Phospholipid-assisted protein folding: phosphatidylethanolamine is required at a late step of the conformational maturation of the polytopic membrane protein lactose permease

Mikhail Bogdanov and William Dowhan¹

Department of Biochemistry and Molecular Biology, University of Texas-Houston, Medical School, Houston, TX 77225, USA

¹Corresponding author

Previously we presented evidence that phosphatidylethanolamine (PE) acts as a molecular chaperone in the folding of the polytopic membrane protein lactose permease (LacY) of *Escherichia coli*. Here we provide more definitive evidence supporting the chaperone properties of PE. Membrane insertion of LacY prevents its irreversible aggregation, and PE participates in a late step of conformational maturation. The temporal requirement for PE was demonstrated *in vitro* using a coupled translation–membrane insertion assay that allowed the separation of membrane insertion from phospholipid-assisted folding. LacY was folded properly, as assessed by recognition with conformation-specific monoclonal antibodies, when synthesized in the presence of PE-containing inside-out membrane vesicles (IOVs) or in the presence of IOVs initially lacking PE but supplemented with PE synthesized *in vitro* either co- or post-translationally. The presence of IOVs lacking PE and containing anionic phospholipids or no addition of IOVs resulted in misfolded or aggregated LacY, respectively. Therefore, critical folding steps occur after membrane insertion dependent on the interaction of LacY with PE to prevent illicit interactions which lead to misfolding of LacY. **Keywords:** cell-free phospholipid biosynthesis/membrane protein folding/phosphatidylethanolamine/phospholipid-assisted folding

Introduction

Newly synthesized polypeptides fold into their native structures via partially folded intermediates that display hydrophobic regions transiently on their surfaces. These regions can interact with one another, resulting in aggregates that may become insoluble. The high concentrations of interacting protein surfaces inside cells result in a need for molecular chaperones (Hendrick and Hartl, 1995; Ellis and Hartl, 1996; Hartl, 1996) to prevent incorrect interactions between these surfaces. A well-established class of such molecular chaperones is the proteins that recognize and bind transiently to these surfaces, thus preventing their premature or inappropriate interaction which lead to misfolding and self-aggregation. Molecular chaperones can bind to proteins either co-translationally (Fedorov and Baldwin, 1997) or post-translationally (Hendrick and Hartl, 1993), and thereby assist in correct folding at multiple stages of protein assembly.

Although there is significant progress in our understanding of protein folding for water-soluble proteins, there is little information available for the folding of multispansing polytopic integral membrane proteins. Large hydrophobic membrane proteins in *Escherichia coli* are synthesized in the cytoplasm and may interact with cytoplasmic molecular chaperones (Bochkareva *et al.*, 1996) which prevent aggregation. These proteins are delivered to the membrane where the final stages of folding take place. During membrane assembly, integral membrane proteins must interact with other proteins within the membrane, the phospholipid bilayer as solvent and individual phospholipid molecules either as permanent ligands or transiently during folding, which collectively results in the correct folding of a native membrane protein.

The role of the phospholipid bilayer as an important structure-forming environment has been established (Selinsky, 1992; In't Veld *et al.*, 1993; Marsh, 1993); however, the role of individual phospholipid molecules as specific participants in the process of protein assembly has been largely ignored. Can phospholipids act as specific permanent or transient ligands during the assembly of a membrane protein to either prevent unproductive folding or direct the proper folding of a membrane protein? Conceptually, there is considerable similarity between interaction of protein folding intermediates with either amphipathic phospholipid molecules or protein molecular chaperones (Zardeneta and Horowitz, 1992). What is lacking is a large body of evidence supporting such a role for phospholipids.

In order to determine the role that individual phospholipids play in the folding of membrane proteins in *E. coli*, we have employed LacY (lactose permease) as a model for studying the folding and assembly of such proteins. LacY is particularly suited for this purpose because of the extensive molecular genetic and structural information available (Varela and Wilson, 1996; Kaback *et al.*, 1997) as well as its homology with the major facilitator superfamily of transport proteins (Marger and Saier, 1993).

We have presented experimental evidence that the phospholipid phosphatidylethanolamine (PE) fulfills the minimum requirements to be a non-protein molecular chaperone in the folding of LacY (Bogdanov *et al.*, 1996). PE corrects *in vitro* a LacY folding defect caused by *in vivo* assembly in PE-deficient membranes and, once properly folded, PE is no longer required to maintain the 'native' conformation. These results were obtained using a novel blotting technique (Eastern–Western) employing phospholipid during renaturation of proteins on solid supports in the standard Western blotting procedure.

Although the refolding of denatured full-length protein provides important information on the mechanism of protein folding, it is not an ideal model, since the refolding of the protein starts from the complete polypeptide chain,

for lanes 5–8 with mAb4B11 (data not shown), indicating that the discontinuous epitope recognized by this antibody is only present in membrane-integrated LacY. Unlike mAb4B1 (Bogdanov *et al.*, 1996), mAb4B11 recognizes LacY assembled in both PE-containing and PE-deficient cells (lanes 9 and 10, respectively). Therefore, the formation of epitope 4B1 not only requires expression of LacY in PE-containing cells but the integration of LacY into the membrane where association with PE must occur. In addition, integration into the membrane is required for the proper folding of domains C8 and C10 (defining epitope 4B11) but in a PE-independent manner. These findings are consistent with different conformations of LacY depending on whether it is embedded in the lipid bilayer of the membrane (either with or without PE) or present in the lipid-free cytoplasm. Based on these results for *in vivo* expressed LacY, these two conformation-specific antibodies were used to determine whether membranes with and without PE are required co- or post-translationally for the proper assembly of LacY *in vitro*.

***In vitro* translation and insertion assay of LacY**

A translation system for the *in vitro* expression of LacY either in the presence or absence of inside-out membrane vesicles (IOVs) was developed using a plasmid-borne copy of the *lacY* gene under T7 promoter control. In order to control the make up and PE content of added IOVs, membrane- and phosphatidylserine (PS) synthase-free S30 fractions were prepared, as described in Materials and methods, from *E. coli* strain AD905 (*pss93::kan degP*) carrying the null allele of the *pssA* gene. This strain cannot synthesize PE due to the lack of PS synthase (DeChavigny *et al.*, 1991) which in wild-type cell lysates is almost exclusively bound to ribosomes (Dutt and Dowhan, 1977). Addition of an S30 fraction from wild-type cells containing PS synthase would result in the formation of PE in the IOVs which contain PS decarboxylase (Dowhan, 1997). The IOVs tested in these experiments were prepared either from PE-containing [strain AD93 (*pss93::kan*) carrying plasmid pDD72 (*pss*⁺)] or PE-deficient (strain AD93 lacking plasmid pDD72) cells grown in the absence of isopropyl- β -D-thiogalactopyranoside (IPTG), which resulted in no detectable LacY, as previously described (Bogdanov and Dowhan, 1995). LacY synthesis was initiated from the transcript of plasmid pT7-5 (carrying the *lacY* gene) in the presence of the above S30 fraction. After 5 min, the reaction mixture was either supplemented (co-translational membrane assembly) or not supplemented with the indicated IOVs. Synthesis was continued for an additional 60 min, followed by analysis of the translation-membrane insertion mix by SDS-PAGE and immunoblotting either with pAb, mAb4B11 or mAb4B1 (Figure 3)

