A Small Hydrophobic Domain Anchors Leader Peptidase to the Cytoplasmic Membrane of Escherichia coli*

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Leader peptidase is an enzyme of the Escherichia coli cytoplasmic membrane which removes amino-terminal leader sequences from many secreted and membrane proteins. Three potential membrane-spanning segments exist in the first 98 amino acids of leader peptidase. We have characterized the topology of leader peptidase based on its sensitivity to protease digestion. Proteinase K and trypsin treatment of right-side-out inner membrane vesicles and spheroplasts yields protected fragments of approximately 80 and 105 amino acid residues, respectively. We have shown that both fragments are derived from the amino terminus of the protein and that the smaller protected peptide can be derived from the larger. Removal of the third potential membrane-spanning segment (residues 82-98) does not affect the size of the proteinase K-protected fragment but does reduce the size of the trypsin-protected peptide. Because the proteinase K-protected fragment is about 9000 daltons, is derived from the amino terminus of leader peptidase, and its size is not affected when amino acids 82-98 are removed from the protein, it must extend from the amino terminus to approximately residue 80. Likewise, the trypsin-protected fragment must extend from the amino terminus to about residue 105. These data suggest a model for the orientation of leader peptidase in which the second hydrophobic stretch (residues 62-76) spans the cytoplasmic membrane and the third hydrophobic stretch resides in the periplasmic space.

The delivery of newly synthesized proteins to their correct cellular compartments involves several steps (Wickner and Lodish, 1985). Proteins destined for translocation across the cytoplasmic membrane of Escherichia coli must first bind to the membrane in a way which allows subsequent assembly steps. Transport through the hydrophobic interior of the outer membrane is often followed by the removal of leader or signal sequences from the amino-terminal portion of membrane and secreted proteins. This endoproteolytic processing of precursor proteins into their mature forms is catalyzed by leader peptidase.

Isolation of the gene which encodes leader peptidase (Date and Wickner, 1981) has facilitated the characterization of this protein and has allowed us to study the enzyme's membrane assembly requirements. These studies have shown that, like other proteins of the cytoplasmic membrane, leader peptidase is synthesized without a cleaved amino-terminal leader sequence (Wolfe et al., 1983). The assembly of this protein is dependent upon the presence of an electrochemical potential across the inner membrane and functional secA and secY gene products (Wolfe et al., 1985). Studies aimed at determining which domains of leader peptidase are essential for insertion into the cytoplasmic membrane have demonstrated that the last 182 amino acids of the protein are required (Dalbey and Wickner, 1986). Moreover, efficient assembly requires the presence of at least two of the three hydrophobic stretches in the protein.

Mechanisms proposed for the assembly of leader peptidase must take into consideration its orientation across the cytoplasmic membrane. Previous studies on the topology of this enzyme have shown that it is anchored with a small portion of its amino-terminal domain exposed to the cytoplasmic surface and a larger polar carboxy-terminal domain in the periplasmic space (Wolfe et al., 1983). These experiments were not, however, able to establish which of the three hydrophobic stretches (Fig. 1A, boxed sequences) span the cytoplasmic membrane. Based on protease protection studies conducted on right-side-out inner membrane vesicles and spheroplasts, we report that the third hydrophobic stretch of amino acids (residues 82-98) is exposed to the periplasm and that the second hydrophobic stretch (residues 62-76) plays a critical role in the anchoring of leader peptidase to the cytoplasmic membrane. Our working model for the topology of this enzyme is depicted in Fig. 1B.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin (tosylphenylalanyl chloromethyl ketone treated) and soybean trypsin inhibitor were from Worthington. Phenylmethylsulfonyl fluoride, chymotrypsin, proteinase K, and indoleacrylic acid were from Sigma. CNBr-activated Sepharose 4B was obtained from Pharmacia P-L Biochemicals, and Staphylococcus aureus V8 proteinase was purchased from Cooper Biomedical.

