Phospholipid-assisted Refolding of an Integral Membrane Protein

MINIMUM STRUCTURAL FEATURES FOR PHOSPHATIDYLETHANOLAMINE TO ACT AS A MOLECULAR CHAPERONE*

(Received for publication, July 31, 1998, and in revised form, February 4, 1999)

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Escherichia coli-derived phosphatidylethanolamine (PE) or PE with fully saturated fatty acids was able to correct in vitro a defect in folding in the lipid-dependent epitope 4B1 of lactose permease (LacY) resulting from in vivo assembly in the absence of PE. PE plasmalogens, PE with two unsaturated fatty acids, and lyso-PE, which all do not favor bilayer organization, did not support proper refolding. Proper refolding occurred when these latter lipids were mixed with a bilayer-forming lipid (phosphatidylglycerol), which alone could not support refolding. L-Phosphatidylserine (PS; natural diastereomer) did support proper refolding. PE derivatives of increasing degrees of methylation were progressively less effective in supporting refolding, with phosphatidylcholine being completely ineffective. Therefore, the properties of nonmethylated aminophospholipids capable of organization into a bilayer configuration are essential for the recovery of the native state of epitope 4B1 after misassembly in vivo in the absence of PE. Neither D-PS (sn-glycero-1-phosphate backbone) nor P-n-S (n-serine in the head group) is competent in supporting proper refolding unless used in binary mixtures with phosphatidylglycerol. The detailed characterization of phospholipid-assisted refolding reported here further supports a specific rather than nonspecific role for PE in structural maturation of lactose permease in vivo (Bogdanov, M., and Dowhan, W. (1998) EMBO J. 17, 5255–5264).

How a polytopic membrane protein inserts into the membrane and adopts its native conformation remains one of the major unresolved questions of biology. During membrane assembly, integral membrane proteins must interact with other proteins and with the lipid bilayer itself, resulting in their proper conformational maturation. The role phospholipids play in forming a membrane bilayer and a hydrophobic environment for membrane protein folding and assembly is well accepted (1, 2); however, the role specific phospholipids play in assisting or directing folding of membrane proteins has not been extensively explored. In order to determine the role that individual phospholipids play in the folding of membrane proteins in

Escherichia coli, we are using the lactose permease (LacY) as a model system. LacY is one of the most intensively studied integral membrane proteins for which there is both extensive structural information (3, 4) and monoclonal antibodies (mAbs) directed against several extramembrane epitopes whose recognition depends on secondary or tertiary structural organization (5, 6). The organization of extramembrane and transmembrane domains of LacY is characteristic of the major facilitator superfamily of transport proteins (7), making results obtained from studies on LacY applicable to a variety of other transport proteins.

Employing a novel blotting technique (Eastern-Western) in which proteins are exposed to phospholipids bound to a solid support during renaturation from SDS in the standard Western blotting procedure, we previously presented the following experimental evidence (8, 9) that the phospholipid phosphatidylethanolamine (PE) acts as a non-protein molecular chaperone in the folding of LacY: (i) LacY appears to fold in vivo with the assistance of PE as assessed by recognition by a conformation-sensitive mAb directed against periplasmic loop P7 (Phe245–Gly254), which is flanked by transmembrane domains VII and VIII and defines epitope 4B1 (Fig. 1); (ii) LacY assembled initially in vivo in the presence of PE either retains native-like conformation of this epitope throughout or has sufficient “conformational memory” to reform this epitope after SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis in the absence of added lipid; (iii) after SDS-PAGE and Western blotting, no phospholipid can be detected associated with LacY (less than one molecule of PE per 75 LacY monomers); (iv) LacY initially assembled in vivo in the absence of PE can form the native-like conformation of epitope 4B1 during refolding from SDS in the presence of specifically PE but not phosphatidylglycerol, CL, or phosphatidylethanolamine (PE, PG, CL, and PC, respectively); (iv) extensive denaturation of LacY using urea-SDS eliminates epitope 4B1 in LacY originally assembled in PE-containing membranes and prevents its refolding in the presence of PE; (v) the requirement for PE in the folding of LacY has been demonstrated in an in situ assembly system using in vitro protein synthesis coupled with in vitro phospholipid synthesis in the presence of membranes initially lacking PE; (vi) PE is not required either prior to or concomitant with membrane insertion but is required in a late step of conformational maturation to attain native structure. Thus, PE corrects