If LacY was synthesized using an S30 extract without co-translational addition of IOVs, then no monomeric form was detected and only multimeric forms were detected by the site-directed pAb (Figure 3, lane 1 versus lanes 2–5). If IOVs were present co-translationally, then a diffuse immunoreactive band was also observed in the 31–33 kDa range that corresponds to lactose permease (Dunten *et al.*, 1993); the basis for the diffuse nature of these bands is discussed below. An apparently stronger signal was detected for monomeric LacY assembled *in vitro* in PE-deficient (lanes 4 and 5) IOVs versus PE-containing IOVs

Fig. 3. Assembly of *in vitro* synthesized LacY into IOVs. Transcription of the *lacY* gene using the T7 RNA polymerase-dependent promoter of plasmid pT7-5 was followed by *in vitro* translation using a membrane- and PS synthase-free S30 extract either in the absence (lanes 1 and 6) or in the presence of IOVs (lanes 2–5 and 7–14) prepared either from PE-containing (+PE, AD93/pDD72) or PE-deficient (–PE, AD93) cells grown in the absence of IPTG. Each reaction mixture was analyzed by SDS-PAGE followed by immunodetection with either pAb, mAb4B11 or mAb4B1 as indicated.

(lanes 2 and 3), as we had observed previously for LacY assembled *in vivo* in the absence or presence of PE (Bogdanov *et al.*, 1996). The last five residues in the C-terminal portion of transmembrane (TM) helix XII are necessary for proper folding of LacY (Roepe *et al.*, 1989). The pAb used here and mAb4A1OR share a common overlapping epitope in the C-terminus of LacY. In the latter case, the mAb recognition of LacY on Western blots is sensitive to conformational changes in the C-terminus brought about by a mutation in LacY resulting in energy uncoupling of lactose transport (Herzlinger *et al.*, 1985). LacY assembled in PE-deficient membranes is uncoupled similarly (Bogdanov and Dowhan, 1995), which might explain the differences in sensitivity of LacY to pAb dependent on the presence or absence of PE during its assembly. More important was the observation that mAb4B11 (lanes 6–10) only recognized the monomeric form of LacY, as observed for *in vivo* assembly (Figure 2), with the same apparent sensitivity independent of the presence of PE. This supports a correct *in vitro* co-translational membrane assembly of LacY at least with respect to domains C8 and C10 independent of the presence of PE during assembly. Site-directed pAb was not suitable for comparing LacY insertion yields between PE-containing and PE-deficient IOVs (Figure 3, lanes 2–5). However, mAb4B11 can be used as an immunological probe to discriminate between inserted and uninserted LacY molecules as well as an indicator of comparable membrane insertion efficiency of LacY whether or not PE is present. In this co-translational assay, 15–18% of the *in vitro* synthesized LacY was fully integrated into IOVs from wild-type cells as judged by quantitative Western blot analysis (Bogdanov and Dowhan, 1995). pAb and ³⁵S-labeled protein A were used to estimate the percentage of integrated LacY (lanes 2) relative to total LacY (lane 1) (data not shown).

Direct binding studies and *in vitro* refolding experiments had provided independent evidence for the preferential recognition by conformation-sensitive mAb4B1 of the P7 periplasmic domain of LacY assembled *in vivo* in the cells containing PE (Bogdanov *et al.*, 1996). A similar dependence on PE was observed *in vitro* when mAb4B1

recognition of LacY co-translationally assembled in PE-containing IOVs (Figure 3, lanes 11 and 12) was compared with LacY assembled into PE-deficient IOVs (Figure 3, lanes 13 and 14). Probing with mAb4B1 after removal of the mAb4B1 verified the presence of the same amount of LacY in lanes 11–14 (data not shown). Therefore, these results confirm the *in vivo* assembly data indicating the dependence on PE for the formation of the native conformation of epitope 4B1, and also indicate that the insertion efficiency of LacY into the membrane is not dependent on PE.

In our experiments, LacY displayed diffuse multiple species with different mobilities. These properties of LacY have been seen numerous times (Kaback, 1986), but the high salt content of the *in vitro* system resulted in even broader bands. Since LacY maintains a high degree of secondary structure in SDS (Bogdanov *et al.*, 1996) and displays an aberrant mobility on SDS gels for a protein of $M_r = 46\,000$ (Kaback, 1986), the multiple bands most likely represent different conformations of LacY due to incomplete denaturation under the conditions of SDS–PAGE.

PE corrects co-translationally the misfolding of LacY assembled *in vitro* in IOV without PE

We next wished to determine whether addition of PE to LacY assembled *in vitro* in PE-deficient IOVs could result in the formation of native epitope 4B1. Previously we had shown that addition of a dispersion of PE to LacY assembled in membranes *in vivo* in the absence of PE prior to solubilization with SDS did not result in recognition of LacY by mAb4B1 after Western blot analysis (Bogdanov *et al.*, 1996). Therefore, we employed a cell-free system of phospholipid biosynthesis that utilizes intact inner membranes of *E. coli* (Sparrow *et al.*, 1984) which should reflect more accurately *in vivo* conditions for delivery of PE to membranes. We took advantage of the fact that the absence of PE in PE-deficient cells results in an elevated level of acidic phospholipids including phosphatidic acid (PA) of up to 8% of total phospholipid composition. As previously shown (Sparrow *et al.*, 1984), the addition of CTP and either L-serine or *sn*-glycerol-3-phosphate to PA-enriched *E. coli* membranes results in the formation of PE or phosphatidylglycerol (PG), respectively. Membranes lacking PS synthase, as is the case for IOVs from PE-deficient mutants, can be reconstituted by the addition of partially purified PS synthase which normally is associated with ribosomes in cell lysates of wild-type cells (Dutt and Dowhan, 1977; Louie *et al.*, 1986). A membrane-free PS synthase-enriched fraction (PSS) (see Materials and methods) converted nearly all of the PA (pre-labeled with ^{32}P by *in vitro* exchange reaction with radioactive ATP) in IOVs lacking PE to PE in 1 h, dependent on the addition of CTP and L-serine (Figure 4); *E. coli* membranes contain PS decarboxylase which is necessary for this conversion (Dowhan, 1997). These membranes also convert PA to PG and cardiolipin (CL) if CTP and *sn*-glycerol-3-phosphate are added (data not shown).