**Bacteria and Plasmids**—E. coli strains MC1061 (Dalbey and Wickner, 1986; and HJMI14 (Wickner and Killick, 1977) have been described elsewhere. Details of the construction of pRD8 are given in Dalbey and Wickner (1985). A description of the mutagenesis procedures used to obtain pRD382-98, pRDam79, and pRDam105 will be published separately (Dalbey and Wickner, 1987).

**Growth and Labeling Conditions**—Cells were grown at 37 °C to midlog phase in M9 minimal media (Miller, 1972) containing 0.5% fructose, 0.1 mg/ml ampicillin, and 50 μg/ml of each amino acid but methionine. Arabinose (0.2%) was added to induce synthesis of wild type or mutant leader peptidase. When appropriate, cells were pulse-labeled with [35S]methionine (1000 Ci/mmol, Amersham Corp.).

**Preparation of Spheroplasts and Inner Membranes**—Inner membranes from E. coli strain MC1061 were prepared by the method of

8806
Osborn et al. (1972). Spheroplasts were made from a second E. coli strain, HJM114. 0.02-liter cells were grown as described above except that the period of induction in the presence of arabinose was increased to 2 h. After induction cells were chilled to 4°C and centrifuged at 5000 x g for 5 min. (Subsequent steps were conducted at 4°C.) Cell pellets were resuspended in 1 ml of 0.75 M sucrose, 10 mM Tris-Cl, pH 8.0, to which 0.05 ml of 2 mg/ml lysozyme was added. After 2 min of incubation, 2 ml of 10 mM EDTA, pH 8.5, was added dropwise with shaking over a 5-min period. MgSO₄ was added to a final concentration of 20 mM.

**Protease Treatment of Inner Membrane Vesicles**—Inner membrane vesicles and spheroplasts were incubated at 37°C for 1 h in the presence of 0.2 mg/ml proteinase K or trypsin in 0.05 M Tris-Cl, pH 8.0. Control digests were conducted in the presence of 1% Triton X-100. Reactions were stopped either by the addition of 20 mM PMSF (2 mM) or soybean trypsin inhibitor (2 mg/ml).

**Construction of trpE Leader Peptidase Fusion Plasmids**—Four fusion plasmids were constructed using sequences from the lep gene and the trpE vectors created by Koerner. These plasmids are diagrammed in Fig. 4A and have been designated pLP1-68, pLP70-141, pLP141-221, and plp221-323. The numbers refer to amino acids in leader peptidase encoded by the plasmids. Three types of fusion vectors were used to construct these plasmids: pATH 1 was used for plp221-323; pATH 3 for pLP70-141 and pLP141-221; and pATH 10 was used for pLP1-68. These vectors can be distinguished by the arrangement of restriction sites within the 3' end of the trpE gene. The restriction enzymes listed in Fig. 4A were used to liberate leader peptidase gene sequences from pRD8 DNA. The resulting DNA fragments were isolated from agarose gels by adsorption onto NA45 DEAE paper (Schleicher & Schuell). Where appropriate, sticky ends of the fragments were filled in by incubation with DNA polymerase I (large fragment, New England Biolabs) and deoxynucleotide triphosphates (Schleicher & Schuell) and specific regions of leader peptidase (see above) were induced by lytically digested membranes. This fragment of leader peptidase is approximately 32,000 daltons or 5,000 daltons smaller than its full length counterpart. It has been designated TRF 1. The resulting inner membrane vesicles used in these studies were mostly sealed in the right-side-out orientation. Proteolytic digests of spheroplasts have been included to verify results obtained with membrane vesicles.

