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In Vitro Insertion of Leader Peptidase into *Escherichia coli* Membrane Vesicles

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Leader peptidase is an integral protein of the *Escherichia coli* cytoplasmic membrane whose topology is known. We have taken advantage of this knowledge and available mutants of this enzyme to develop a genetic test for a cell-free protein translocation reaction. We report that leader peptidase inserted into inverted plasma membrane vesicles in its correct transmembrane orientation. We have examined the in vitro membrane assembly characteristics of a variety of leader peptidase mutants and found that domains required for insertion in vivo are also necessary for insertion in vitro. These data demonstrate the physiological validity of the in vitro insertion reaction and strengthen the use of this in vitro protein translocation reaction for the dissection of this complex sorting pathway.

Cell-free protein translocation reactions have provided important insight into the metabolic requirements for the translocation of proteins across membranes (17). Within recent years, a complex translocation reaction for *Escherichia coli* has been described (3, 14). Using this system, Chen and Tai have made the important finding (4) that ATP is needed for translocation into inverted inner membrane vesicles (IMVs). Geller et al. (10) have shown that both the electrochemical potential and ATP are required for optimal assembly of the precursor form of the outer membrane protein A (pro-OmpA) into *E. coli* IMVs. In recent reports, Crooke and Wickner (5, 6) have demonstrated that trigger factor, a cytosolic protein, must interact with pro-OmpA to assure its proper folding into a translocation-competent conformation. Since this in vitro reaction is a major new avenue for investigating bacterial protein export, we thought it necessary to establish that it faithfully reflects the properties of the intact cell. We chose leader peptidase as a model protein to test the in vitro membrane assembly reaction for several reasons: (i) abundant information is available from in vivo studies on the biogenesis of this protein (7–9, 18–20); (ii) the topology of leader peptidase across the cytoplasmic membrane has been determined (13, 20); (iii) a number of mutants are available whose in vivo membrane insertion characteristics have been investigated (7–9); and (iv) in addition to an internal, uncleaved signal sequence, leader peptidase contains novel "translocation poison" (16) and "hydrophobic helper" (7, 8, 16) regions. An analysis of in vitro translocation of mutant leader peptidase molecules which lack these functions may indicate whether these functions can be studied in such a cell-free system.

Leader peptidase has three hydrophobic domains (residues 1 through 22, 62 through 76, and 83 through 98) and is an integral protein of the cytoplasmic membrane. Residues 23 through 61 of leader peptidase comprise a polar, cytoplasmic domain, while the majority of the polypeptide chain (residues 77 through 323) is exposed to the periplasmic space. The second hydrophobic region (residues 62 through 76) anchors the protein to the membrane (13) and serves as the internal, uncleaved signal sequence for membrane assembly (7–9). The topology of leader peptidase in inverted IMVs is shown in Fig. 1c. Previous studies (19) have demonstrated that when inverted IMVs are treated with trypsin, a proteolytic fragment of approximately 32,000 daltons remains protected from complete digestion (Fig. 1d). This fragment has been named TRF for trypsin resistant fragment. The 5,000-dalton fragment degraded during this treatment is derived from the amino terminus of leader peptidase (19). Treatment of vesicles with proteinase K yields approximately the same TRF as treatment with trypsin (K. Moore and W. Wickner, unpublished results). Our assay (Fig. 1) consists of an in vitro transcription-translation-translocation reaction carried out in the presence of [^{35}S] methionine, followed by incubation with proteinase K to generate TRF.