LacY was expressed *in vitro* co-translationally as described in Figure 3 either in the presence of PE-deficient (–PE) IOVs, without or with *in vitro* synthesis of PE (Figure 5, lanes 1 and 3, respectively), or in the presence of PE-containing (+PE) IOVs (Figure 5, lanes 4).

Fig. 4. Cell-free phospholipid biosynthesis using IOVs isolated from PE-deficient cells. IOVs derived from strain AD93 lacking PE but containing [^{32}P]PA were supplied with PS synthase-enriched fraction, CTP and dCTP, and serine as indicated. After 1 h of incubation, the lipids were isolated and analyzed by TLC.

Fig. 5. Assembly of *in vitro* synthesized LacY into IOVs coupled with *in vitro* synthesis of phospholipid. A membrane- and PS synthase-free S30 extract from strain AD905 and membrane-free PS synthase-enriched fraction (PSS) were used in this experiment. IOVs made from either PE-containing (+PE) or PE-deficient (–PE) cells (as in Figure 3) were added co-translationally to the *in vitro* LacY expression system described in Figure 3. Each reaction mixture was supplemented co-translationally with components necessary for *in vitro* PE biosynthesis (see Figure 4) or PG biosynthesis (*sn*-glycerol-3-phosphate, G-3-P) as indicated. For the results in lanes 7 and 8, the transcription reaction was stopped by using addition of LiCl to remove CTP prior to initiation of translation without or with PE synthesis. All samples were analyzed as described in Figure 3, using mAb4B1.

Western blot analysis, monomeric LacY was recognized by mAb4B1 only from membranes containing PE originally or from IOV supplied with PE by *in vitro* biosynthesis (lanes 4 and 3, respectively); no significant detection of LacY assembled in PE-deficient IOVs was observed (lane 1). Coupling *in vitro* biosynthesis of either zwitterionic phospholipids (PE and PS) or anionic phospholipids (PG and CL) with assembly of LacY resulted in no change in recognition of LacY assembled in IOVs previously containing PE (data not shown). Furthermore, *in vitro* biosynthesis of anionic phospholipids (lane 2) in IOVs originally lacking PE did not result in the recognition of monomeric LacY by mAb4B1. Addition of all components necessary for *in vitro* PE synthesis except PSS as a control showed no appearance of the native epitope 4B1 (Figure 5, lane

Fig. 6. Post-translational reconstitution of *in vitro* assembled LacY with *in vitro* synthesized PE. LacY was inserted co-translationally into PE-containing (+PE) or PE-deficient (-PE) IOVs as described in Figure 3. After 60 min, the reaction mixtures were supplemented as indicated (see Figure 5) and phospholipid synthesis was continued for 60 min. Samples were analyzed using mAb4B1.

5). Finally the use of a PSS-like fraction (PSS*) lacking PS synthase (Figure 5, lane 6) or the absence of added serine and CTP (Figure 5, lane 7 versus 8) resulted in no LacY detectable by mAb4B1. These results confirm the direct dependence on PE biosynthesis and the absence of PE-containing membranes in the PSS fraction. When mAb4B1 was stripped from the nitrocellulose sheet and the sheet probed again with mAb4B1, the same amount of LacY was detected in all cases (data not shown), indicating that PE is not a determinant for the yield of insertion. Therefore, when *in vitro* synthesized LacY was inserted into PE-containing IOVs or assembled in PE-deficient membranes during co-synthesis *in vitro* of PS and PE (but not PG and CL), the permease could be recognized by mAb4B1, indicating the proper folding of domain P7.

PE corrects post-translationally the misfolding of LacY assembled either *in vitro* or *in vivo* without PE

In vitro protein synthesis was carried out in the presence of IOVs either with (Figure 6, lanes 1–2) or without (lanes 3–4) PE until membranes were saturated with newly made LacY before *in vitro* PE synthesis was initiated. mAb4B1 recognized monomeric LacY assembled in IOVs containing PE (lane 1) but not in those lacking PE (lanes 3–4). Introduction of PE by *in vitro* synthesis had no effect on the level of mAb4B1 recognition of LacY assembled in IOVs containing PE (lane 2), but had a dramatic effect on recognition of LacY from IOVs originally lacking PE (lanes 5 and 6). Similar effects of *in vitro* PE synthesis on the formation of native epitope 4B1 were observed using LacY assembled initially *in vivo* in either PE-containing (Figure 7, lanes 1 and 2 versus lanes 3 and 4, no effect) or PE-deficient (Figure 7, lanes 5 and 6 versus 7 and 8, large effect) membranes. When mAb4B1 was stripped from the sheet and the sheet probed again with mAb4B1, the same amount of LacY was detected in all lanes in Figures 6 and 7 (data not shown), indicating that membrane insertion was not dependent on PE.

Therefore, PE can bring about the proper folding of LacY first assembled either *in vivo* or *in vitro* in membranes lacking PE even when added via cell-free biosynthesis after assembly into the membrane bilayer. These results suggest that PE can promote the transition of fully integ-

Fig. 7. Reconstitution of *in vivo* assembled LacY with *in vitro* synthesized PE. IOVs in which LacY had been expressed *in vivo* were isolated from PE-containing (+PE) and PE-deficient (-PE) cells. The indicated supplements were added as described in Figure 6 for *in vitro* phospholipid biosynthesis, and samples were analyzed using mAb4B1; duplicate samples are shown.

rated LacY during a late step in assembly from a state approximating its final tertiary structure to the native state.

PE acts as molecular chaperone

In order to classify a biological molecule as a molecular chaperone, the molecule must assist protein folding through a transient non-covalent interaction and not be required to maintain the final native structure. The possibility that residual PE is bound to LacY from PE-containing cells after Western blotting analysis, and thereby is essential to maintain the conformation of epitope 4B1, was re-investigated in a more quantitative manner than previously reported (Bogdanov *et al.*, 1996). Strain AD93/pDD72/pT7-5 induced by IPTG for LacY expression was labeled by growth in the presence of $^{32}\text{P}_i$ (10 $\mu\text{Ci/ml}$) in minimal media. Total membranes were prepared, 25 μg of membrane protein were subjected to SDS-PAGE and the protein was transferred to nitrocellulose sheets by electroblotting. Beta imaging (90 min) using an Instant Imager[®] and autoradiography (12 h) of the nitrocellulose sheet detected no radioactivity associated either with the position of LacY or anywhere on the nitrocellulose sheet.