An inner membrane fraction was prepared from cells (pRD8/MC1061) which carry a multicopy plasmid with the leader peptidase gene under arabinose promoter regulation. Addition of arabinose to this strain induces the synthesis of leader peptidase. Induced and uninduced cells were labeled with [35S]methionine, converted to spheroplasts and sonicated, and subjected to isopycnic sucrose density centrifugation (Osborn et al., 1972). The resulting inner membrane vesicles from induced cells (Fig. 2A, lane 1) were digested with a variety of proteases; chymotrypsin (lanes 2 and 3), trypsin (lanes 4 and 5), proteinase K (lanes 6 and 7), and Staphyloccocus aureus
V8 protease (lanes 8 and 9). Protein-resistant fragments fell into two size classes with approximate molecular weights of 11,000 for trypsin and V8 protease (Fig. 2A, lanes 4 and 8) and 9,000 for chymotrypsin and proteinase K (lanes 2 and 6). These resistant fragments were not observed in digests conducted in the presence of detergent (lanes 3, 5, 7, and 9). Both classes of resistant fragments were found in tight association with membrane fractions (data not shown). Studies conducted using increasing amounts of protease on these IMVs demonstrated that both the 9,000- and the 11,000-dalton protected fragments are final digestion products (data not shown). These protected fragments were not observed in digests of IMVs from uninduced cells (Fig. 2B) and, therefore, are derived from leader peptidase.

In order to test whether these two proteolytic species were related, inner membrane vesicles from induced cells (Fig. 3, lane 1) were treated first with trypsin (lane 2) and then subsequently with proteinase K (lane 3). The third lane shows that the trypsin-resistant fragment is converted to the proteinase K-resistant fragment when both enzymes are used. These results demonstrate that the smaller of the two fragments (the proteinase K-Resistant Fragment or KRF I) is derived from the larger fragment (the trypsin-resistant fragment or TRF II).

To assess directly which regions of leader peptidase were yielding the proteolytic fragments TRF II and KRF I, we prepared antibodies which recognize residues 1-68, 70-141, 141-221, and 221-323 of leader peptidase. These antibodies were obtained from rabbits injected with TrpE-leader peptidase fusion proteins and were purified by affinity chromatography as described under "Experimental Procedures." The efficacy of this purification procedure can be seen in Fig. 4B. (Residues 70-141 were not immunogenic, and, therefore, antibodies which recognize this region were not included in these experiments.) Identical Western blots of induced fusion proteins were probed with three affinity-purified antibodies. Each of the antibodies was able to recognize full length leader peptidase (lanes 1, 6, and 11) as well as the fusion proteins used in their preparation (lanes 2, 8, and 14). However, these antibodies were not able to recognize other fusion proteins related to leader peptidase (lanes 3, 4, 7, 9, 12, and 13) or a fusion protein consisting of the trpE gene product and an amino acid sequence unrelated to leader peptidase (lanes 5, 10, and 15).

To confirm that TRF II and KRF I are derived from the amino terminus of leader peptidase, Western blots of IMVs digested with trypsin and proteinase K were probed with

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**Fig. 2.** Protease digests of inner membrane vesicles. 0.05-liter pRD8/MC1061 cells were grown to $A_{660} = 0.2$ and incubated either in the presence (A) or absence (B) of 0.2% arabinose for 1 h. Cultures were labeled for 2 min with 1 mCi of [35S]methionine and chased for 2 min with 0.2 mg/ml unlabeled methionine. These radioactive cultures were then converted to spheroplasts and sonicated. Inner membranes were isolated by sucrose gradient centrifugation. 20 μl of these IMVs were incubated in a total reaction volume of 30 μl for 1 h at 37 °C without protease (lane 1) or with a final concentration of 0.2 mg/ml of the following proteases: chymotrypsin (lanes 2 and 3); trypsin (lanes 4 and 5); and proteinase K (lanes 6 and 7). S. aureus V8 protease was used at a final concentration of 1 mg/ml (lanes 8 and 9). Lanes 3, 5, 7, and 9 represent digests conducted in the presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and fluorography as described under "Experimental Procedures." LPase, leader peptidase.

