

# Phosphatidylethanolamine mediates insertion of the catalytic domain of leader peptidase in membranes

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**Abstract** Leader peptidase is an integral membrane protein of *E. coli* and it catalyses the removal of most signal peptides from translocated precursor proteins. In this study it is shown that when the transmembrane anchors are removed *in vivo*, the remaining catalytic domain can bind to inner and outer membranes of *E. coli*. Furthermore, the purified catalytic domain binds to inner membrane vesicles and vesicles composed of purified inner membrane lipids with comparable efficiency. It is shown that the interaction is caused by penetration of a part of the catalytic domain between the lipids. Penetration is mediated by phosphatidylethanolamine, the most abundant lipid in *E. coli*, and does not seem to depend on electrostatic interactions. A hydrophobic segment around the catalytically important residue serine 90 is required for the interaction with membranes.

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**Key words:** Leader peptidase; Membrane protein; Protein-lipid interaction; Protein secretion; Phosphatidylethanolamine; Insertion

## 1. Introduction

For recognition by the export machinery, proteins are usually synthesized as precursors with amino terminal extensions called leader or signal sequences. Signal sequences contain a hydrophobic core region which is preceded by an amino terminal positively charged domain [1]. After translocation signal sequences are removed by the action of leader (signal) peptidases which cleave 5–7 residues downstream of the hydrophobic core of the signal sequence [1]. In *E. coli*, signal sequences are removed by Lep (also called leader peptidase or signal peptidase I) which is encoded by the *LepB* gene [2]. It requires a turn inducing residue (glycine or proline) at position –6 and small residues, preferably alanine, at positions –3 and –1 with respect to the cleavage site [3,4].

Lep is comprised of an amino terminal part containing two transmembrane helices (H1 and H2) separated by a highly positively charged cytosolic loop (P1), and a large, carboxy-

terminal periplasmic domain (P2) (Fig. 1A). Site-directed mutagenesis studies have suggested that the proteolytic mechanism involves a serine at position 90 and a lysine at 145, and that all residues required for cleavage are located in the P2 domain [5,6]. Serine 90 is positioned in a mildly hydrophobic segment (H3).

The substrates for Lep are membrane bound. There is compelling evidence that during translocation the amino terminus of the signal peptide stays at the cytosolic side of the membrane [7,8], while the hydrophobic core is probably too short to span the membrane. This means that in order to reach its substrate, the catalytic site of Lep must be in or very close to the membrane. One of the possible ways to achieve this is by a direct interaction between the catalytic domain and lipid constituents of the membrane.

In this paper interactions between the periplasmic domain of leader peptidase and lipids are reported. The ability of different Lep constructs to interact with biomembranes and artificial membranes has been studied. In particular, one construct ( $\Delta 2-75$ ) with very good catalytic activity [9] and lacking the membrane-anchoring H1-P1-H2 domain shows a pronounced ability to penetrate into pure lipid bilayers and monolayers with a specificity for the zwitterionic lipid phosphatidylethanolamine. The results are discussed in relation to the mode of action of Lep.

## 2. Materials and methods

### 2.1. Isolation and purification of proteins and lipids

Two truncated forms of Lep,  $\Delta 2-75$  and  $\Delta 2-98$ , were isolated essentially as described before [9,10] but detergent was left out. The proteins were stored in 20 mM Tris-HCl, pH 7.4. Total lipid extracts from the inner membrane were prepared by extraction [11] and further purified by column chromatography on a silica column (silicagel 30–60  $\mu$ m, Baker BV). After equilibration in chloroform, phospholipids were eluted in a chloroform/methanol 1:1 (v/v) mixture and converted to their sodium salts [12]. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA).

