Insertion and folding of polytopic membrane proteins is an important unsolved biological problem. To study this issue, lactose permease, a membrane transport protein from *Escherichia coli*, is transcribed, translated, and inserted into inside-out membrane vesicles *in vitro*. The protein is in a native conformation as judged by sensitivity to protease, binding of a monoclonal antibody directed against a conformational epitope, and importantly, by functional assays. By exploiting this system it is possible to express the N-terminal six helices of the permease (N6) and examine changes in conformation during insertion into the membrane. Specifically, when N6 remains attached to the ribosome it is readily extractable from the membrane with urea, whereas after release from the ribosome or during insertion of additional helices, those polypeptides are not urea extractable. Furthermore, the accessibility of an engineered Factor Xa site to Xa protease is reduced significantly when N6 is released from the ribosome and inserted into the membrane. Moreover, in contrast to full-length permease, N6 is degraded by FtsI protease *in vitro*, and N6 with a single Cys residue at position 148 does not react with N-ethylmaleimide. Taken together, the findings indicate that N6 remains in a hydrophilic environment until it is released from the ribosome or additional helices are translated and continues to fold into a quasi-native conformation after insertion into the bilayer. Furthermore, there is synergism between N6 and the C-terminal half of permease during assembly, as opposed to assembly of the two halves as independent domains.

Most inner membrane proteins in *Escherichia coli* are targeted to the membrane by the signal recognition particle (SRP) pathway and insert into the membrane via the Sec machinery (1–4). It has been suggested that bacterial SRP (Ffh protein and 4.5 S RNA) binds to the hydrophobic region of nascent membrane proteins, and subsequently, the ribosome-nascent chain complex and SRP interact with the SRP receptor (FtsY) at the membrane surface. Recently, it has been proposed that FtsY is a primary membrane-anchoring site for the ribosome, and SRP binds to nascent protein after FtsY-ribosome binding (5). The Sec machinery is comprised of SecY, SecE, and SecG proteins in the cytoplasmic membrane (6–9) and contributes to the topology of some membrane proteins (10). It has been shown (11, 12) that the Sec machinery and YidC exist as a complex, and several inner membrane proteins interact with YidC during insertion. Thus, YidC appears to play a key role in inner membrane protein insertion (12). SecD, SecF, and YajC form another heterotrimERIC complex that binds to the SecYEG complex (13). In addition, it has been reported (14) that YidC interacts directly with SecDF rather than SecYEG, which forms an even larger complex with SecYEG. Furthermore, SecA, a cytosolic protein with ATPase activity, is also important for insertion of inner membrane proteins with large periplasmic loops (15, 16). Although some details regarding the mechanism of integral membrane protein insertion have been elucidated, there are still many issues that require resolution, particularly those related to insertion of polytopic membrane proteins. Examples include folding of intermediates, interaction between helices during translation/insertion, the order and timing of helices exiting from the translocon into the lipid bilayer, and topological determinants.

In an effort to study some of these issues we have chosen to use the lactose permease of *E. coli* (LacY) as a model system. LacY is a member of the Major Facilitator Superfamily (17) and catalyzes the coupled stoichiometric translocation of galactosides and H⁺ (symport) (18). The protein has been solubilized from the membrane and purified to homogeneity in a completely functional state (19, 20) and is a 12-helix bundle with the N and C termini on the cytoplasmic face of the membrane (21–23). In addition, LacY is physiologically (24) and structurally a monomer in the membrane (25–27). Analysis of an extensive library of mutants, particularly Cys replacement mutants (28), in conjunction with a battery of site-directed biochemical and biophysical techniques has led to the formulation of a tertiary-structure model (29) as well as a hypothesis for the mechanism of lactose/H⁺ symport (30).