**Fig. 3.** Protease digests of IMVs show that TRF II and KRF I are related. IMVs were isolated as described in the legend to Fig. 2. [35S]Methionine-labeled IMVs from induced cells (40 μl) were incubated with trypsin at a final concentration of 0.2 mg/ml for 30 min at 37 °C. Half of the reaction mixture was removed, and soybean trypsin inhibitor was added to 2 mg/ml (lane 2). The remaining portion was digested with 0.2 mg/ml proteinase K for an additional 30 min at 37 °C. The reaction was terminated by the addition of PMSF at a final concentration of 2 mM (lane 3). 20 μl of IMVs were incubated without protease for 1 h at 37 °C (lane 1). SDS-PAGE and fluorography were used to visualize radioactive polypeptides. LPase, leader peptidase.
affinity-purified antibodies which recognize residues 1–68, 141–221, and 221–323 (Fig. 5, A, B, and C, respectively). Both resistant fragments can only be recognized by those antibodies directed against the amino-terminal region of leader peptidase (Fig. 5A, lanes 2 and 4). Antibodies which react with carboxy-terminal sequences are only able to recognize leader peptidase from IMVs which have not been subjected to proteolysis (Fig. 5, B and C, lane 1).

To verify that the inner membrane vesicles described in the previous experiments were sealed in the right-side-out orientation, we performed proteolytic digests on spheroplasts. pRD8/HJM cells were grown in the presence of arabinose, converted to spheroplasts, and digested with increasing amounts of trypsin in the absence (Fig. 6A, lanes 1–4) or presence (lane 5) of detergent. Digested and undigested spheroplasts were subjected to Western blot analysis using the antibody directed against residues 1–68 of leader peptidase as a probe. Incubation of spheroplasts with 0.02 mg/ml trypsin for 1 h at 37 °C was sufficient to convert all of the full length leader peptidase to the trypsin-resistant form (lane 3). Digestions conducted with a higher concentration of enzyme (0.2 mg/ml) did not result in further digestion of this resistant fragment (lane 4).

Similar experiments were performed to check whether the proteinase K-protected fragment obtained from spheroplasts was the same KRF I seen in the digests of IMVs. Spheroplasts were treated with proteinase K and subjected to Western blot analysis using the antibody directed against residues 1–68 of leader peptidase as a probe. The resulting proteinase K-resistant fragment (Fig. 6B, lane 4) has the same electrophoretic mobility as the one generated from proteinase K digestion of IMVs (lane 2). This observation is confirmed by the fact that protected peptides from the two samples (KRF I from spheroplasts and IMVs) migrate as one band when mixed (lane 3). The same kind of mixing experiment was conducted with KRF I from spheroplasts (lane 9) and TRF II from inner membrane vesicles (lane 7). The difference in the apparent molecular weights of these two peptide species can be seen in lane 8.

To determine whether the third hydrophobic stretch of amino acids in leader peptidase is exposed to the periplasm, inner membrane vesicles were prepared from a strain (pRDΔ82-98) which overproduces a mutant leader peptidase missing residues 82–98. These pRDΔ82-98/MC1061 vesicles were digested with trypsin and proteinase K and compared with identical digests of wild type pRD8/MC1061 IMVs. Western blot analysis, again using affinity-purified antibodies
FIG. 5. Trypsin and proteinase K-protected fragments are derived from the amino terminus of leader peptidase (L.Pase). IMVs were prepared as described in the legend to Fig. 2 except the volume of the starting culture was 1 liter and the cells were not labeled with radioactive amino acids prior to harvest. 5-μl IMVs were treated without protease (lane 1) or with 0.2 mg/ml trypsin (lanes 2 and 3) or 0.2 mg/ml proteinase K (lanes 4 and 5) for 1 h at 37°C. Reactions were stopped by adding soybean trypsin inhibitor (2.5 mg/ml) or PMSF (2 mM). Lanes 3 and 5 represent digestions conducted in the presence of 1% Triton X-100. Samples were analyzed by SDS-PAGE and Western blotting techniques. Identical gels were transferred to nitrocellulose filters and probed with affinity-purified antibodies which recognize amino acids 1-68 (A), 141-221 (B), or 221-323 (C) in leader peptidase. which recognize residues 1-68 of leader peptidase as a probe, shows that this mutant protein (Fig. 7, lane 2) migrates slightly faster than its wild type counterpart (lane 1). Bands smaller than the full length pRDΔ82-98-encoded leader peptidase (lane 2) represent membrane-associated in vivo generated degradation products.5 As predicted from the model in Fig. 1B, the size of the trypsin-resistant fragment is smaller in the pRDΔ82-98/MC1061 vesicles (Fig. 7, lane 4) than in the pRD8/MC1061 vesicles (lane 3). When both types of vesicles were treated with proteinase K, protected fragments with identical electrophoretic mobilities were observed (lanes 5 and 6). Therefore, the size of the proteinase K-protected fragment has not been affected by the removal of the third hydrophobic stretch of amino acids whereas the size of the trypsin-protected fragment is reduced. Since KRF I is about 9,000 daltons, reacts with antibody to residues 1-68, and yet is not altered by removal of residues 82-98, it must extend from within a few amino acids of the amino terminus leader peptidase to approximately residue 80. By the same logic, TRF II must extend from very close to the amino terminus to approximately residue 105.