### 2.2. Subcellular fractionation

An overnight culture of *E. coli* strain MC1061 [13] bearing a plasmid encoding the H2-CC construct [14] was diluted into fresh M9 medium [15] supplemented with ampicillin (50  $\mu$ g/ml), 0.2% fructose, thiamin and all amino acids except for methionine. Expression was induced during early-exponential phase with 0.2% arabinose and cells were labelled with 50  $\mu$ Ci [<sup>35</sup>S]methionine. After 2 min cells were harvested by centrifugation for 2 min at 13 krpm in an Eppendorf centrifuge, washed and resuspended in 30 mM Tris-HCl, pH 7.5. An equal volume of the same buffer with 40% sucrose (w/v) and EDTA (final concentration 0.1 mM) was added and the mixture was incu-

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**Abbreviations:** CC, cleavage cassette; DTT, dithiothreitol; LUVETS, large unilamellar vesicles made by extrusion techniques; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

bated at room temperature for 10 min. Cells were pelleted and the pellet was quickly resuspended in ice cold 0.5 mM MgCl<sub>2</sub> and incubated on ice for 10 min. After centrifugation for 8 min the supernatant was separated from the pellet. Both pellet and supernatant fractions were immunoprecipitated with Lep and  $\beta$ -lactamase antibodies and analysed by SDS-PAGE followed by autoradiography.

Inner and outer membrane fractions of MC1061 expressing H2-CC were essentially prepared as described in the next section. Cells were grown in LB medium supplemented with ampicillin (50  $\mu$ g/ml) and induced with 0.2% arabinose in early exponential phase. After one hour of induction the cells were harvested by 15 min centrifugation at 6 krpm in a GSA rotor (Sorvall).

### 2.3. Vesicle isolation and preparation

Inverted and right-side out inner membrane vesicles and outer membranes of *E. coli* strain MC1061 were isolated according to published procedures [16,17]. The identity of the fractions was confirmed by lipopolysaccharide staining and immunodetection of OmpA, Lep and SecY. Large unilamellar vesicles (LUVETs) were prepared by means of extrusion through a polycarbonate filter (Nucleopore; 0.2  $\mu$ m pore size) of a rehydrated (10 mM Tris-HCl, 50 mM NaCl, pH 7.5) total lipid extract from the inner membrane of MC1061.

### 2.4. Vesicle binding assay

LUVETs corresponding to 200 nmol lipid were incubated with the indicated amounts of protein (Fig. 3B) in 300  $\mu$ l 10 mM Tris-HCl, 50 mM NaCl, pH 7.5, for 1 h at room temperature. Inner membrane vesicles and outer membranes were incubated in 300  $\mu$ l 50 mM triethanolamine/HAc, 250 mM sucrose, 1 mM DTT, pH 7.5, to maintain the same environment as during isolation. Vesicles were pelleted by centrifugation for 30 min at  $236 \times 10^3 g$  at room temperature in a TLA 100.3 rotor using a TL 100 ultracentrifuge (Beckmann Instruments Inc., Palo Alto, CA, USA). Pelleting efficiencies of the vesicles were calculated after phosphorus determination [18] on supernatant and pellet. The amount of bound protein was determined after SDS-PAGE and Coomassie Brilliant Blue staining of pellet and supernatant fractions. The intensities of the bands were quantified by densitometry (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) and compared to calibration curves of the same proteins which were run on the same gels. The amount of bound protein was corrected for the pelleting efficiency of the vesicles which was always above 80%. Control experiments without vesicles revealed that more than 99% of the added proteins was recovered in the supernatant.

### 2.5. Monolayer experiments

The Wilhelmy plate method was used to measure protein induced changes in surface pressure of a monomolecular layer of phospholipids at constant surface area [19]. Surface pressures were measured at  $26 \pm 1^\circ\text{C}$  using a Cahn 2000 microbalance while continuously stirring the subphase with a magnetic bar. Unless stated otherwise, a subphase of 5 ml 10 mM Tris-HCl, 50 mM NaCl, pH 7.5, was placed in a teflon trough. The monomolecular lipid layers were spread from a chloroform/methanol 3:1 (v/v) solution at the air/buffer interphase to give initial surface pressures between 18 and 35 mN/m. Lower initial surface pressures were not used since both proteins gave rise to surface pressures of 18 mN/m in the absence of a lipid monolayer. In all experiments saturating amounts of protein (for both proteins 4  $\mu$ g/ml) were added in the subphase from a hole in the edge of the trough. The surface pressure was measured until a constant level was reached.

