A Phospholipid Acts as a Chaperone in Assembly of a Membrane Transport Protein*

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A mutant of Escherichia coli lacking phosphatidyethanolamine (PE) and a monoclonal antibody (mAb 4B1) directed against a conformationally sensitive epitope (4B1) of lactose permease were used to establish a novel role for a phospholipid in the assembly of a membrane protein. Epitope 4B1 is readily detectable in spheroplasts and right-side-out membrane vesicles from PE-containing but not from PE-deficient cells expressing lactose permease. Lactose permease from PE-containing membranes, but not from PE-deficient membranes, subject to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot analysis is recognized by mAb 4B1. If total E. coli phospholipids or PE (but not phosphatidyicholine, phosphatidylglycerol, or cardiolipin) are blotted on nitrocellulose sheets (Eastern blot) prior to transfer of proteins from SDS-polyacrylamide gels, the permease from PE-deficient cells regains its recognition by MAb 4B1. Therefore, PE is required during assembly to form epitope 4B1, but, once formed, sufficient “conformational memory” is retained in the permease to either retain or reform this epitope in the absence of PE. Lactose permease lacking epitope 4B1 can be induced to form the epitope if partially denatured and then reanimated in the presence of PE specifically. These results establish for the first time a role for PE as a molecular chaperone in the assembly of the lactose permease.

Only a limited number of reports address the role of the native phospholipid environment in the function and assembly of membrane proteins. Clearly, the amphipathic environment of the membrane is an important determinant in the folding and maintenance of membrane protein structure. However, what has not been widely considered is a role for individual phospholipids in determining the folding pathway for membrane proteins independent from the maintenance of the final structure, i.e. to act in the capacity of a non-protein molecular chaperone.

Mutants of Escherichia coli are available in which membrane phospholipid composition can be varied in a way that is difficult to achieve in vitro (DeChavigny et al., 1991; Dowhan, 1992). Such “phospholipid mutants” were used to study the in vivo role of PE in lactose permease (lacY gene product) function (Bogdanov and Dowhan, 1995). The lactose permease of E. coli is an extensively studied prototype of most secondary transport systems found in both prokaryotic and eukaryotic organisms (Kaback et al., 1994). PE is not required for energy-independent downhill translocation of substrate mediated by lactose permease, but appears to be essential for H⁺-coupled active lactose accumulation in vivo (Bogdanov and Dowhan, 1995). These results parallel the earlier observation (Chen and Wilson, 1984; Page et al., 1988) with purified permease reconstituted into proteoliposomes where PE was also found to be required specifically for active transport.

How phospholipids affect membrane protein assembly and function is still largely unknown. Do the properties of the target membrane phospholipids affect assembly and function? Do individual phospholipids act as conformational determinants? In the present work, we address the possibility that membrane phospholipid composition, and specifically PE, determines the conformation of the lactose permease. The experiments described here combine structural and functional information on the lactose permease with the use of a conformationally sensitive monoclonal antibody (Sun et al., 1996) directed against an epitope in the periplasmic loop (L(VII/VIII)) of the permease between helices VII and VIII (Fig. 1). Monoclonal antibodies have been used effectively to detect folding intermediates of soluble proteins in solution (Fedorov et al., 1992), but have not been used extensively to follow folding of membrane proteins as described in this report. The proper assembly of lactose permease, as assessed by the presence of the conformational epitope, into the membranes of an E. coli mutant lacking PE (DeChavigny et al., 1991) was compared to assembly in wild type cells to establish a requirement for PE. We also introduce a novel adaption of the Western blot procedure termed an “Eastern-Western” in which phospholipids are applied to the surface of nitrocellulose sheets (Taki et al., 1994) prior to transfer of proteins from SDS-polyacrylamide gels by electroblotting. This combination of protein and ligand on a solid support allows the monitoring by a conformation-specific monoclonal antibody of the involvement of a specific phospholipid in the refolding of a denatured protein. The information gathered from these approaches indicate that PE plays an important role in facilitating the proper assembly of the lactose permease into the membrane and thereby demonstrates for the first time that a phospholipid can act as a molecular chaperone.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—E. coli strain AD93 (pss93::kan, PE-deficient) cannot make either PE or phosphatidylserine except when carrying plasmid pDD72 (pss−, PE-containing) (DeChavigny et al., 1993). All strains (mutant and wild type for PE formation) were grown at 30°C in LB medium containing 50 mM MgCl₂ and, except as noted, carried multiple copies of the lacY gene on plasmid pT7-5/ lacY1.

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The abbreviations used are: PE, phosphatidylethanolamine; IPTG, isopropyl-1-thio-β-D-galactoside; RSO, right-side-out; ISO, inside-out; DTT, dithiothreitol; mAb, monoclonal antibody; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; PIPES, 1,4-piperazineethanesulfonic acid.