Amber mutations in the leader peptidase gene which encode truncated versions of leader peptidase were used to estimate the sizes of TRF II and KRF I. Two amber mutants were made available for our studies5 which encode the first 78 and 104 amino acids in leader peptidase. Plasmids which carry these two mutants, pRDam79 and pRDam105, were transformed independently into MC1061, grown in the presence of 0.2% arabinose, and labeled for 1 min with [35S]methionine. Total cell lysates from induced cells were electrophoresed either separately (Fig. 8, lanes 3 and 6, for amber fragments 104 and 78 amino acids long, respectively) or in mixtures (lanes 2 and 5) with protease-protected fragments from 35S-labeled IMVs (lanes 1 and 4). Lane 2 represents a mixture of total proteins from pRDam105/MC1061 and TRF II from IMVs digested with trypsin. Lane 5 represents a mixture of cell lysates from pRDam79/MC1061 and KRF I from IMVs. These results indicate that the observed length of TRF II is very close to 104 amino acids and KRF I is slightly larger than 78 amino acids in length.

DISCUSSION
It has not been established how proteins such as leader peptidase, synthesized without cleavable leader sequences, are able to assemble into the inner membrane of E. coli. It has been postulated that internal uncleaved signal sequences are required for translocation to occur (Blobel, 1980). It has also been suggested (Wickner and Lodish, 1985) that spontaneous insertion domains may be responsible for the integration of these proteins into the lipid bilayer. All hypotheses which attempt to explain the assembly of a given protein into a lipid bilayer must, however, take into consideration its final orientation in the membrane.

Studies described by Wolfe et al. (1983) demonstrated that leader peptidase is anchored to the cytoplasmic membrane by a segment near the amino-terminal end of the polypeptide. This conclusion was based on an experiment in which inverted vesicles, prepared by the sonication of spheroplasts, yielded a smaller form of leader peptidase upon trypsin digestion. (While these vesicles were obtained by sonicating spheroplasts, the sealed inverted vesicles represented only about 10% of the total population of vesicles.) The authors postulated that this trypsin-resistant fragment (TRF I) resulted

5 R. E. Dalbey and W. Wickner, unpublished results.
from the loss from one end of the protein of a peptide approximately 5000 daltons in molecular mass. Two-dimensional thin layer chromatography was used to establish that this peptide was derived from the amino terminus of leader peptidase. However, these studies did not establish which regions of the protein span the cytoplasmic membrane.

Three potential membrane-spanning regions are predicted from analysis of the amino acid sequence of leader peptidase (Eisenberg et al., 1984, Fig. 1A, boxed sequences). We have shown here that the second hydrophobic stretch (residues 62–76) spans the cytoplasmic membrane and that the third hydrophobic sequence of amino acids (residues 83–98) resides in the periplasmic space. This model is based on the following findings. 1) Right-side-out membrane vesicles and spheroplasts protect a 9000-dalton peptide from complete digestion when treated with proteases which exhibit wide substrate specificities (i.e. proteinase K and chymotrypsin); 2) this protected fragment is derived from the amino terminus of the protein; and 3) its apparent size is not affected by the removal of amino acid residues 82–98. This hydrophobic stretch of 15 amino acids is shorter than those generally expected for membrane-spanning regions. Our data indicate that the proteinase K-protected fragment is several amino acids longer than the 78-amino acid amber fragment, suggesting that some of the residues between amino acids 77 and 82 are included in the membrane anchor region. However, we cannot be sure whether the residues Pro-Gly-Trp-Leu-Glu between amino acids 57 and 61 or the amino acids between residues 76 and 83, Arg-Ser-Phe-Ile-Tyr-Glu, are included in the membrane anchor region. At this time little is known about the inclusion of ions or ion pairs in membrane-spanning domains. This is due to the fact that the topology of few transmembrane proteins is known with accuracy.