IMVs used in these translocation assays were prepared from *E. coli* K-12 D10 (*rna-10 relA1 spoT1 metB1*) by the method of Chang et al. (2) including the modifications suggested by Goodman et al. (11). To assess the proportion of vesicles in these reactions which were sealed in an inverted orientation, we incubated vesicles in buffer alone (Fig. 2, lane 2) or with increasing concentrations of trypsin (lanes 3 to 5). Following a 1-h digestion at 0°C, trypsin was inactivated with soybean trypsin inhibitor and the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12) and immunoblot analysis (15). A polyclonal antibody to leader peptidase was used as a probe for these blots. At the highest concentration of trypsin (10-mg/ml final concentration; Fig. 2, lane 5), all of the full-length leader peptidase (37,000 daltons) was converted to the TRF form. When right-side-out, sealed vesicles were treated with trypsin, a protected fragment of 11,000 daltons was detected (20). The 11,000-dalton protected fragment was not observed in this vesicle preparation. In addition, the TRF was not detected when the nonionic detergent Triton X-100 was included in the reaction (Fig. 2, lane 6). We conclude that these membranes are sealed in an inverted orientation.

The mutant leader peptidases included in the following experiments have been studied with respect to their in vivo insertion into the *E. coli* cytoplasmic membrane (7–9, 16). Their characteristics are summarized in Fig. 3. These pro-

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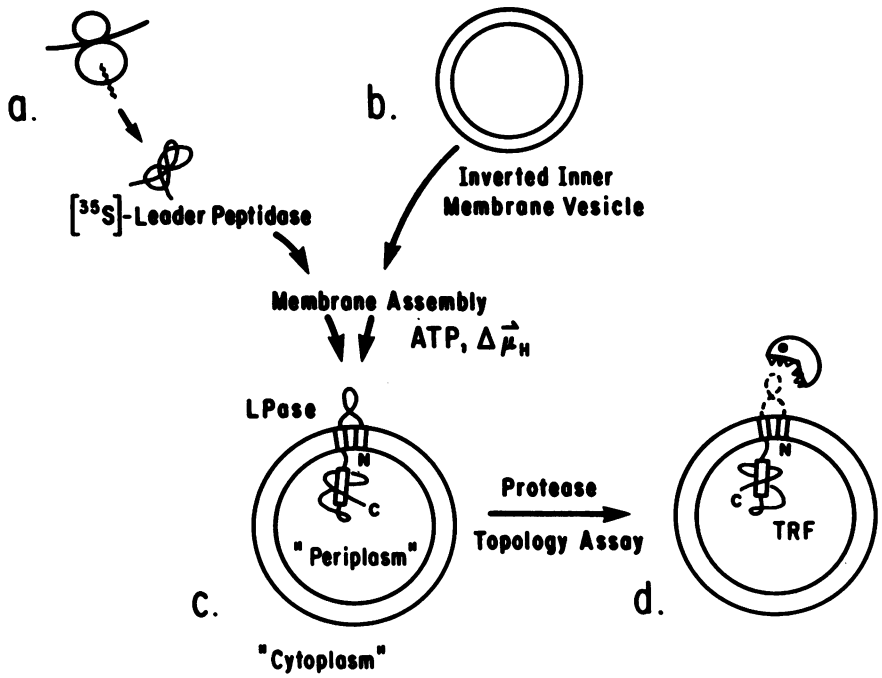

FIG. 1. Synthesis and membrane insertion of leader peptidase in a cell-free translocation reaction. (a) ^{35}S-labeled leader peptidase was synthesized in a cell-free reaction in the presence of *E. coli* IMVs (b). (c) Integration of leader peptidase in the in vivo orientation (10). The lumen of the vesicles corresponds to the periplasm of the intact cell. Rectangles represent hydrophobic domains. (d) Dotted lines show the polar region subject to proteolytic digestion.