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* This work was supported in part by National Institutes of Health Grant GM20478 (to W. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: LacY, lactose permease; PAGE, polyacrylamide gel electrophoresis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; PMME, monomethyl derivative of PE; PDME, dimethyl derivative of PE; mAb, monoclonal antibody; H2, inverted hexagonal II; CL, cardiolipin; D-PS, PS with sn-glycero-1-phosphate backbone; P-n-S, PS with n-serine in the head group.
in vitro a LacY folding defect caused by either in vivo or in vitro assembly in PE-deficient membranes, but once this epitope is formed in vivo, PE is not required to maintain its conformation. Based on these results, we proposed as a general principle that phospholipids can act as molecular chaperones of non-protein origin that specifically mediate the folding of multispansing polypitic membrane proteins, thereby extending the definition of chaperones to other biomolecules in addition to proteins. What are the minimum requirements of a phospholipid to facilitate membrane protein folding? In this study, we establish a specific requirement for a nonmethylated aminophospholipid of natural chirality and preference for lamellar organization for a specific requirement for a nonmethylated aminophospholipid. Based on these results, we proposed as a general principle that PE (sn-glycerol-3-phosphate isomer) of PS and is not inhibited by the n-isomer (13). A solution of 1.4 mM rac-(C16:0)PS and 8.6 mM Triton X-100 in 0.5 mL of 0.1 M potassium phosphate (pH 7.0) was supplemented with sufficient crude phosphatidylecerine decarboxylase (0.2 units) to convert all of the n-PS to PE as judged by pilot experiments using l-(C16:0)PS as nonmethylated. Phosphatidylethanolamine (PE) was further purified with chloroform/methanol/water (1:2:0.5), and PE was separated from d-(C16:0)PS by silica gel thin layer chromatography as described below.

Membrane preparation and SDS-PAGE—Inside-out membrane vesicles, the source of membrane protein in all experiments, were prepared as described previously (14), using a French press to break cells at 8,000 psi in the absence of 200 mM MgCl₂. Protein was determined by the BCA assay according to the manufacturer’s suggestions. Inside-out membrane vesicles were adjusted to the concentration of the SDS gel-loading buffer of 2.8% SDS, 10% glycerol, 100 mM dithiothreitol and heated at 37 °C for 15 min and centrifuged to remove any insoluble material prior to being subjected to SDS-PAGE in 12.5% polyacrylamide as described previously (8).

**Experimental Procedures**

**Materials**—For natural occurring phospholipids, no fatty acid composition is indicated, although the acyl chains are generally 16 carbons or longer and fully saturated at the 1-ω-position and unsaturated at the 2-ω-position. For all synthetic diacyl-phospholipids, the indicated fatty acids are the same in both positions of the glycerol backbone unless otherwise indicated, and the stereochemical configuration of the glycerophosphate backbone and the head groups are the natural configuration unless otherwise indicated. Unless otherwise noted, unsaturated fatty acids are of the natural cis configuration. Monounsaturated fatty acids are Δ9, Δ12, and diunsaturated fatty acids are Δ9, Δ12. E. coli PE, bovine heart CL, bovine brain phosphatidylserine (PS), egg yolk PC, egg yolk lyso-PE, and 1-oleoyl-sn-glycerol-3-lysophosphoethanolamine (lyso-(C18:1)PE) were supplied by Sigma. E. coli PG was purchased from Matreya, Inc., and the N-methyl (C18:1)PME) and N,N-di- (C18:1) PDME derivates of PE came from Avanti Polar Lipids, Inc. rac-(C16:0)PS (rac referring to the configuration of glycerophosphate in the backbone) and (C16:0)PS were purchased from Sigma. (C12:0)PE, (C18:0)PE, (C18:1)PE, and (C18:2)PE were purchased from Avanti Polar Lipids; both PG derivatives were rac with respect to the glycerol head group. Cyclic antibiotic Ro90–0198 isolated from Streptococcus that specifically interacts with PE has been described elsewhere (10). PE plasmalogens and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-d-serine (P-S) were kindly provided by Drs. F. Palka (Technische Universit鋐t, Graz, Austria) and R. Pidcock (Ontario, Canada), respectively. Antibodies directed against a continuous epitope formed by periplasmic domain P7 (mAb 4B1) and a discontinuous epitope consisting of cytosolic domains C8 and C10 (mAb 4B1) were generously provided by Dr. H. R. Kaback (UCLA). Nitrocellulose sheets (pore size 0.45 μm) for immunoblotting were purchased from Schleicher and Schuell. Immobilon-P (polyvinylidene difluoride) sheets were obtained from Millipore Corp. Bovine serum albumin (BSA) gel thin layer chromatography plates were purchased from EM Science and Merck. The ECL kit was obtained from Amersham Pharmacia Biotech.