The asymmetric orientation of leader peptidase offers a unique opportunity for assessing the orientation of sealed vesicles. Kaback and co-workers have shown that the majority...
of the markers for the inner membrane are largely exposed on the cytoplasmic surface of the bilayer (Owen and Kaback, 1979). Leader peptidase, as presented here, exhibits characteristic digestion patterns when sealed vesicles of either orientation are subjected to treatment with trypsin. Inverted vesicles yield a 32,000-dalton protected fragment (TRF I), and right-side-out vesicles yield a protected fragment of 11,000 daltons (TRF II). To date, only the coat protein of M13 phage (Chang et al., 1979; Wickner, 1976) has afforded similar opportunities for studying membrane orientation.

The orientation of leader peptidase also poses interesting questions of how the structure of the cytoplasmic domain may influence the enzymatic activity of the periplasmic domain (Zimmerman et al., 1982). Studies conducted in vitro using protease-treated inverted membrane vesicles have shown that removal of the amino terminus of leader peptidase inactivates leader peptide-processing activity (Ohno-Iwashita et al., 1984). In addition, mutants in leader peptidase which are missing residues 4–50 of the mature protein are able to assemble into the inner membrane but fail to cleave precursor proteins to their mature forms in vitro. The function of this amino-terminal domain on the opposite side of the membrane from the catalytic domain is not known.

The results described in this paper are consistent with the observation (Dalbey and Wickner, 1987) that residues 62–76 are essential for the assembly of leader peptidase into the inner membrane. When these amino acids are removed from the polypeptide using genetic techniques, the mutant protein fails to insert across the bilayer. In addition, a fusion protein composed of residues 51–82 of leader peptidase and the mature part of OmpA is capable of translocation across the inner membrane. Therefore, it appears that the second hydrophobic domain functions both as a signal and a membrane anchor sequence for leader peptidase, as is the case for influenza virus neuraminidase (Blok et al., 1982).

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Fig. 7. **Topological localization of the third hydrophobic stretch of amino acids in leader peptidase (LPase).** IMVs were made from an *E. coli* strain pRD82-98/MC1061. Proteolytic digests of these vesicles were compared with digests of pRD8/MC1061 vesicles. Odd-numbered lanes represent IMVs from pRD8 vesicles, and even-numbered lanes show IMVs from pRD8-98 vesicles. Undigested membranes can be seen in lanes 1 and 2. Vesicles treated with 0.2 mg/ml trypsin for 1 h at 37 °C are shown in lanes 3 and 4. Proteinase K-digested vesicles (0.2 mg/ml, 1 h, 37 °C) are seen in lanes 5 and 6. PMSF (2 mM) or soybean trypsin inhibitor (2 mg/ml) was used to stop proteolytic reactions. These samples were subjected to Western blot analysis using affinity-purified antibodies which recognize residues 1–68 of leader peptidase as a probe.

Fig. 8. **Molecular weight assessment of TRF II and KRF I.** Cells (MC1061) carrying the plasmids pRDam79 and pRDam105 were grown and labeled with [35S]methionine using the conditions described under “Experimental Procedures.” Total cell lysates of pRDam105/MC1061 (lane 3) and pRDam79/MC1061 (lane 6) are shown. Lanes 1 and 4 represent IMVs digested with trypsin (0.2 mg/ml) and proteinase K (0.2 mg/ml), respectively. Lane 2 is a mixture of the samples run in lanes 1 and 3. Lane 5 represents a mixture of the samples run in lanes 4 and 6. Samples were subjected to SDS-PAGE and visualized by fluorography. a.a., amino acids.

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