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RESULTS AND DISCUSSION

The binding of mAb 4B1 to RSO membrane vesicles and spheroplasts was utilized to determine whether structural differences exist between lactose permease assembled in either PE-deficient or PE-containing membranes. As shown in Fig. 2A, binding of mAb 4B1 to spheroplasts and RSO membrane vesicles from PE-deficient cells is reduced to the level observed for binding to control membranes lacking the permease. High level binding to permease in membranes from PE-containing cells was observed as reported previously (Carrasco et al., 1982, 1984; Herzlinger et al., 1984; Sun et al., 1996). The amount of permease in each membrane preparation was established using Western blot analysis with a site-directed pAb against the C terminus of the permease using either the ECL chemiluminescence-based method (Fig. 2B) or radiolabeled protein A (Fig. 2C) for qualitative or quantitative measurements, respectively. The specific content of permease in spheroplasts (500 and 600 cpm/mg of total membrane protein) and RSO membrane vesicles (6000 and 7000 cpm/mg of membrane protein) was comparable in samples prepared from PE-containing and PE-deficient cells, respectively. Therefore, the marked difference in mAb 4B1 binding to membranes is due specifically to differences in recognition of the epitope. The results are consistent with the hypothesis that membrane phospholipid composition is a determinant for the conformation of lactose permease.

Membrane preparations from PE-containing and PE-deficient cells were then analyzed by Western blot analysis, comparing the sensitivity of SDS-denatured permease to detection with mAb 4B1 and pAb (Fig. 3A). As reported previously (Carrasco et al., 1982), mAb 4B1 binds to permease from PE-deficient cells after being transferred to a solid support. Permease from PE-deficient cells exhibits greatly reduced binding of mAb 4B1, although probing with pAb verified the presence of ample amounts of permease (Fig. 3B). Therefore, permease assembled in PE-containing membranes retains sufficient “conformational memory” to reform the native structure of the epitope after removal of SDS. The results confirm the absence of the conformational epitope in PE-deficient membranes and also suggest that once the information is imparted to form the epitope, it cannot be removed. Transfer of proteins from gels to nitrocellulose sheets (Schleicher & Schuell, pore size of 0.45 μm) using a semidyed electrophoretic system (Milliprep-SDE electrophoretic apparatus) and detection by antibody were carried out as described previously (Bogdanov and Dowhan, 1995) except as follows. The gel was incubated for 10 min at room temperature in cathode buffer 1 (electrophoretic system) prior to transfer. Bound antibody (mAb 4B1 or pAb) was visualized by either the ECL detection system (Amersham with supplied peroxidase-labeled anti-mouse or anti-rabbit antibody, respectively) and exposure to x-ray film or 35S-protein A and analysis with a Betagene Imager as described previously (Bogdanov and Dowhan, 1995).

In the Eastern-Western blot procedure, bands of phospholipids (0.05–0.10 mg) on silica gel thin layer plates were developed using chloroform/methanol/water/30% ammonium hydroxide (120:75:6:2, v/v/v); for total E. coli phospholipids, no chromatography was performed. Plates were dipped in isopropyl alcohol/0.2% aqueous CaCl₂/methanol (40:20:7, v/v/v) for 20 s. A nitrocellulose sheet was placed on top of the silica gel surface followed by a glass microfiber sheet (Whatman, United Kingdom). Transfer of phospholipid was accomplished by pressing on the stack with a metallic heating block at 130 °C for 10 s (modification of Taki et al., 1994)). Western blot transfer (phospholipid side facing the acrylamide gel) of proteins was accomplished as described above.
The surprising finding is that lactose permease from PE-deficient membranes subjected to Eastern-Western blotting with either PE or E. coli phospholipids regains the ability to bind mAb 4B1. In marked contrast, neither the E. coli anionic phospholipids PG and CL nor PC, which is also zwitterionic but not present in E. coli, promote the formation of the epitope. This specificity for PE is consistent with the earlier results of Chen and Wilson (1984) who showed that only PE and not the other three phospholipids listed above could restore active transport to lactose permease reconstituted in proteoliposomes. Therefore, restoration of mAb 4B1 binding specifically by PE indicates that PE plays a positive role as a structural determinant, rather than acting to exclude a negative determinant in the form of anionic phospholipids.

Subjecting membrane preparations to SDS-PAGE in the presence of 5 M urea eliminates detection by mAb 4B1 in both Western and Eastern-Western blots with permease from both PE-containing and PE-deficient cells (data not shown). Lactose permease retains about 70% of its helical structure in SDS as estimated from circular dichroism measurements,2 and recent evidence indicates that denaturants such as SDS induce alterations in helical packing within the permease with little or no change in secondary structure (Jung et al., 1994). However, urea disrupts the secondary structure of proteins by stabilizing the random coil state (Schein, 1990). In the case of the permease, urea appears to eliminate structural elements that either carry conformational memory after assembly in vivo or have potential to form the 4B1 epitope. The possibility that residual PE is bound to permease from PE-containing cells was eliminated by labeling membrane phospholipids by growth in the presence of 32P, followed by SDS-PAGE. Autoradiography of the gel revealed no difference in the labeling pattern between samples from PE-containing cells with or without lactose permease. However, bands in

2 D. L. Foster, M. Boublik, and H. R. Kaback, unpublished data.
Lactose permease in PE-deficient membranes is unable to reassociate PE or wild type liposomes (12.5 mg of total membrane protein plus 50 mg of membranes are solubilized in the presence of PE-containing liposomes. Therefore, the 4B1 epitope once formed is maintained in the presence of PE rather than simply exposure of the denatured protein to PE.