**Bacterial Strains, Plasmids, and Growth Conditions**—Strains carrying the pssY3:kan null allele cannot synthesize PE and require either growth medium containing 50 mM MgCl₂ for viability or a functional plasmid-borne copy of the pssA gene (plasmid pPD72, temperature-sensitive for replication) to restore wild type phospholipid composition (11). Plasmid pPT5–5 (12) carries the intact lacY gene under control of both lacOP and the T7 RNA polymerase promoter (pT7) and was used for in vivo high level expression of LacY. Cells were grown in LB-rich medium, and ampicillin (100 μg/mL) was included in the growth medium for maintenance of plasmid pT7–5. Since replication of plasmid pPD72 is temperature-sensitive and mutants lacking PE require MgCl₂ for viability, all strains (mutant and wild type) were grown at 30 °C in 50 mM MgCl₂. Overexpression of the lacY gene on plasmid pPT5–5 directed by the lacOP promoter was carried out in mutant and wild type strains grown exponentially in the presence of 1 mM isopropl β-thio-galactosidase. Strain JA200/pPSD2b carrying a copy of the pssA gene (encodes phosphatidylecerine decarboxylase) and a tetR marker on a multicopy plasmid was grown in LB medium containing 25 μg/ml tetracycline (13) and was used to prepare membranes enriched in phosphatidylecerine decarboxylase.

**Enzymatic Preparation of Δ-(C16:0)PS—E. coli strain JA200/pPSD2b was used to prepare a Triton X-100 membrane extract containing amplified levels of phosphatidylecerine decarboxylase as described previously (13). Phosphatidyleserine decarboxylase is specific for the n-isomer (sn-glycerol-3-phosphate isomer) of PS and is not inhibited by the n-isomer (13). A solution of 1.4 mM rac-(C16:0)PS and 8.6 mM Triton X-100 in 0.5 mL of 0.1 M potassium phosphate (pH 7.0) was supplemented with sufficient crude phosphatidylecerine decarboxylase (0.2 units) to convert all of the n-PS to PE as judged by pilot experiments using l-(C16:0)PS as nonmethylated. Phosphatidylethanolamine (PE) was further purified with chloroform/methanol/water (1:2:0.5), and PE was separated from d-(C16:0)PS by silica gel thin layer chromatography as described below.
Periplasmic or cytoplasmic side of the inner membrane of the transmembrane domains joined by hydrophilic domains on the membrane insertion of LacY (9).

Formation of this epitope is dependent on a continuous, PE-independent epitope (4B11) formed by the last two cytoplasmic loops, C8 and C10 (6, 9). Formation of this epitope is dependent on membrane insertion of LacY (9).

mAb 4B11 has been shown (6) to recognize a conformation-sensitive, discontinuous, PE-dependent epitope (4B11) formed by the last two cytoplasmic loops, C8 and C10 (6, 9). Formation of this epitope is dependent on membrane insertion of LacY (9).

Phospholipids with fully saturated fatty acids normally adopt a bilayer (lamellar) organization irrespective of hydration, pH, ionic strength, or divalent cation nature and concentration (17). Among unsaturated fatty acid-containing phospholipids, only PE adopts the non-bilayer inverted hexagonal II phase (HII) at physiological temperatures, pH values, and salt concentrations, while divalent cations induce the HII phase for CL. PE's with combined long chain and more extensively unsaturated fatty acids such as PE plasmalogen adopt the HII phase almost exclusively. Lyso phospholipids generally adopt a micellar organization. Phospholipids that adopt the bilayer phase exclusively can stabilize HII-preferring lipids (i.e., unsaturated PE) in an overall bilayer organization in mixed binary systems (18). The proportions of bilayer lipid required to achieve this can vary substantially (usually > 20%), with 30 mol % of PG mixed with (C18:1)PE being lamellar in its physical state. To distinguish between the potential contributions of individual phospholipid structure and the physical state of the lipid, we further investigated whether this difference between saturated and unsaturated phospholipid in supporting refolding might be explained by different phase-promoting properties of these lipids.