Phospholipids were extracted from the cells subjected to Western blot analysis, and serial dilutions were separated by thin-layer chromatography (TLC) and subjected to autoradiography and beta imaging for the same times as indicated above to determine the minimum radioactivity detectable with these methods. Beta imaging and autoradiography were easily able to detect 10 c.p.m. (as determined by direct scintillation counting of lipids eluted from plates) of [^{32}P]PE in 90 min and 12 h, respectively (data not shown). The specific activity of PE (based on chemical analysis of phosphate present) was 25 000 c.p.m./nmol. These same membranes (5–25 μg of protein) were again subjected to SDS-PAGE and the protein transferred to nitrocellulose sheets by electroblotting. In the same experiment, known amounts of purified LacY were treated in a similar manner over a range similar to the amount present in the membrane samples. These blots were subjected to Western blot analysis using mAb4A10R that recognizes the C-terminus of LacY independently of structure (Carrasco *et al.*, 1982). Treatment with [^{35}S]protein A was used to quantify the amount of LacY in the membrane samples relative to the known amounts of pure LacY (Bogdanov and Dowhan, 1995). Both

Fig. 8. Effect of PE on yield of epitope 4B1. LacY from PE-containing (+PE) or PE-deficient (–PE) cells was subjected to SDS–PAGE followed by Eastern–Western blotting either without PE (lanes 1–3 and 7–9) or with PE (lanes 4–6 and 10–12). In each set of three samples, 3, 6 and 12 μ g of the total membrane protein were subjected to SDS–PAGE and analyzed using either mAb4B1 or mAb4B11 as indicated.

homogeneous LacY and the LacY in the membrane samples showed a linear relationship between amount of sample analyzed and radiolabeled protein A bound. Based on this analysis, 5% of the membrane protein was LacY, which is consistent with estimates of 3–5% previously reported (Newman *et al.*, 1981) (H.R.Kaback, personal communication). Thus the 25 μ g of membrane protein subjected to SDS–PAGE and Western blot analysis contained ~ 0.03 nmol of LacY which, if associated with [32 P]PE in a 1:1 molar ratio, should have contained minimally 750 c.p.m. Since we would have detected 10 c.p.m. of [32 P]PE easily, there is considerably less than one molecule of PE per 75 LacY molecules after Western blot analysis.

To rule out the possibility that we are only able to detect the small percentage of LacY molecules that have residual PE bound, we carried out the following experiment based on the following assumptions. First, we have established clearly that exposure of LacY lacking epitope 4B1 to PE during electroblotting (Eastern–Western procedure) dramatically increases the signal observed with mAb4B1. Secondly, we have demonstrated that mAb4B11, which recognizes a discontinuous epitope defined by cytoplasmic domains C8 and C10 (Figure 1), recognizes the monomeric membrane-integrated form of LacY with the same efficiency whether or not assembly occurred in the presence of PE (Figure 2). If we were only able to detect a small population of LacY containing epitope 4B1 due to trace amounts of PE, then subjecting LacY from PE-containing cells to the Eastern–Western procedure in the presence of PE should amplify dramatically the signal produced by mAb4B1. However, such treatment, if anything, reduced the signal by 50% (Figure 8A, lanes 1–3 versus 4–6), suggesting that the reconstitution of LacY from cells

lacking PE has been underestimated. The same result was obtained using mAb4B11 (lanes 7–9 versus 10–12), indicating that the same proportion of LacY is detected irrespective of the lipids that may be present. In a related approach, the same amount of membranes from PE-containing (Figure 8B, lanes 1–3 and 7–9) and PE-deficient (Figure 8B, lanes 4–6 and 10–12) cells, known to contain the same amount of LacY (Bogdanov and Dowhan, 1995), were subject to Western blotting analysis except that the LacY from PE-deficient cells was supplemented with PE during electroblotting. The signal with both antibodies for the PE-supplemented PE-deficient sample was about half that of the PE-containing sample, indicating that the amount of LacY, reconstituted by PE is about the same as the total amount of LacY and that reconstituted LacY and LacY from PE-containing cells give the same signal.

Taken together, these results strongly indicate that LacY originally assembled in membranes containing PE either maintains the conformation of epitope 4B1 or contains sufficient conformational memory to reform epitope 4B1 independently of residual PE. Therefore, PE fulfills the minimum requirements to be classified as a molecular chaperone, at least with respect to the folding of epitope 4B1.

Discussion

A cell-free translation–insertion assay was developed to study assembly of a polytopic membrane protein into a native, correctly folded and integrated conformation dependent on individual membrane phospholipids. The method took advantage of the availability of conformation-specific mAbs that recognize native-like epitopes of LacY. Using this system, we demonstrated that translation of LacY in the presence of PE-containing IOVs results in the appearance of an integrated and properly folded LacY at least in the region encompassing TM domains VII–XI. Up to 20% of *in vitro* synthesized LacY was assembled into IOVs, which is comparable with the yield seen with *in vitro* protein translocation (Muller and Blobel, 1984; Chen *et al.*, 1985) and *in vitro* membrane protein integration (Moore *et al.*, 1988; Ahrem *et al.*, 1989; van Klompenburg *et al.*, 1997) experiments. However, in all of the previously reported *in vitro* assays for membrane protein integration, there were no indicators of whether native structure was achieved since only yield of insertion could be tested. A particularly novel aspect of the work presented here was the ability to analyze factors necessary to attain a native conformation of a domain of a membrane protein assembled *in vitro*.

The *in vitro* phospholipid biosynthesis system employed here accurately reflects the conditions of an intact inner membrane of *E.coli* (Sparrow *et al.*, 1984) where phospholipid biosynthesis occurs on the cytoplasmic side of the membrane (Raetz and Dowhan, 1990). Employing a similar cell-free biosynthetic approach (Huijbregts *et al.*, 1998), 35–65% of newly synthesized PE was translocated with a half-time of <1 min from the cytoplasmic to periplasmic leaflet of IOVs originally devoid of PE. Therefore, this mode of delivery of PE to membranes makes the lipid available to all areas of the membrane. Such an approach should be widely applicable to studies of the possible involvement of phospholipids in pre-

protein translocation across the membrane, transmembrane signaling, etc.

Probing with mAb4B11, which is specific for membrane-associated LacY independent of membrane phospholipid composition, indicated that a comparable amount of LacY was assembled with or without PE both *in vitro* into IOVs and *in vivo* into membranes. These results strongly suggest that PE is not required for integration of LacY into the membrane and are also consistent with our previous results where the membrane content of LacY expressed *in vivo* was the same in both PE-containing and PE-deficient cells (Bogdanov and Dowhan, 1995). LacY assembled *in vitro* into IOVs lacking PE does not contain lipid-dependent epitope 4B1, but this epitope does form with either co- or post-translational biosynthesis of new PE (but not PG and CL) in IOVs originally lacking PE. Therefore, PE appears to be required after membrane insertion to direct the attainment of native conformation. In the *in vitro* phospholipid biosynthetic system, both PE and PS are present, while *in vivo* the levels of PS are extremely low, which supports PE as the physiologically important molecule. However, we have shown that PS mimics the effects of PE in reconstituting epitope 4B1 using the Eastern–Western blotting procedure (data not shown). This result is consistent with the observation that either PE or PS must be present during reconstitution of LacY into liposomes in order to obtain mediated active transport of lactose involving net translocation of protons (Chen and Wilson, 1984; Page *et al.*, 1988). In the presence of only anionic phospholipids or only PC, reconstitution of facilitated downhill diffusion but not active transport was observed (Page *et al.*, 1988).