teins have been designated either as xR, where x is the position of the amino acid which has been replaced by arginine, or Δx-y, indicating the deletion of residues from x to y (inclusive). Plasmids encoding wild-type leader peptidase or leader peptidase mutants were used as templates in DNA-directed protein synthesis reactions using the S-30 system of Zalkin et al., (21). Synthesis mixtures (25 μl) contained 0.8% arabinose, 9 μg of AraC protein, 1.5 μg of DNA, 20 μCi of Tran^{35}S-label (1,087 Ci/mmol; ICN Pharmaceuticals Inc.), and 2.4 mg of the IMVs described above per ml. PEG 6000 was omitted. After a 1-h synthesis at 37°C, samples were chilled to 0°C and portions were removed for proteinase K treatment in either the presence (Fig. 4, lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33) or absence (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, and 32) of detergent. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, and 31 represent 15% of the protein synthesized in the various synthesis mixtures. Following protease treatment, all samples were immunoprecipitated with antiserum to leader peptidase and visualized by SDS-PAGE. The protected wild-type TRF can be seen in lane 2. Removal of the amino terminus resulted in the loss of two of the eight methionine residues present in the full-length protein. Because we labeled proteins with [^{35}S]methionine, conversion of leader peptidase to the TRF form resulted in a decrease in radioactivity in protected leader peptidase peptides. Each of the lanes in Fig. 4 was quantified with a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments). In the experiment shown in Fig. 4, 40% of the wild-type leader peptidase molecules were able to insert with their correct transmembrane topology, an efficiency which is comparable to that seen for other proteins in this translocation reaction.

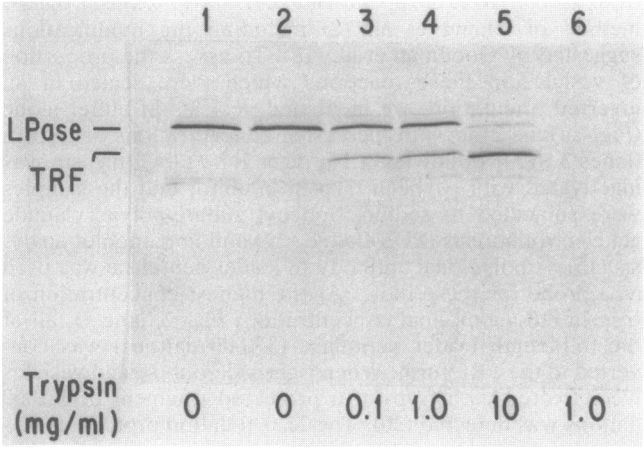

FIG. 2. Trypsin treatment of IMVs. Inner membranes from *E. coli* D10 (10 μl, 20 mg/ml) were incubated with increasing concentrations of trypsin (lanes 3 to 5) for 1 h at 0°C. Lane 2 contains undigested IMVs, and lane 6 contains IMVs digested with 1 mg of ml trypsin per ml in the presence of 1% Triton X-100. Digestions were stopped by the addition of 20 mg of soybean trypsin inhibitor per ml. Purified leader peptidase (LPase) (10 ng) was included as a standard (lane 1). Samples were subjected to SDS-PAGE and immunoblot analysis.

As seen in vivo (7–9), substitution of an arginyl residue at position 9 (R9; Fig. 4, lanes 19 to 21), 68 (R68; lanes 22 to 24), or 91 (R91; lanes 31 to 33) or removal of the third apolar domain (Δ83-98; lanes 13 to 15) did not prevent integration of leader peptidase into inverted vesicles. These mutants assembled with efficiencies of 28.3, 68.2, 18.5, and 34.4% for leader peptidases 9R, 68R, 91R, and Δ83-98, respectively. Removal of the first hydrophobic domain and the translocation poison sequence (16) inhibited translocation but did not