## 3. Results

The catalytic domain of Lep is localised in the periplasm [20]. To study possible interactions of this domain with membranes *in vivo*, a mutant was required in which the catalytic P2 domain could be separated from the membrane anchoring H1-P1-H2 domain. To this end, we used a construct (H2-CC) with a Lep cleavage site engineered downstream of H2 (Fig. 1A). This construct can be efficiently expressed and is cleaved *in vivo*, probably by the native population of Lep molecules in the membrane [14]. When spheroplasts were prepared the cleaved form was accessible to proteases ([14], results not

shown). Periplasmic fractions of pulse labelled cells expressing H2-CC were isolated by osmotic shock and screened by immunoprecipitations. While most ( $75 \pm 6\%$ ) of the periplasmic marker protein  $\beta$ -lactamase was recovered in the soluble periplasmic fraction, the cleaved domain of Lep was found predominantly in the pellet ( $84 \pm 7\%$ ) (results not shown), suggesting that the catalytic domain has an intrinsic ability to associate with membranes.

To investigate more precisely the localisation of the periplasmic domain, membrane fractions were prepared from cells expressing H2-CC. Inner and outer membranes were separated on a sucrose gradient and the different fractions were analysed by SDS-PAGE. Antibodies against Lep were used to stain blots made from the different fractions (Fig. 1B, insert). Two bands reacted with the antibody. One of 36 kDa corresponding to uncleaved, full-length Lep, was found mainly in the inner membranes around fraction 8 and to a lesser extent in the outer membrane (Fig. 1B, circles) as reported before [21]. A band with an apparent molecular weight of approx-

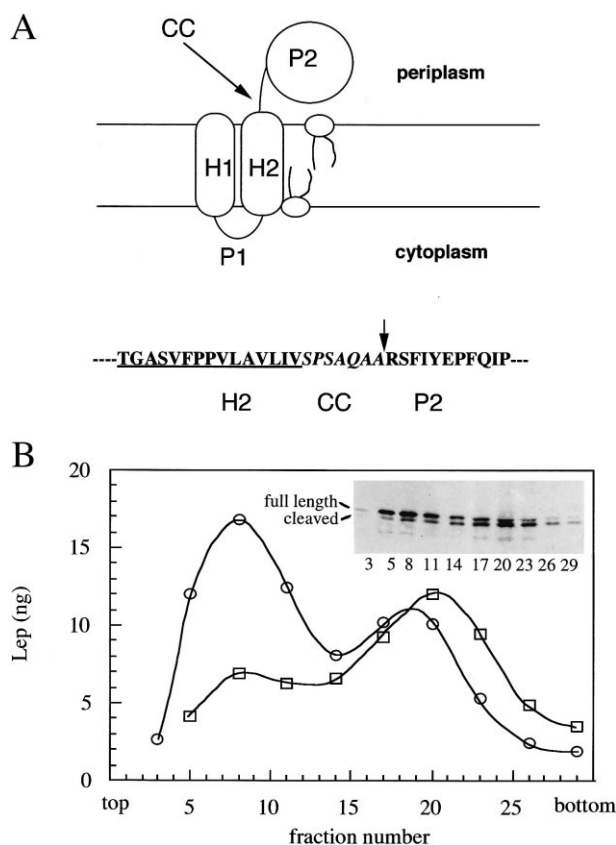


Fig. 1. The P2 domain of Lep fractionates both with the inner and outer membrane. A: Orientation of leader peptidase in the inner membrane and indication of the position of an engineered cleavage cassette (CC) behind the second transmembrane helix. The amino acid sequence of the region around H2 of a construct bearing a cleavage cassette is depicted. H2 (from residue 68 to 76) is underlined and the cleavage cassette between residues 76 and 77 is depicted in italics. The most likely cleavage site is indicated by an arrow. B: The amount of full-length Lep and of the cleaved P2 domain was determined in fractions which were collected from a sucrose gradient on which *E. coli* membranes were separated. The amounts of full length Lep (circles) and cleaved form (squares) are plotted against the fraction number. The inner and outer membranes were found around fractions 8 and 20m respectively. The insert shows the result of the corresponding Western blot decorated with Lep antibody.

imately 30 kDa, which is the size expected for the cleaved P2 domain, was associated with both membranes with a slight preference for the outer membrane (Fig. 1B, squares).