Biochemical data support an *in vivo* co-translational mode for LacY assembly into the cytoplasmic membrane (Stochaj and Ehring, 1987). We were unable to detect post-translational membrane insertion of LacY, which could be explained by the fact that TM segments have a very strong tendency to aggregate and require the presence of molecular chaperones for post-translational integration (Bochkareva *et al.*, 1996). *In vitro* synthesis of PE in IOVs added post-translationally also had no stimulatory effect on post-translational insertion of LacY (M.Bogdanov and W.Dowhan, unpublished data), which further supports the misfolding of LacY when translated in the absence of membranes. High overproduction of LacY *in vivo* in the presence of PE also results in a significant portion of the protein remaining in soluble species which, once formed, cannot integrate into the membrane (Roepe and Kaback, 1989).

In wild-type *E.coli*, assembly of LacY occurs into membranes containing an abundance of PE so that a separation between membrane insertion and phospholipid-assisted folding cannot be observed. By using mutants lacking PE coupled with the ability to add PE both co- and post-translationally to LacY integrated into membranes, we have been able to observe an intermediate along the folding pathway which requires interaction with PE in order to effect a high yield of the properly folded protein. Although failure to insert into the membrane appears to be irreversible, the intermediates that we have trapped within the membrane in the absence of PE can still be rescued by the post-translational addition of PE. Therefore, these intermediates must closely resemble the final native

conformation of LacY; this conclusion is also supported by the fact that LacY assembled in membranes lacking PE is still functional in facilitated diffusion of substrate (Bogdanov and Dowhan, 1995). What remain unanswered are the details and stoichiometry of the interaction of PE with LacY and possibly with other membrane proteins of *E.coli*. LacY may form a PE-binding site as a folding intermediate during or after membrane insertion, and the occupancy of this site transiently by PE may stabilize a folding intermediate in a 'near native' conformation, thus either preventing misfolding or directing proper folding. This process would be analogous to the evolving concept of molecular chaperones that can prevent aggregation (Georgopoulos and Welch, 1993), can sterically direct proper folding (Peralta *et al.*, 1994; Ellis, 1998) and can be non-protein in nature (Ellis, 1997).

The fact that PE is required for phospholipid-assisted folding of LacY both *in vivo* and *in vitro* is now well documented (Bogdanov and Dowhan, 1995; Bogdanov *et al.*, 1996). What has been questioned is whether PE can be classified as a true molecular chaperone (Ellis, 1997), i.e. can the conformation of epitope 4B1 be maintained after removal of PE? This question is distinct from whether PE is required for function of LacY or even whether in the presence of PE the lipid associates with LacY through a specific site. Although demonstration of the complete lack of PE is difficult, results reported here indicate that no significant amount of PE is associated with LacY after Western blot analysis. Based on these results, we conclude that PE is required during phospholipid-assisted folding of LacY but is not required to maintain the final conformation of epitope 4B1, which fulfills the minimum requirements of a chaperone. Our results cannot rule out that PE *per se* is required either for maintenance of proper structures of domains other than 4B1 or is involved directly in net turnover of the permease required for energy-dependent active transport of substrate. Native LacY reconstituted into PG/CL liposomes carries out facilitated but not active transport (Chen and Wilson, 1984; Page *et al.*, 1988), suggesting additional requirements for PE beyond the formation of epitope 4B1.

In conclusion, we have demonstrated the *in vitro* synthesis of a polytopic membrane protein followed by its insertion and assembly into native-like organization at least with respect to several of its domains. Membrane insertion for LacY and early steps of its conformational maturation appear to be independent of membrane phospholipid composition. However, late steps in the final organization of the protein into a fully functional transporter involve a specific phospholipid-assisted folding process analogous to assisted folding carried out by protein chaperones. Recent reports show a growing body of evidence that specific lipids assist in the folding and conformational maturation of proteins. Characterization of PG-dependent conformational changes of *E.coli* DegP protein leading to protection of unfolded protein against self-aggregation at relatively low temperatures indicates that phospholipids could bind to certain key areas of unfolded proteins, keep critical interactive domains apart, and prevent misfolding (Skorko-Glonek *et al.*, 1997). This phenomenon was not observed in the presence of PE or CL, demonstrating that folding of DegP was PG specific. Similarly, the glycolipid portion of lipopolysaccharide

appears to facilitate the conformational maturation of outer membrane proteins (De Cock and Tommassen, 1996) as they pass through the inner membrane but not as they assemble into the outer membrane. With increasing awareness of the principle of specific lipid-assisted folding of proteins will come even more examples.

Materials and methods

Materials

[γ - 32 P]ATP, [35 S]protein A and peroxidase-labeled antibody enhanced chemiluminescence detection kit (ECL) came from Amersham Corp. T7 RNA polymerase, ribonuclease inhibitor (RNasin), ribonucleotides and complete amino acid mixtures were purchased from Promega. Spermidine-HCl, ATP, GTP, creatine phosphate, phosphoenolpyruvate (PEP) and PEP kinase came from Sigma. NADH and dCTP were purchased from Boehringer Mannheim, and *sn*-glycerol 3-phosphate was purchased from Serva. Nitrocellulose sheets for immunoblotting were purchased from Schleicher and Schuell. Purified LacY and antibodies directed against a C-terminal dodecapeptide of LacY (pAb), the C-terminus of LacY (mAb4A10R), a continuous epitope formed by periplasmic domain P7 (mAb4B1) and a discontinuous epitope consisting of cytosolic domains C8 and C10 (mAb4B11) were generously provided by Dr H.R. Kaback (University of California, Los Angeles).

Bacterial strains and plasmids

Strains (AD93 and AD905) carrying the *psa93::kan* null allele (PE-deficient) require either a functional plasmid-borne copy of the *psaA* gene (encodes PS synthase on plasmid pDD72 which is temperature sensitive for replication) or growth media containing 20–50 mM MgCl₂ for viability (DeChavigny *et al.*, 1991). Strains wild-type for phospholipid metabolism carried the *psa93::kan* null allele and plasmid pDD72 (PE-containing). Plasmid pT7-5 (Bibi and Kaback, 1990) carries the intact *lacY* gene under control of both *lacOP* and the T7 RNA polymerase promoter [p(T7)], and was used for *in vitro* transcription/translation experiments and for *in vivo* overexpression. Plasmid pGP1-2 containing the T7 RNA polymerase gene was used in conjunction with the above *lacY* plasmid to maximize LacY overproduction *in vivo* in strain JF618 (*E. coli* Genetic Stock Center); due to a defect in cytidine synthase, growth medium was supplemented with 50 μ g/ml cytidine.