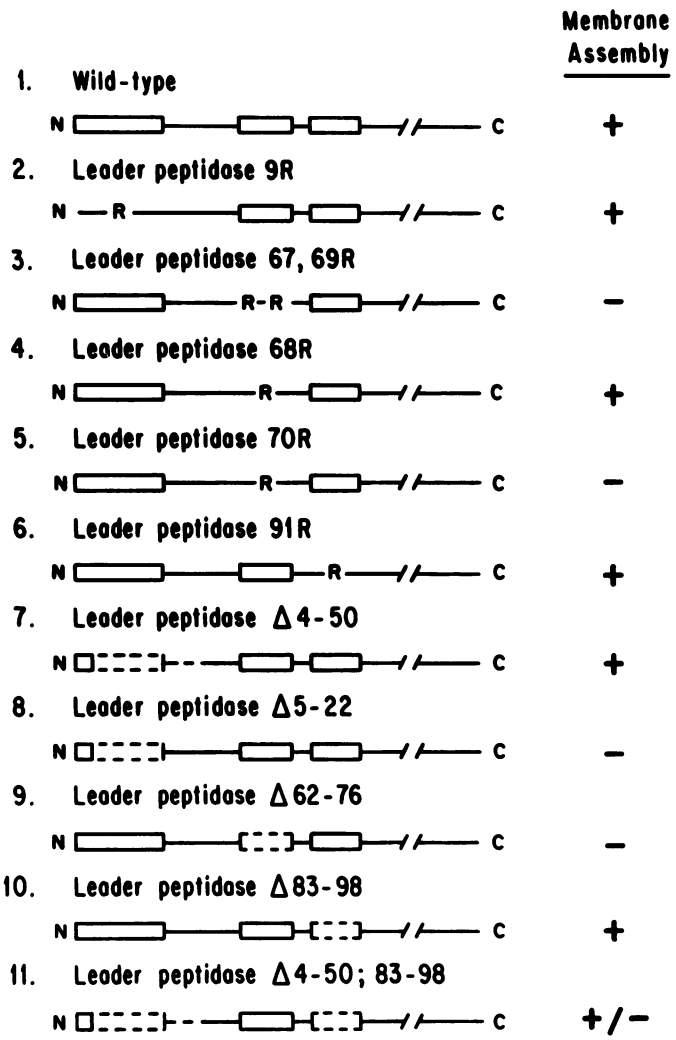

FIG. 3. Leader peptidase mutants and their in vivo membrane insertion characteristics. Mutants of leader peptidase have been designated either by the positions at which arginine residues have been inserted or by the residues which have been deleted (see text). Deleted residues are indicated by dashed lines.

inhibit it entirely (Fig. 4, lanes 4 to 6), with 5% of the mutant proteins correctly assembled. Deletion of both regions 4 through 50 and 83 through 98 further reduced the number of assembly-competent molecules to 1.2% (Δ4-50, 83-98′, lanes 16 to 18). As also found in vivo, removal of the second hydrophobic stretch of amino acids (Δ62-76; lanes 10 to 12), which we have shown to be the internal, uncleaved signal region, prevented leader peptidase insertion into vesicles. Likewise, when the signal sequence was interrupted by arginine residues at positions 67 and 69 (R67 and 69; lanes 25 to 27) or at residue 70 (R70; lanes 28 to 30), membrane insertion could not be achieved. Finally, removal of the first hydrophobic domain, leaving the translocation poison sequence, resulted in a diminished capacity for membrane insertion, with only 1.8% of the synthesized protein protected.

Taken together, these results suggest that each of the mutants tested has the same capacity to insert into the *E. coli* plasma membrane in vitro as in vivo. We also note that the difference in mobility on SDS-PAGE between wild-type leader peptidase (Fig. 4, lane 1) and its TRF (lane 2) was far greater than that between leader peptidase Δ4-50 (lane 4) and its TRF (lane 5). This demonstrates that it is the amino terminus of leader peptidase that is exposed on the surface of the IMVs after assembly, confirming that the protein assembles into the membrane with the correct orientation. This assembly reaction also requires the function of the *secY-prlA*-encoded protein (Fig. 5) as previously established for the membrane assembly of leader peptidase in vivo (18).

