vivo fail to refold into active enzymes until their own prosequences are added. Moreover prosequences are not interchangeable in this reaction. In vivo, the propetide of subtilisin imparts steric information during folding of the protease domain that, after folding is complete, is no longer required to maintain the native conformation.

The late folding steps usually correspond to rearrangements of the polypeptide chain within an already compact or near native conformation (34). The lipid bilayer also affects certain folding events in the final stage of α-helix formation, which appear to be slow and rate-limiting steps in folding. This would suggest that folding intermediates trapped by interaction with the membrane bilayer could undergo conformational changes leading to properly folded integral membrane proteins (16). PE appears not to be preventing protein aggregation but rescues LacY from or prevents a reversible misfolding event. The order of folding events in the native membrane containing PE is not known. However, in the absence of PE LacY appears to be trapped within an energy minimum for the native folding pathway. Therefore, a lipochaperone could either remove the energy barrier (see Fig. 2A) or prevent the formation of the energy barrier (see Fig. 2B), thereby allowing attainment of the final native structure.

**How Are Lipochaperones Released from Substrate Protein?**

To fulfill a requirement for molecular chaperone action, lipid must be released from the protein or no longer interact in a manner necessary to maintain protein structure (52, 53). For LacY this criterion is difficult to establish in the native membrane where PE comprises 70% of the phospholipid. However, it is clear that epitope 4B1 of LacY once formed maintains its conformation in the absence of PE, and formation of this epitope is dependent on late folding events requiring PE rather than exposure of the mature or totally unfolded protein to PE (24, 25).

The evidence for dissociation of lipid from protein after folding is clear for several other examples of lipochaperone action. DegP appears to require PG for proper folding during passage through the inner membrane of E. coli but is no longer associated with lipid after being released into the periplasm (38). Similarly mitochondrial rhodanese is no longer associated with CL after import into the mitochondrial matrix (54). In the case of PhoE LPS chaperone activity appears to occur in the inner membrane to facilitate the formation of the monomers competent for trimerization in the outer membrane where interaction with LPS appears not to be required. An outer membrane protein chaperone and phospholipid may be required for final release of LPS and maturation into the native trimer (54). A preference for PE in this late step of assembly was recently observed.3

**Do Protein and Non-protein Molecular Chaperones Cooperate in Vivo?**

Reconstitution experiments (55) demonstrated that active GroEL-GroES chaperonin heterooligomers are able to associate with the phospholipid bilayer and may work in concert forming “lipochaperonin” that could assist membrane protein folding as well as the folding of water-soluble proteins that transiently interact while in the molten globular state (55). Components of the protein import machinery, which are required for the efficient post-translational translocation of precursor proteins into the mitochondrial matrix (56), could form a “lipochaperonin” complex with CL as suggested from the studies of lipid-dependent unfolding of proteins imported into mitochondria (57). Therefore coupled protein and phospholipid folding machinery may have co-evolved in different membrane systems.

**Lipochaperones and Protein Quality Control**

Molecular chaperones and proteins together or proteases with intrinsic chaperone activity could eliminate misfolded or partially folded proteins derived from low rates of folding, stress, or biosynthetic errors (58). Incubation of macrophages with sterols is associated with a change in lipid composition in the membrane of a non-lysosomal compartment resulting in the degradation of lipoprotein ApoE. The authors postulated that interaction with cel-

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3 H. de Cock, personal communication.
lular membranes of altered phospholipid composition could modulate the folding or reorganization of ApoE, thereby making it susceptible to proteostatic degradation (59).

**Protein Misfolding: Lipids as Molecular Anti-chaperones**

The list of diseases that are fully or partly due to defects in protein folding is growing (60). Alzheimer’s disease is characterized by deposits composed largely of the β-amyloid peptide. A growing number of observations indicates that some pathophysiological effects of β-amyloid peptide plaques could be the result of peptide-lipid interactions. This peptide binds to ganglioside GM1-containing membranes (61) and upon binding undergoes a rapid conformational transition from a random coil to a β-sheet structure, which is not induced by zwitterionic or anionic phospholipids or the oligosaccharide moiety of the ganglioside (62). Ganglioside-rich domains in neural membranes may act as templates that promote self-aggregation of β-amyloid peptides to form the mature amyloid plaque (63). Post-translational refolding of normal prion protein (PrPc) into a β-sheet-rich abnormal conformer (PrP Sc) is is also involved in disease (64). The molten globular state can be induced upon interaction with hydrophobic proteins with lipids increases the solubility of folding intermediates (64) and thus prevents irreversible aggregation and promotes proper folding. However, the results reviewed here strongly suggest a more specific role for lipids in protecting membrane proteins. Whether lipids remain in specific association with proteins after attainment of final structure or whether they simply define their role as a structural element or chaperone, respectively. More important is that lipids can assist in folding by interacting with non-native intermediates consistent with the most fundamental definition of molecular chaperones. The limited but broad spectrum of examples of lipid-assisted folding suggests that this is a widespread phenomenon with many similarities to the role of protein molecular chaperones. The molten globular state can be induced upon interaction with lipids and very likely could be an intermediate in proper insertion or translocation across the membrane (30, 31).

**Perspectives**

It is likely that examples of lipid-assisted protein folding will increase in the near future as the role of individual lipids in the folding of different proteins is further documented. There is no doubt that interaction of hydrophilic proteins with lipids increases the solubility of folding intermediates (64) and thus prevents irreversible aggregation and promotes proper folding. However, the results reviewed here strongly suggest a more specific role for lipids in properly protecting membrane proteins. Whether lipids remain in specific association with proteins after attainment of final structure or whether they simply define their role as a structural element or chaperone, respectively. More important is that lipids can assist in folding by interacting with non-native intermediates consistent with the most fundamental definition of molecular chaperones. The limited but broad spectrum of examples of lipid-assisted folding suggests that this is a widespread phenomenon with many similarities to the role of protein molecular chaperones.

The molten globular state can be induced upon interaction with lipids and very likely could be an intermediate in proper insertion or translocation across the membrane (30, 31). In this case unfolding of protein is concomitant with membrane insertion or translocation. Alternatively, lipids can participate in the fine-tuning of structure already folded into a near native compact state. In this case insertion and folding are mechanistically distinct and uncoupled events (24). The consequence of the latter is 2-fold; some proteins could be awakened from a “silent” form by this interaction whereas other proteins could become activated for degradation or to cause adverse effects associated with disease.

Future research should now focus on a more detailed understanding of the specific mechanism underlying lipid-assisted folding. What structural features of membrane proteins account for their dependence on lipid assistance? How are lipochaperones released from inserted membrane proteins or excluded from the immediate domain of polypeptides residing in the membrane? Could lipids hold the nascent chain in a conformation that permits interactions with other components of the insertion, folding, and translocation machinery?

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**Minireview:** Lipids as Molecular Chaperones