The ability of the periplasmic domain of Lep to bind to membranes was confirmed by vesicle binding experiments. For this purpose we made use of a purified, enzymatically active construct ( $\Delta 2-75$ , see Fig. 3A) lacking the H1-P1-H2 domain [9]. By means of ultracentrifugation experiments the binding of  $\Delta 2-75$  to right-side-out inner membrane vesicles and outer membranes was determined. In the absence of membranes,  $\Delta 2-75$  was quantitatively recovered in the supernatant after ultracentrifugation (Fig. 2, upper panel, lanes 1–3). Right-side out inner membrane vesicles (Fig. 2, upper panel, lanes 4–6) contain many different proteins as judged by Coomassie Brilliant Blue staining of gels, while outer membranes (Fig. 2, lower panel, lanes 1–3) show a characteristic pattern with only two dominant bands. Both types of vesicles were pelleted efficiently. When  $\Delta 2-75$  was incubated with vesicles prior to centrifugation, a significant fraction of the molecules sedimented with the vesicles (Fig. 2, upper panel, lanes 7–9 and lower panel, lanes 4–6). Thus,  $\Delta 2-75$  is apparently capable of binding to both inner and outer membranes while the native population of Lep is found mostly in the inner membrane. This suggests that the periplasmic domain does not require specific inner membrane components for binding. To investigate the possibility that the P2 domain recognises the lipid component of membranes, the binding of 5  $\mu$ g  $\Delta 2-75$  to inner membranes and to unilamellar lipid

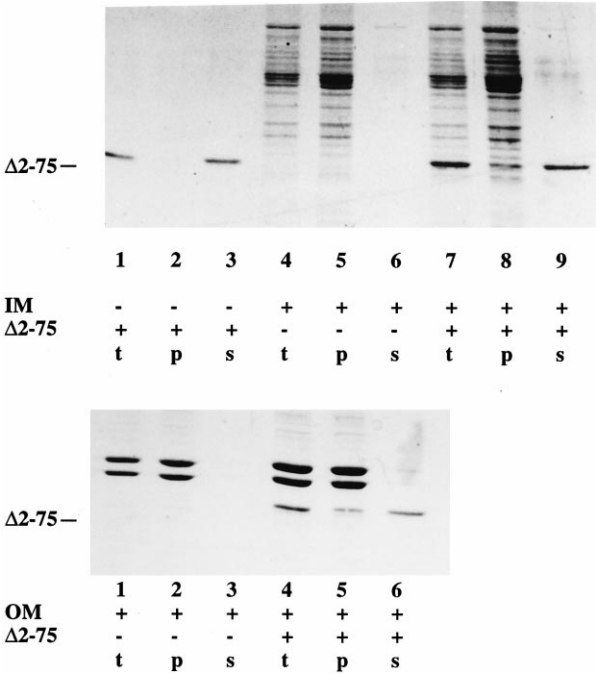


Fig. 2. Purified  $\Delta 2-75$  associates both with inner and outer membrane vesicles. Samples with (upper panel, lanes 1–3 and 7–9; lower panel, lanes 4–6) or without  $\Delta 2-75$  (upper panel, lanes 4–6; lower panel, lanes 1–3) were incubated in the absence (upper panel, lanes 1–3) or presence of membranes before ultracentrifugation. After centrifugation samples were split in pellet (p) and supernatant (s) fractions and compared to the total (t) amount before centrifugation. The upper panel shows incubations with inner membranes and the lower panel with outer membranes. Gels were stained with Coomassie Brilliant Blue.