Consistent with the physical state of PE being an important determinant for renaturation, those forms of PE that can readily assume a bilayer or lamellar structure (19) at room temperature (Fig. 2 and Table I, saturated fatty acids) supported renaturation, while those derivatives that tend to form micellar structures or the non-bilayer HII arrangement did not support renaturation. However, HII-forming lipids in binary mixtures with a lamellar or bilayer-forming lipid such as PG supported renaturation of LacY irrespective of the fatty acid composition of PG. The same was true for lyso-PE, but a higher proportion of PG was required probably to convert micellar to lamellar organization. PE plasmalogen, which also favors the HII phase over the lamellar phase, required a higher mol % of PG to be effective. There may be some advantage of saturated fatty acids over unsaturated fatty acids, since binary mixtures with (C18:1)PE were not as effective as with (C16:0)PG in supporting renaturation by (C18:1) or (C18:2)PE. Although (18:1Δ9trans)PE by itself was not as effective as (C18:0)PE, it did support significant refolding, especially when compared with (18:1Δ9cis)PE, which was only effective in the presence of PG. The basis for the reduced effectiveness of
shown in Fig. 3 (out facilitated but not active transport of substrate). Also which is not present in PS, LacY only carries out facilitated diffusion of substrates. PC, presence of PC lacks full function (29, 30) (expected finding may explain why native LacY reconstituted in the presence of PC resulted in loss of epitope 4B1 (Fig. 3, lanes 4–6). Therefore, PC appears to act as an antagonist in the maintenance of native LacY structure. This unexpected finding may explain why native LacY reconstituted in the presence of PC lacks full function (29, 30) (i.e. only carries out facilitated but not active transport of substrate). Also shown in Fig. 3 (lanes 1–3) is an increase in the apparent yield of epitope 4B1 when LacY originally assembled in the presence of PE is subjected to Eastern-Western blotting in the presence of E. coli-derived PG/CL (in a molar ratio of 20:1; lanes 1–3), or with egg yolk PC (lanes 7–9). In each set of three samples, 3, 6, and 12 µg of total membrane protein was subjected to SDS-PAGE and analyzed using mAb 4B1.

### TABLE I

| PE derivative<sup>a</sup> | Supplement | Relative 4B1 intensity | Phase<sup>b</sup> | Reference |
|--------------------------|------------|------------------------|------------------|-----------|
| E. coli<sup>c</sup>      |            | ++++                   | L                | 19        |
| 12:0                     |            | ++                     | L                | 20        |
| 12:0                     | 50% (16:0)/PG | ++++                  | L                | 22        |
| 12:0                     | 80% (16:0)/PG | ++++                  | L                | 21        |
| 18:0                     |            | ++++                   | L                | 22        |
| 18:0                     | 50% (16:0)/PG | ++++                  | L                | 18        |
| 18:0                     | 80% (16:0)/PG | ++                    | L                | 18        |
| 18:0 (trans)             |            | +++                    | L                | 24        |
| (16:0/18:0 or 18:1)Lyso<sup>d</sup> |           |                        |                  |           |
| (16:0/18:0 or 18:1)Lyso<sup>d</sup> | 50% (16:0)/PG | –                      | M                | 25        |
| (16:0/18:0 or 18:1)Lyso<sup>d</sup> | 85% (16:0)/PG | ++++                  | L                | 26        |
| Plasmalogen              |            | –                      | H<sub>R</sub>    | 27        |
| Plasmalogen              |            | –                      | H<sub>R</sub>    | 28        |
| (18:1)PDME               |            | +++                    | L                | 21        |
| (18:0)PC                 |            | –                      | L                |           |
| (18:0)PC                 | 50% (16:0)/PG | –                      | L                |           |
| PC<sup>c</sup>           | 85% (16:0)/PG | –                      | L                |           |

<sup>a</sup> Indicates both fatty acids unless otherwise noted.