Growth of bacteria

Cells were grown in LB (liquid broth) rich medium and supplemented with ampicillin (100 μ g/ml) or kanamycin (40 μ g/ml) for maintenance of plasmid pT7-5 or pGP1-2, respectively. Since replication of plasmid pDD72 is temperature sensitive and mutants lacking PE require MgCl₂ for viability, all strains (mutant and wild-type for phospholipid composition) were grown at 30°C in 50 mM MgCl₂. Overexpression of the *lacY* gene on plasmid pT7-5 from the *lacOP* promoter was carried out in mutant and wild-type strains grown exponentially in the presence of 1 mM IPTG. Overexpression from the T7 promoter in strain JF618 harboring plasmids pGP1-2 and pT7-5 was carried out by induction of the T7 RNA polymerase/promoter system with a temperature shift to 42°C for 25 min followed by addition of rifampicin (100 μ g/ml) and continued incubation at 42°C for an additional 10 min (Tabor and Richardson, 1985). The culture was incubated at 37°C for 1 h before harvesting.

Immunodetection of proteins

In vivo and *in vitro* synthesized LacY was analyzed by SDS-PAGE and immunoblotting with pAb and mAb followed by ECL fluorescence detection. Samples were suspended in SDS gel sample buffer, heated for 15 min at 37°C, centrifuged to remove any insoluble material, subjected to SDS-PAGE (12.5% gel), electroblotted to nitrocellulose sheets and immunodetected as described previously (Bogdanov and Dowhan, 1995; Bogdanov *et al.*, 1996). Stripping and sequential reprobing of nitrocellulose sheets with antibodies was performed according to the suggestions of manufacturer of the ECL kit. Phospholipids were applied to nitrocellulose sheets (Eastern blot) prior to electroblotting of proteins as previously described (Bogdanov *et al.*, 1996).

In vitro transcription, translation and membrane insertion of LacY

In vitro transcription from *lacY* placed downstream of a T7 promoter in plasmid pT7-5 was carried out at 37°C in a 10 μ l transcription mix

containing 20 mM triethanolamine (TEA) acetate buffer (pH 7.5), 10 mM Mg(acetate)₂, 30 mM NH₄ acetate, 0.1 mM potassium EDTA, 0.8 mM spermidine-HCl, 2 mM dithiothreitol (DTT), 10 U of T7 RNA polymerase, 1.5 μ g of plasmid DNA, 1 U of RNasin and 2.5 mM rNTPs. After 1 h, the mix was placed on ice to stop the reaction and used for translation as described below. In order to remove CTP during subsequent translation of mRNA, the reaction was stopped by the addition of 40 μ l of 9 M LiCl to precipitate and isolate mRNA as previously described (Govezensky *et al.*, 1994). All experiments using *in vitro* transcribed mRNA were done using both methods of stopping the reaction, with identical results. Unless otherwise indicated, the experiments reported here used the former method of halting transcription.

A membrane- and PS synthase-free S30 fraction of a cell homogenate was prepared from *E. coli* strain AD905 (*psa93::kan degP*) grown in LB containing 0.2% of glucose as described (Lesley *et al.*, 1991). Cells were lysed using a French press and membranes removed by centrifugation (Bochkareva *et al.*, 1996) of 0.3 ml of the lysate layered over 4.7 ml of 22% sucrose at 4°C for 110 min at 45 000 r.p.m. (SW50.1 rotor, Beckman L5-65 centrifuge). The samples (top 3.5 ml) were then concentrated to 10 mg of protein per ml using either a Centrprep 10 (Amicon) or an Ultrafree 15 (PBGC 10,000 NMWL, Millipore) centrifuge filter device. The resulting S30 lysate was incubated for 80 min at 37°C with 0.1 vol. of pre-incubation mix containing 0.75 M Tris acetate (pH 8.2), 7.5 mM DTT, 21.3 mM Mg(acetate)₂, 75 μ M of each amino acid, 6 mM ATP, 14 mg/ml of PEP and 16 mg/ml of PEP kinase and then dialyzed versus three changes of buffer (Lesley *et al.*, 1991).

Synthesis of LacY was carried out in a 30 μ l translational mixture containing the above 10 μ l of *lacY* transcription mix, 50 μ g of protein from a membrane- and PS synthase-free S30 crude extract (3 μ l), 0.1 mM final concentration each of 20 amino acids, 10 mM TEA acetate buffer (pH 7.5), 0.1 mM potassium EDTA, 15 mM NH₄ acetate, 5 mM Mg(acetate)₂, 0.4 mM spermidine-HCl, 1 mM DTT, 3 mM ATP, 0.625 mM GTP, 10 mM creatine phosphate, 10 mM PEP, 60 μ g/ml PEP kinase, 0.1 mg/ml *E. coli* tRNA, 1 mM NADH and 20 mg/ml polyethylene glycol (6000/8000 *M_w*). Competency of the translation system was determined by inclusion of 30 μ Ci of [35 S]Met in place of cold Met and measuring incorporation of [35 S]Met after precipitation of 2 μ l samples on 3MM Whatman filter disks using hot 10% trichloroacetic acid (TCA) containing 0.1% methionine.

IOVs were prepared from PE-containing and PE-deficient cells as described previously (Bogdanov and Dowhan, 1995) using a French press to break cells at 8000 p.s.i. in buffer with a final concentration of 20 mM Mg²⁺. IOVs were added to a final concentration of 10 μ g per 30 μ l of translation mix as indicated. The reaction was stopped by the addition of SDS sample buffer.

In vitro phospholipid biosynthesis

Membrane vesicles with elevated PA levels have been used successfully for *in vitro* assays of late steps in phospholipid biosynthesis (Sparrow *et al.*, 1984). To assay the utilization of PA and formation of individual phospholipids in IOVs from strain AD903, the PA was pre-labeled by direct transfer of terminal phosphate from [γ - 32 P]ATP (Weissbach *et al.*, 1971) followed by incubation with 2.5 mM each of CTP and dCTP, 1 mM L-serine, 5 mM NADH, and a PS synthase-enriched fraction (PSS, 10 μ g of crude protein) isolated (see below) from PS synthase-overproducing strain JA200/pPS3155- λ (Ohta *et al.*, 1981). Alternatively, 1 mM *sn*-glycerol 3-phosphate was added to the translation mixture to initiate biosynthesis of PG/CL. At various times, aliquots were removed and the radioactive lipid product was extracted and separated on boric acid-impregnated silica TLC plates as described previously (Fine and Sprecher, 1982), using chloroform/methanol/water/concentrated ammonium hydroxide (120/75/6/2) and then quantified using an Instant Imager[®] (Packard Instrument Company).