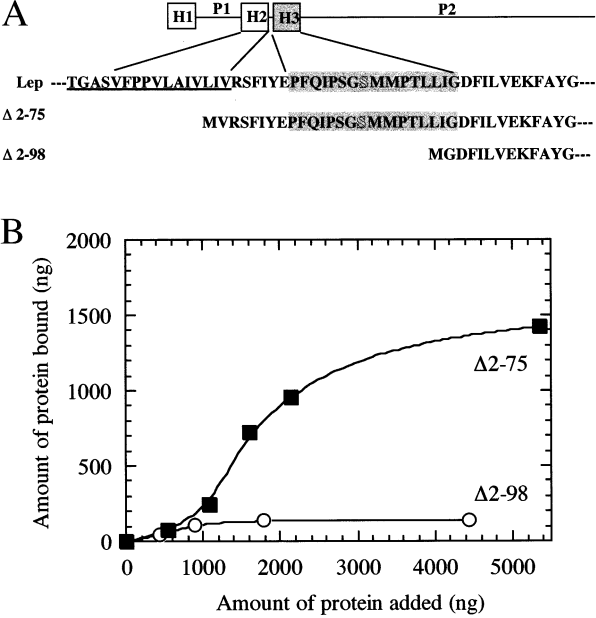


Fig. 3.  $\Delta 2-75$  binds more efficiently to lipid vesicles than  $\Delta 2-98$ . A: The second transmembrane segment H2 is underlined and a third hydrophobic segment (H3) coloured gray. The catalytically important serine-90 is shown in white. B: Binding of  $\Delta 2-75$  (closed squares) and  $\Delta 2-98$  (open circles) to lipid vesicles was determined as described in Section 2.

vesicles made from purified inner membrane lipids were compared. The same amount of lipid phosphorus was used for both types of vesicles. Right side out inner membrane vesicles bound  $1.7 \pm 0.3 \mu$ g of  $\Delta 2-75$  while the lipid vesicles bound  $1.6 \pm 0.3 \mu$ g, implying that membrane binding of  $\Delta 2-75$  is mediated by lipids.

To gain insight into the part of  $\Delta 2-75$  responsible for membrane activity, the lipid binding ability of  $\Delta 2-75$  was compared to that of construct  $\Delta 2-98$  (Fig. 3A) which lacks the mildly hydrophobic H3 segment. Fig. 3B shows efficient binding of  $\Delta 2-75$  to the lipid vesicles (closed squares). The slightly sigmoidal binding curve indicates that some cooperativity is involved in the binding process. Strikingly, binding of  $\Delta 2-98$  (open circles) is greatly reduced suggesting that the interaction of the periplasmic domain of Lep is mediated via the H3 region.

The association of  $\Delta 2-75$  with lipid vesicles can be caused by binding to the surface as well as by insertion of a part of the protein between the lipids. To study the nature and specificity of the interaction of the periplasmic domain of Lep with lipids we made use of monolayer experiments. Lipids isolated from purified inner membranes of *E. coli* were spread at the air-water interface to an initial surface pressure of 22 mN/m (Fig. 4A). When a solution of  $\Delta 2-98$  was injected under the monolayer a fast but small increase in surface pressure was observed which stabilised around 30 min. In contrast  $\Delta 2-75$  gave rise to a much larger pressure increase, indicating a more efficient interaction with the lipids, in accordance with the vesicle binding data. Since proteins that only interact with the lipid head groups without penetration between the lipids do not give rise to a pressure increase [22], the results show that  $\Delta 2-75$  inserts efficiently between the phospholipids in the monolayer.

The limiting surface pressure is defined as the maximal ini-

tial surface pressure at which a protein can cause an increase in surface pressure. This parameter is a measure of the membrane-penetrating ability of the protein. In biological membranes the packing densities of the lipids correspond to surface pressures between 31 and 35 mN/m [23]. To investigate whether the P2-constructs are able to penetrate into membranes with physiological lipid packing densities, monolayers with different initial surface pressures were made. Fig. 4B shows that the limiting surface pressure for  $\Delta 2-98$  (squares) is below the physiological threshold. In contrast, extrapolation of the curve for  $\Delta 2-75$  to zero surface pressure increase reveals that this construct is able to insert into monolayers with initial pressures as high as 38 mN/m, suggesting that the P2 domain is capable of penetrating into biological membranes *in vivo*.