<sup>b</sup> L, lamellar; M, micellar; H<sub>R</sub>, inverted hexagonal II. A question mark indicates uncertainty.

<sup>c</sup> Composition primarily 1-<i>sn</i> 16:0 and 2-<i>sn</i> 18:1.

<sup>d</sup> Either egg-derived (mixture of indicated fatty acids) or synthetic.

<sup>e</sup> Soy bean PC (1-<i>sn</i>, 16:0/18:0 or 18:1)Lyso<sub>d</sub> trans.

<sup>f</sup> Defining the Head Group Specificity of PE—Consistent with the above requirement (29, 30) of either PE or PS for reconstitution of full LacY function <i>in vitro</i>, refolding of LacY (assembled in membranes lacking PE) in the presence of bovine brain PS led to recovery of epitope 4B1 (Table II and Fig. 4B, lanes 3 and 4). Therefore, PS is as effective as PE in reconstituting epitope 4B1. These results are also consistent with the lack of active transport mediated by LacY in cells lacking PE (14) but with the ability to actively transport lactose in mutants in which 95% of the PE has been replaced by PS (31). The importance of the amino group of PE for the proper refolding of LacY was further tested by shielding the phosphatidylethanolamine head group of PE by the peptide antibiotic Ro09-0198, which accommodates the head group of PE specifically and does not interact with other phospholipids including PS (10). No refolding of LacY was observed when solid support was preblotted with PE treated with Ro09-0198 (Fig. 4A, lanes 1–3 versus lanes 7–9). In a control experiment, the specificity of Ro09-0198 for PE and its lack of interference with antibody detection were demonstrated by the lack of effect of the anti-phosphatidylethanolamine monoclonal antibody 4B1, which is specific for the head group.

**Fig. 3. Negative effect of PC on epitope 4B1.** LacY from PE-containing cells was subjected to Eastern-Western blotting without phospholipid (lanes 4–6), with E. coli-derived PG/CL (in a molar ratio of 20:1; lanes 1–3), or with egg yolk PC (lanes 7–9). In each set of three samples, 3, 6, and 12 µg of total membrane protein was subjected to SDS-PAGE and analyzed using mAb 4B1.
bioric on PS-assisted refolding of LacY (Fig. 4B, lanes 5 and 6) and on the detection of LacY assembled in PE-containing cells (data not shown). Stripping of mAb 4B1 as described under “Experimental Procedures” and reprobing with mAb 4B1 verified the presence of ample amounts of LacY in all lanes (data not shown). mAb 4B1 recognizes a discontinuous epitope defined by cytoplasmic domains C8 and C10 (Fig. 1) with the same efficiency whether or not assembly occurred in the presence of PE (9).

**Effectiveness of PS Analogues on Refolding**—The ability of PS analogues to promote proper refolding of LacY was investigated (Table II). Bovine brain PS and L-(16:0)PS were able to support refolding in binary mixtures with PG although PS normally adopts a lamellar organization irrespective of fatty acid composition (17). The unnatural diastereomers P-D-S and D-PS were also not able to support renaturation unless in binary mixtures with PG (Fig. 5); rac-PS did support renaturation but with about 50% of the efficiency when compared with the natural diastereomer. Although we were not able to investigate the dependence on fatty acid composition extensively, the lack of effectiveness of P-D-S appears not to be due to its fatty acid composition, since it is similar to that of bovine brain PS, which does support refolding. These differences between diastereomers are quite surprising as is the difference between the saturated and unsaturated derivations of PS. These results may be due to subtle differences in the physical properties between diastereomers and between PSs with different fatty acid compositions as discussed below.

### Table II

**Refolding of LacY with PS derivatives**

| Lipid          | Supplement | Relative 4B1 intensity |
|----------------|------------|------------------------|
| PS bovine brain | + + + +     |                        |
| (18:1)PS       | -          |                        |
| (18:1)PS       | + + + +     |                        |
| P-D-S          | + + + +     |                        |
| L-(16:0)PS     | + + + +     |                        |
| rac-(16:0)PS   | -          |                        |
| D-(16:0)PS     | 80% (16:0)PG | + + + +                |
| D-(16:0)PS     | 80% (16:0)PG | + + + +                |