PSS was prepared by breakage of cells with a French pressure cell followed by isolation of a supernatant fraction by centrifugation at 36 000 *g_{max}* for 1 h. Membranes were removed first by centrifugation through 22% sucrose as described above for preparation of the S30 fraction; the resulting fraction was then subjected to a second centrifugation after addition of EDTA to 10 mM and KCl to 0.25 M. A mock PSS* fraction was prepared in an identical manner from strain AD93 lacking PS synthase.

To initiate phospholipid synthesis in the translation experiments, pre-labeling of IOVs with [γ - 32 P]ATP was omitted. *In vitro* protein synthesis was initiated and IOVs were added 5 min later unless otherwise indicated. *In vitro* phospholipid synthesis was initiated by the indicated additions (including NADH) either at the same time as IOVs were added or 1 h

after addition of IOVs as indicated. The reaction was stopped after 1 h by addition of SDS sample buffer.

Miscellaneous procedures

Protein was determined by the BCA assay according to the manufacturer's suggestions. Labeling of phospholipids *in vivo* with $^{32}\text{PO}_4$ was carried out as described previously (DeChavigny *et al.*, 1991). Lipid phosphorus was determined as described previously (Chalvardjian and Rudnicki, 1970). The TLC plates or dried nitrocellulose sheets were scanned for radiolabel and quantified using an Instant Imager[®] (Packard Instrument Company), or radiolabeled areas of plates were scraped and counted using liquid scintillation.

Acknowledgements

We are most grateful to Professor H.R.Kaback (UCLA) for providing us with purified LacY and antibodies specific for LacY without which this work would not have been possible. This work was supported in part by NIH grant GM20478.

References

- Ahrem, B., Hoffschulte, H.K. and Muller, M. (1989) *In vitro* membrane assembly of a polytopic, transmembrane protein results in an enzymatically active conformation. *J. Cell Biol.*, **108**, 1637–1646.
- Bibi, E. and Kaback, H.R. (1990) *In vivo* expression of the lacY gene in two segments leads to functional lac permease. *Proc. Natl Acad. Sci. USA*, **87**, 4325–4329.
- Bochkareva, E., Seluanov, A., Bibi, E. and Girshovich, A. (1996) Chaperonin-promoted post-translational membrane insertion of a multispanning membrane protein lactose permease. *J. Biol. Chem.*, **271**, 22256–22261.
- Bogdanov, M. and Dowhan, W. (1995) Phosphatidylethanolamine is required for *in vivo* function of the membrane associated lactose permease of *Escherichia coli*. *J. Biol. Chem.*, **270**, 732–739.
- Bogdanov, M., Sun, J., Kaback, H.R. and Dowhan, W. (1996) A phospholipid acts as a molecular chaperone in assembly of a membrane transport protein. *J. Biol. Chem.*, **271**, 11615–11618.
- Carrasco, N., Tahara, S.M., Patel, L., Goldkorn, T. and Kaback, H.R. (1982) Preparation, characterization and properties of monoclonal antibodies against the lac carrier protein from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **79**, 6894–6898.
- Chalvardjian, A. and Rudnicki, E. (1970) Determination of lipid phosphorus in the nanomolar range. *Anal. Biochem.*, **36**, 225–226.
- Chen, C.-C. and Wilson, T.H. (1984) The phospholipid requirement for activity of the lactose carrier of *Escherichia coli*. *J. Biol. Chem.*, **259**, 10150–10158.
- Chen, L., Rhoads, D. and Tai, P.C. (1985) Alkaline phosphatase and OmpA protein can be translocated posttranslationally into membrane vesicles of *Escherichia coli*. *J. Bacteriol.*, **161**, 973–980.
- De Cock, H. and Tommassen, J. (1996) Lipopolysaccharides and divalent cations are involved in the formation of an assembly-competent intermediate of outer membrane protein PhoE of *E.coli*. *EMBO J.*, **15**, 5567–5593.
- DeChavigny, A., Heacock, P.N. and Dowhan, W. (1991) Sequence and inactivation of the *pss* gene of *Escherichia coli*: phosphatidylethanolamine may not be essential for cell viability. *J. Biol. Chem.*, **266**, 5323–5332.
- Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity: why are there so many lipids? *Annu. Rev. Biochem.*, **66**, 199–232.
- Duntun, R.L., Sahin-Toth, M. and Kaback, H.R. (1993) Role of the charge pair aspartic acid-237–lysine-358 in the lactose permease of *Escherichia coli*. *Biochemistry*, **32**, 3139–3145.
- Dutt, A. and Dowhan, W. (1977) Intracellular distribution of enzymes of phospholipid metabolism in several gram-negative bacteria. *J. Bacteriol.*, **132**, 159–165.
- Ellis, R.J. (1997) Do molecular chaperones have to be proteins? *Biochem. Biophys Res. Comm.*, **238**, 687–692.
- Ellis, R.J. (1998) Steric chaperones. *Trends Biochem. Sci.*, **266**, 43–45.
- Ellis, R.J. and Hartl, F.-U. (1996) Protein folding in the cell: competing models of chaperonin function. *FASEB J.*, **10**, 20–26.
- Fedorov, A.N. and Baldwin, T.O. (1997) Cotranslational protein folding. *J. Biol. Chem.*, **272**, 32715–32718.
- Fedorov, A.N., Friguet, B., Djavadi-Ohanian, L., Alakhov, Y.B. and Goldberg, M.E. (1992) Folding on the ribosome of *Escherichia coli* tryptophan synthase b subunit nascent chains probed with conformation-dependent monoclonal antibody. *J. Mol. Biol.*, **228**, 351–358.
- Fine, J.B. and Sprecher, H. (1982) Unidimensional thin-layer chromatography of phospholipids on boric acid-impregnated plates. *J. Lipid. Res.*, **23**, 660–663.
- Frillingos, S. and Kaback, H.R. (1996) Monoclonal antibody 4B1 alters the pKa of a carboxylic acid at position 325 (helix 10) of the lactose permease of *Escherichia coli*. *Biochemistry*, **35**, 10166–10171.
- Georgopoulos, C. and Welch, W.J. (1993) Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell. Biol.*, **9**, 601–634.
- Goldberg, M.E. (1991) Investigating protein conformation, dynamics and folding with monoclonal antibodies. *Trends Biochem. Sci.*, **16**, 358–362.
- Govezensky, D., Bochkareva, E.S., Zamir, A. and Girshovich, A.S. (1994) Chaperones as potential gene regulatory factors. *J. Biol. Chem.*, **269**, 14003–14006.
- Hartl, F.-U. (1996) Molecular chaperones in cellular protein folding. *Nature*, **381**, 571–580.
- Hendrick, J.P. and Hartl, F.-U. (1993) Molecular chaperone functions of heat shock proteins. *Annu. Rev. Biochem.*, **62**, 349–384.
- Hendrick, J.P. and Hartl, F.-U. (1995) The role of molecular chaperones in protein folding. *FASEB J.*, **19**, 1559–1569.
- Herzlinger, D., Carrasco, N. and Kaback, H.R. (1985) Functional and immunochemical characterization of a mutant of *Escherichia coli* energy uncoupled for lactose transport. *Biochemistry*, **24**, 221–229.
- Huijbregts, R.P.H., de Kroon, A.I.P.M. and de Kruijff, B. (1998) Rapid transmembrane movement of newly synthesized phosphatidylethanolamine across the inner membrane of *Escherichia coli*. *J. Biol. Chem.*, **273**, 18936–18942.
- In't Veld, G., Driessen, A.J.M. and Konings, W.N. (1993) Bacterial solute proteins in their lipid environment. *FEMS Microbiol. Rev.*, **12**, 293–314.
- Kaback, H.R. (1986) Active transport in *Escherichia coli*: passage to permease. *Annu. Rev. Biophys. Chem.*, **15**, 279–319.
- Kaback, H.R., Voss, J. and Wu, J. (1997) Helix packing in polytopic membrane proteins: the lactose permease of *Escherichia coli*. *Curr. Opin. Struct. Biol.*, **7**, 537–542.
- Lesley, S.A., Brow, M.A.D. and Burgess, R.R. (1991) Use of *in vitro* protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies. *J. Biol. Chem.*, **266**, 2632–2638.
- Louie, K., Chen, Y.-C. and Dowhan, W. (1986) Substrate-induced membrane association of phosphatidylserine synthase from *Escherichia coli*. *J. Bacteriol.*, **165**, 805–812.
- Marger, M.D. and Saier, M.H.J. (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.*, **18**, 13–20.
- Marsh, D. (1993) The nature of the lipid–protein interface and the influence of protein structure on protein–lipid interactions. In Watts, A. (ed.), *New Comprehensive Biochemistry: Protein–Lipid Interactions*, Elsevier, Amsterdam, Vol. 25, pp. 41–66.
- Moore, K.E., Dalbey, R.E. and Wickner, W. (1988) *In vitro* insertion of leader peptidase into *Escherichia coli* membrane vesicles. *J. Bacteriol.*, **170**, 4395–4398.
- Muller, M. and Blobel, G. (1984) *In vitro* translocation of bacterial proteins across the plasma membrane of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **81**, 7421–7425.
- Newman, M.J., Foster, D.L., Wilson, T.H. and Kaback, H.R. (1981) Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J. Biol. Chem.*, **256**, 11804–11808.
- Ohta, A., Waggoner, K., Louie, K. and Dowhan, W. (1981) Cloning of genes involved in membrane lipid synthesis. Effect of amplification of phosphatidylserine synthase in *Escherichia coli*. *J. Biol. Chem.*, **256**, 2219–2225.
- Page, M.G., Rosenbusch, J.P. and Yamato, I. (1988) The effects of pH on proton sugar symport activity of the lactose permease purified from *Escherichia coli*. *J. Biol. Chem.*, **263**, 15897–15905.
- Peralta, D.D., Hartman, D.J., Hoogenraad, N.J. and Hoj, P.B. (1994) Generation of a stable folding intermediate which can be rescued by the chaperonins GroEL and GroES. *FEBS Lett.*, **339**, 45–49.
- Raetz, C.R. and Dowhan, W. (1990) Biosynthesis and function of phospholipids in *Escherichia coli*. *J. Biol. Chem.*, **265**, 1235–1238.
- Roepe, P.D. and Kaback, H.R. (1989) Characterization and functional reconstitution of a soluble form of the hydrophobic membrane protein lac permease from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **86**, 6087–6091.