The ability to reconstitute segments of intracellular sorting pathways in cell-free systems is essential for complete characterization of these complex processes. In fact, use of in vitro systems may be necessary to uncover required components whose participation in membrane assembly would be difficult to demonstrate in vivo, as has been the case for the requirement for ATP. The study presented here shows that the wild-type and mutant leader peptidases assemble with their physiological orientations across the cytoplasmic membrane in a cell-free system. These results provide evidence that events observed in this in vitro translocation system reflect the properties of the intact cell. The faithful reflection of the in vivo roles of hydrophobic helper and poison domains in our in vitro assembly reaction opens the door to biochemical analysis of their roles in translocation.

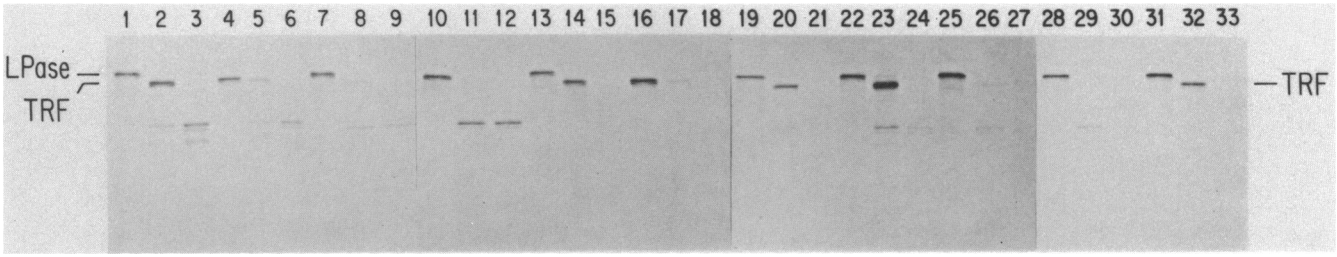

FIG. 4. Insertion of leader peptidase and mutants into *E. coli* inverted IMVs. Samples were digested (lanes 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, and 18) with 0.035 U of proteinase K (Sigma) per ml. Where indicated (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 33) Triton X-100 was present during the digestion at a final concentration of 1%. Samples were immunoprecipitated with anti-leader peptidase (LPase) antiserum and analyzed by SDS-PAGE and fluorography (1). Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, and 31 represent 15% of the proteins subjected to protease treatment in the adjacent lanes. Wild-type and mutant insertion reactions are presented in the following order: wild type leader peptidase (lanes 1 to 3), leader peptidase Δ4-50 (lanes 4 to 6), leader peptidase Δ5-22 (lanes 7 to 9), leader peptidase 62-76 (lanes 10 to 12), leader peptidase Δ83-98 (lanes 13 to 15), leader peptidase Δ4-50;83-98 (lanes 16 to 18), leader peptidase R9 (lanes 19 to 21), leader peptidase R68 (lanes 22 to 24), leader peptidase R67,69 (lanes 25 to 27), leader peptidase R70 (lanes 28 to 30), and leader peptidase R91 (lanes 31 to 33).

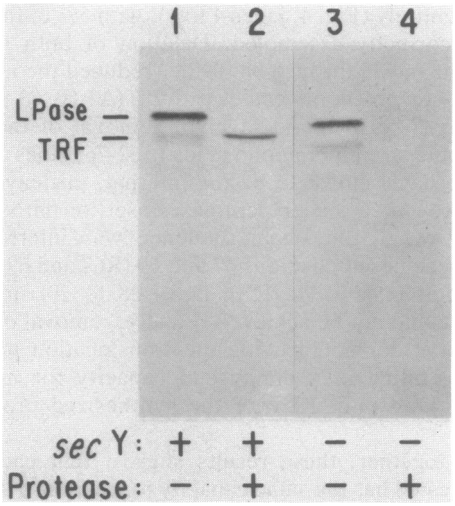

FIG. 5. *secY* requirement of membrane insertion of leader peptidase. The membrane insertion of wild-type leader peptidase (LPase) into DIO IMVs (lanes 1 and 2) or *secY*-deficient vesicles from *E. coli* CJ107 (lanes 3 and 4) was assayed by digestion with protease, as described in the legend to Fig. 4.

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