**DISCUSSION**

The aim of the present study was to explore in more detail the specific properties of the head group, acyl chains, and physical state of phospholipids that support proper refolding of LacY. Although we do not understand the physical and chemical properties of the lipids and proteins once bound to a solid support in the Western blotting procedure, several clear relationships from our studies can be made between the requirements to be effective in refolding of LacY and the physical and chemical properties of solution phospholipids that support refolding: (i) a positively charged head group preferably with a small or ionizable amine; (ii) a fatty acid composition either singly or in binary mixtures that favors an overall bilayer or lamellar organization in solution; and (iii) a distinct preference for naturally occurring diastereomers of the amanolipid in single component systems or the presence of lamellar forming phospholipids of natural chirality in binary mixtures with an unnatural diastereomer of the amanolipid. These relationships clearly extend our previous conclusions supporting a distinct biological specificity for PE in phospholipid-assisted folding of LacY both in vivo and in vitro that goes well beyond simply providing a nonspecific detergent-like or two-phase environment for refolding of LacY.

Since the requirement for naturally occurring PE in the refolding of LacY had been established both in vivo and in vitro, the finding that unsaturated PE as opposed to fully saturated PE cannot facilitate refolding was surprising. This finding raised the question of whether the phase properties of phospholipids were a factor in the refolding process. The refolding effectiveness of the various PE derivatives singly or in binary mixtures with PG is consistent with the importance of the overall macrostructure of the lipid phase and strongly suggests that refolding of LacY is dependent on an overall lamellar macrostructure.

Several reports have documented a preference or specificity for a particular physical organization of phospholipids. Protein translocation across the *E. coli* inner membrane lacking PE could be restored by (C18:1)PE (non-lamellar) but not (C14:0)PE or (C18:1)PC (both lamellar) (32). (18:1Δ9cis)PE (non-lamellar) but not (18:1Δ9trans)PE (lamellar) significantly enhanced the respiratory control ratio of ubiquinol-cytochrome c reductase and ATP-induced membrane potential of the H+-ATPase (33). The photochemical function of rhodopsin (34) and the ATPase activity of *E. coli* SecA protein (35) are enhanced by non-lamellar PE derivatives. However, mammalian Ca2+-ATPase activity (35) is stimulated significantly more by lamellar phase- than HII phase-forming derivatives of PE. Finally, lipopolysaccharide derivatives that tend to organize in nonbilayer structures were better facilitators of folding of PhoE protein *in situ* as it transits the inner membrane of *E. coli* (36).
Phosphatidylethanolamine as a Molecular Chaperone

PS, which is only found in trace amounts in wild type E. coli, may present a more complex special case. Unlike PE, PS contains two chiral centers, one at the 2-sn-position and the other in the serine moiety. Consequently P-PS and L-PS are diastereomers and not enantiomers much like the glycerol-based glycolipids that have multiple chiral centers in the glycerol and sugar moieties. L-(C18:1)PS does not support full function in PC-containing lipid vesicles. Unlike PE, PS contains no stereoselectivity or strict chemical specificity (37, 38). It was suggested that such structural differences might affect packing interactions and alter the effective area individual lipids occupy in respective single component bilayers of these diastereomers (39) that is not apparent in binary mixtures. Finally, the natural 1,2-sn-diastereomer of the glycolipid monoglycosyl diodecylglycerol has a higher propensity to form the lamellar phase than the unnatural 2,3-diastereomer (40) that adopts the HII phase. Moreover, the 1,2-rac mixture of this glycolipid displayed phase properties very similar to the natural diastereomer, indicating that mixtures of diastereomers are not simply additive in their final structural properties. All of these studies including our results support as biologically important the physical properties of the phospholipid macrostructure as well as the chemical properties of individual phospholipid species in biological processes.

The failure of PC to assist proper refolding of LacY assembled in the absence of PE is not surprising, since PC cannot support active transport of LacY in an in vitro reconstituted system (29, 30). However, the fact that PC actively denatures proteins that come in contact with membranes during their assembly. Heightened awareness of lipid-assisted folding of proteins with PE-plasmalogen.

Acknowledgments—We are most grateful to Dr. H. R. Kaback for providing LacY-specific antibodies, without which this work would not have been possible. We also thank Dr. F. Paltauf for suggesting the experiments with PE plasmalogen.

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