- Roepe,P.D., Zbar,R.I., Sarkar,H.K. and Kaback,H.R. (1989) A five-residue sequence near the carboxyl terminus of the polytopic membrane protein lac permease is required for stability within the membrane. *Proc. Natl Acad. Sci. USA*, **86**, 3992–3996.
- Selinsky,B.S. (1992) Protein–lipid interactions and membrane function. In Yeagle,P. (ed.), *The Structure of Biological Membranes*. CRC Press, Boca Raton, FL, pp. 603–651.
- Skorko-Glonek,J., Lipinska,B., Krzewski,K., Zolese,G., Bertoli,E. and Tanfani,F. (1997) HtrA heat shock protease interacts with phospholipid membrane and undergoes conformational changes. *J. Biol. Chem.*, **272**, 8974–8982.
- Sparrow,C.P., Ganong,B.G. and Raetz,C.R.H. (1984) *Escherichia coli* membrane vesicles with elevated phosphatidic acid levels. A detergent-free system for *in vitro* phospholipid synthesis. *Biochim. Biophys. Acta*, **796**, 373–383.
- Stochaj,U. and Ehring,R. (1987) The N-terminal region of *Escherichia coli* lactose permease mediates membrane contact of the nascent polypeptide chain. *Eur. J. Biochem.*, **163**, 653–658.
- Sun,J., Wu,J., Carrasco,N. and Kaback,H.R. (1996) Identification of the epitope for monoclonal antibody 4B1 which uncouples lactose and proton translocation in the lactose permease of *Escherichia coli*. *Biochemistry*, **35**, 990–998.
- Sun,J., Li,J., Carrasco,N. and Kaback,H.R. (1997a) The last two cytoplasmic loops in the lactose permease of *Escherichia coli* comprise a discontinuous epitope for monoclonal antibody. *Biochemistry*, **36**, 274–280.
- Sun,J., Frillingos,S. and Kaback,H.R. (1997b) Binding of monoclonal antibody 4B1 to homologs of the lactose permease of *Escherichia coli*. *Protein Sci.*, **6**, 1503–1510.
- Tabor,S. and Richardson,C.C. (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl Acad. Sci. USA*, **82**, 1074–1078.
- van Klompenburg,W., Ridder,A.N.J.A., van Raalte,A.L.J., Killian,A.J., von Hejine,G. and de Kruijff,B. (1997) *In vitro* membrane integration of leader peptidase depends on the Sec machinery and anionic phospholipids and can occur post-translationally. *FEBS Lett.*, **413**, 109–114.
- Varela,M.F. and Wilson,T.H. (1996) Molecular biology of the lactose carrier of *Escherichia coli*. *Biochim. Biophys. Acta*, **1276**, 21–34.
- Weissbach,H., Thomas,E. and Kaback,H.R. (1971) Studies on the metabolism of ATP by isolated bacterial membranes: formation and metabolism of membrane-bound phosphatidic acid. *Arch. Biochem. Biophys.*, **147**, 249–254.
- Zardeneta,G. and Horowitz,P.M. (1992) Micelle-assisted protein folding. Denaturated rhodanese binding to cardiolipin-containing lauryl maltoside micelles results in slower refolding kinetics but greater enzyme reactivation. *J. Biol. Chem.*, **267**, 5811–5816.

Received May 5, 1998; revised and accepted July 16, 1998