To study the lipid specificity of the P2-membrane interaction, insertion into monolayers of pure lipids were compared. Phosphatidyl ethanolamine (PE) has a zwitterionic headgroup and accounts for approximately 75% of the phospholipids in the *E. coli* inner membrane [24]. The second most abundant lipid is the negatively charged phosphatidyl glycerol (PG) which accounts for about 20% [24]. Insertion of  $\Delta 2-75$  in a monolayer of dioleoyl phosphatidyl ethanolamine (DOPE) and dioleoyl phosphatidyl glycerol (DOPG) as representative lipids was measured as function of the initial pressure. From Fig. 5 it is clear that  $\Delta 2-75$  inserts best (highest pressure increase) into DOPE monolayers (triangles). Strikingly, inser-

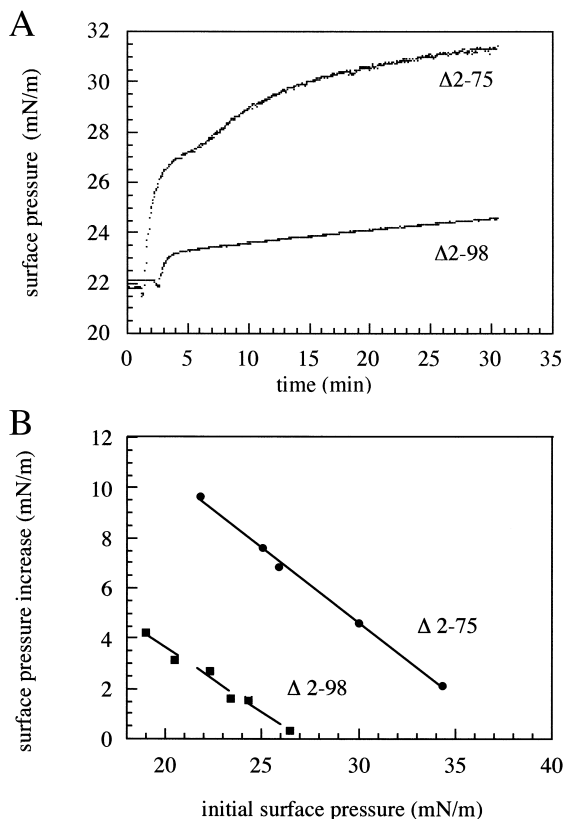


Fig. 4. Interaction of  $\Delta 2-75$  and  $\Delta 2-98$  with monolayers. A: Insertion profile of  $\Delta 2-75$  and  $\Delta 2-98$  into monolayers of *E. coli* inner membrane lipids which were spread at 22 mN/m initial pressure. After injection of protein the changes in surface pressure were followed. B: Ability of  $\Delta 2-75$  and  $\Delta 2-98$  to penetrate into monolayers as function of the initial pressure of the monolayer.

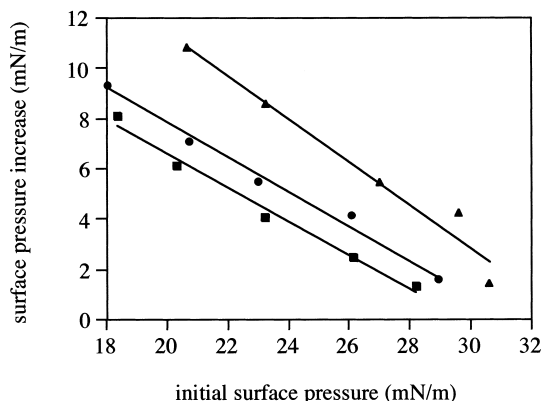


Fig. 5. Specific interaction of  $\Delta 2-75$  with different lipid head group classes. The surface pressure increase which is caused by insertion of  $\Delta 2-75$  into monolayers of DOPE (triangles), DOPC (squares) and DOPG (circles) as function of the initial surface pressure.

tion into monolayers of negatively charged DOPG (circles) was clearly less efficient, indicating that penetration into the monolayer was not mediated by attracting electrostatic interactions in contrast to many other proteins [25]. This was confirmed by measuring the influence of the ionic strength of the subphase on the ability of  $\Delta 2-75$  to interact with monolayers of *E. coli* lipids. Increasing the NaCl concentration up to 1.2 M did not lead to a decrease in the ability of  $\Delta 2-75$  to penetrate into the monolayer (results not shown).