The epitope recognized by mAb 4B1 (see Fig. 1) consists of Phe-247, Phe-250, and Gly-254 on one face of a short α-helical segment in the periplasmic loop between helices VII and VIII (Sun et al., 1996). Treatment of RSO membrane vesicles with mAb 4B1 inhibits all reactions catalyzed by the permease that involve net H+ translocation with no effect on ligand binding and either equilibrium exchange or counterflow of substrate (Carrasco et al., 1982, 1984). The “uncoupling” effect of mAb 4B1 is remarkably similar to the effects of site-directed mutations in Glu-325 (helix X) (Carrasco et al., 1986, 1989), as well as the functional alterations observed in vivo with mutants devoid of PE (Bogdanov and Dowhan, 1995) and in reconstituted proteoliposomes lacking PE (Page et al., 1988; Seto-Young et al., 1985). In this respect, it is particularly interesting that helix VIII contains Glu-269, a residue that is essential for transport and interacts with another essential residue, His-322 (helix X), while helix VII contains Asp-237 and Asp-240 which interact with Lys-358 (helix XI) and Lys-319 (helix X), respectively (Kaback et al., 1994). Since helices VII and VIII interact with helix X which contains Glu-325 and mAb 4B1 mimics the effect of mutations at this position, torsional effects on either of these helices may alter the pKa of one or more residues in helices VII and/or VIII in such a manner as to increase the pKa of Glu-325, thereby limiting deprotonation of the periplasm at the surface of the membrane (Carrasco et al., 1986, 1989; Sun et al., 1996). Bogdanov and Dowhan (1995) postulated that lactose permease in PE-deficient membranes is unable to release a H+ on the inner surface of the membrane. Binding of mAb 4B1 also markedly alters the reactivity of a site-specific Cys replacement of Val-331 (helix X), as well as the fluorescence of this mutant after labeling the Cys with 2-(4'-maleimidobenzyl)maleimide (Sun et al., 1996). Taken together, the data are consistent with a role for PE in either the folding of helices VII and/or VIII their packing within the bilayer. The observation that sufficient conformational memory remains in the permease after removal of PE and renaturation on a solid support to reform the 4B1 epitope indicates that PE does not act per se to maintain the conformation within L(VII/VIII). Rather, PE plays a role in the folding of either the epitope itself or helices VII and VIII which may determine the conformation of the epitope. Further support for this comes from the ability of PE to direct the folding of the epitope during renaturation from SDS of permease originally assembled in the absence of PE. Therefore, PE functions as do protein molecular chaperones in guiding the folding pathway which is a new and novel role for a phospholipid.

What are the special properties of PE which may be related to its specific role in determining protein conformation? PE, unlike PC, has a strong tendency to form nonbilayer structures (Thurmond et al., 1991), and, with its small head group, PE may interact at the interface between phospholipid and protein domains or intercalate within the packed helical structure of membrane proteins; helix VIII contains a region of low information content that has been postulated to be in contact with phospholipids (Hinkle et al., 1990). PE (as well as PC) also exhibits a dipole moment over the ionic head group which can respond to membrane potential (Seelig, 1993). Positive charges have been postulated to interact with π electrons of aromatic side chains of amino acids (Dougherty, 1996). Not only are two Phe residues part of the determinant recognized by mAb 4B1, but the sequence in the region of L(VII/VIII), which is conserved among several other secondary transporters (Frilings et al., 1994), contains 6 Phe residues. Therefore, head group size and electrical properties of PE coupled with the ability to assume nonbilayer structures may combine to influence the final helix structure and packing of the permease and ultimately its function.

In conclusion, the use of monoclonal antibodies in an Eastern-Western blot analysis of other membrane proteins may reveal additional examples of phospholipid-dependent organization of protein structure, especially for those proteins that require the addition of lipids to regain function. Direct assay of enzyme activity after transferring proteins to a solid support in an Eastern-Western blot may prove to be a useful screening technique to survey the role of specific lipids in protein function. The method described here could have broad application for the study of renaturation of proteins from SDS in the presence of either surface-bound ligands or ligands introduced into the electroblootting buffers. Certainly the realization that specific phospholipids can determine the folding pathway of a protein should be taken into account in designing experiments aimed at understanding the mechanism for assembling proteins into membranes.

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Other regions of the gel exhibited significant radioactivity, indicating that the 32P was of sufficiently high specific activity to detect low levels of protein-associated radioactivity such as tightly bound PE. Therefore, the 4B1 epitope once formed is maintained even in the absence of PE. Finally, when PE-deficient membranes are solubilized in the presence of PE-containing liposomes (12.5 mg of total membrane protein plus 50 mg of either PE or wild type E. coli phospholipid), no restoration of mAb 4B1 binding is observed (data not shown), indicating that formation of the epitope is dependent on removal of SDS in the presence of PE rather than simply exposure of the denatured protein to PE.

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