To determine whether the zwitterionic character of DOPE is the only factor responsible for the interaction, dioleoylphosphatidyl choline (DOPC) was also tested. Insertion into DOPC monolayers (squares) was considerably less efficient despite the identical acyl chains, further illustrating the specificity of the interaction of  $\Delta 2-75$  with PE.

#### 4. Discussion

This study reports on the interaction of the catalytic domain of leader peptidase, one of the key enzymes in preprotein translocation, with membranes. Evidence for membrane binding activity was obtained using two approaches. Fractionation studies with the construct H2-CC from which the membrane spanning regions are cleaved from the periplasmic domain *in vivo* showed that the periplasmic domain is associated both with inner and outer membranes indicating no high affinity for specific components of the inner membrane. This finding was confirmed by studies using the purified  $\Delta 2-75$  construct, corresponding to the periplasmic domain of Lep.  $\Delta 2-75$  binds to lipid vesicles and right-side out inner membrane vesicles equally well, suggesting that the catalytic domain does not bind to proteinaceous components present in the inner membrane but directly to the membrane lipids.

The binding to lipids was also demonstrated by the efficient insertion of  $\Delta 2-75$  into monolayers derived from inner membrane lipids. Remarkably, while anionic phospholipids are often important for the insertion of proteins [26,27],  $\Delta 2-75$  displayed best penetration in lipid films made from the zwitterionic lipid phosphatidylethanolamine. Insertion thus does not seem to depend on electrostatic interactions. The limiting surface pressure for insertion into PE monolayers was very close to that of complete extracts of the *E. coli* inner membrane which contain about 75% PE, suggesting that within the

lipid extract the PE component is responsible for insertion of  $\Delta 2-75$ . The preference of the periplasmic domain for binding to the outer membrane (Fig. 1B) is compatible with a specific binding to the PE component, since the periplasmic leaflet of the outer membrane is enriched in PE over the inner membrane [28].

The zwitterionic nature of the PE headgroup is not responsible for the specific interaction with the catalytic domain of leader peptidase because insertion into monolayers of the zwitterionic PC is greatly reduced. In recent years it has become increasingly clear that PE has special properties allowing it to mediate membrane insertion and binding of proteins (for review see [29]). Examples include insertion of the chloroplast precursor protein of ferredoxin [21], the precursor of the *E. coli* pore protein PhoE [30], SecA [27], and blood coagulation factor VIII [31]. Furthermore, PE promotes folding of a periplasmic loop of newly inserted lactose permease [32], it regulates the activity of glycerophosphate acyltransferase [33], it is essential for efficient functioning of preprotein translocase [34], and was recently found in crystals of cytochrome *c* oxidase [35].

The hypothesis has been put forward [36] that PE as non-bilayer lipid with its small headgroup in conjunction with a strong tendency of this lipid to organize in structures with a high intrinsic negative surface curvature when constrained within a bilayer, lowers the lateral pressure in the interface. This could create insertion sites for proteins or for other amphipathic components as the anti-cancer drug doxorubicine [37]. It is this property that may be responsible for insertion of the catalytic domain of leader peptidase into the periplasmic leaflet of the *E. coli* inner membrane.

It is not possible to draw firm conclusions about the part of P2 which is responsible for the interactions with membranes containing phosphatidylethanolamine. However, considering the nature and specificity of the interaction between the periplasmic domain of Lep and lipids it is most likely that membrane association is caused by interaction of a hydrophobic segment within the periplasmic domain with the lipids. Deletion of H3 (residues 83–98) which is the most hydrophobic part within the periplasmic domain indeed diminished both insertion into the lipid monolayers and association with the lipid vesicles.

The insertion into the lipid phase as described in this study has important implications for the mode of action of Lep. Because of the short hydrophobic core and the cytosolic localisation of the N-termini of signal sequences, the catalytic site of Lep must be very close to the membrane. Insertion of the periplasmic domain into the lipid phase and the possible involvement of H3, which carries the catalytic serine-90 residue, implies that the active site of Lep may be at least partially buried in the membrane.

Interestingly, it has been observed that lengthening the hydrophobic core of signal sequences results in reduced processing of preproteins without much effect on translocation [38]. Our data suggest that the signal peptide cleavage site may have been moved too far out of the membrane to be accessible to the active site of leader peptidase under these conditions.

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