Like most inner membrane proteins, LacY is inserted into the membrane co-translationally (31, 32), and insertion involves SRP and FtsY (33, 34) as well as SecY (35). Phosphadiylethanolamine (PE) is also important for the late maturation of LacY and required for proper assembly and function (36). PE can even reverse misfolding of LacY topology after insertion into membranes devoid of PE (37). However, the mechanism by which LacY is inserted into the membrane and folds into its final tertiary conformation is far from clear.
Remarkably, co-expression of LacY in two contiguous, non-overlapping fragments with a covalent discontinuity in either cytoplasmic or periplasmic loops leads to complementation resulting in resistance of the fragments to proteolysis and functional LacY (38–41). Furthermore, a salt bridge between Asp-237 (Helix VII) and Lys-358 (Helix XI) plays an important role in membrane insertion (42, 43), indicating that Helix VII must include a domain of at least 19 residues of XI before insertion of LacY into the bilayer. In addition, the relatively long middle cytoplasmic loop is important for functional expression, possibly acting as a time delay to allow the N-terminal six helices (N₆) to clear the translocon before the last six helices are inserted (44). To analyze these phenomena more completely, as well as the insertion mechanism of LacY, an in vitro transcription/translation/insertion system is described in which LacY appears to be inserted in a native, functional conformation. In this study, we focus on insertion of the N-terminal half of LacY (N₆). 

**EXPERIMENTAL PROCEDURES**

**Materials**—[³⁵S]Met, [¹²⁵I]protein A, and N-[¹⁴C]ethylmaleimide (NEM) were obtained from Amersham Biosciences. Factor Xa protease was obtained from New England Biolabs (Beverly, MA). Site-directed random polyclonal antisera against a dodcpeptide corresponding to the C terminus of LacY (45) was prepared by Berkeley Antibody Co. (Richmond, CA). Monoclonal antibody (mAb) 4B11 was prepared as described (46). Avidin-conjugated horseradish peroxidase was purchased from Pierce. 6-[(N-dansyl)-1-thio-D-galactopyranosyl (Dns₆-Gal) was synthesized as described (47). All other materials were obtained from commercial sources.

**Strains and Plasmids**—E. coli T184 [lacI° O–Y° (A) rpsL, met, thr, recA, hsdM, hsdRI, lacO° Z° (Y° A°)] (48) was used for the preparation of membrane vesicles. E. coli AR796 [MC4100 zdh::Tn10 zyg:3198::Tn10kan (49) or E. coli AR797 [AR796 Hstl(17)] (49) were used as indicated for in vivo expression of the N₆ and C-terminal half LacY (C₆). The lacY cassette gene in plasmid pT7–5 was used for in vitro synthesis of wild type LacY. Plasmids encoding LacY with three tandem Factor Xa protease sites in loop IV/V (53) were constructed as described. Strains and plasmids used are listed in Table I.

**Preparation of Inside-out (ISO) and Right-side-out (RSO) Vesicles**—ISO membrane vesicles were prepared as described (56) with minor modifications (57). ISO membrane vesicles were obtained from the 40% sucrose fraction (50, 45, 40, 35, and 30%; w/w) in 50 mM potassium phosphate (KPi; pH 7.5), 1 mM EDTA. ISO vesicles were frozen in liquid N₂ in 50 mM KPi (pH 7.5), 0.2 mM unlabeled Met was used instead of [³⁵S]Met. After incubation, the vesicles were washed in 500 mM NaCl, 2.0 mM CaCl₂, harvested by centrifugation, and resuspended in the same buffer without or with 1% DDM as indicated. Samples were fractionated by centrifugation on a 50% sucrose cushion and associated proteins and aggregated LacY, ISO vesicles were present during transcription/translation.

**In Vitro Transcription/Translation/Insertion of LacY**—In vitro transcription/translation/insertion reactions were carried out as described (63). ISO vesicles with in vitro synthesized LacY were isolated from a 50% sucrose cushion and pre-equilibrated with 20 mM lactose at 4°C overnight (~50 mg/ml membrane protein). Pre-equilibrated vesicles (~0.1 mg of membrane vesicle) were diluted into a cuvette containing 200 μl of 50 mM KP, (pH 7.5), 6 μM Dns₆-Gal. Fluorescence was recorded on an SLM-Aminco 8100 spectrophotofluorimeter (excitation, 340 nm; emission, 500 nm).

**RESULTS**

**In vitro Transcription/Translation/Insertion of LacY—LacY** was synthesized in vitro directly from a plasmid by using an E. coli S30 extract and [³⁵S]Met in the presence or absence of ISO membrane vesicles. To distinguish between membrane-associated proteins and aggregated LacY, ISO vesicles were fractionated by centrifugation on a 50% sucrose cushion and subjected to SDS/PAGE. The major [³⁵S]-labeled product exhibits an molecular mass of about 33 kDa, which is typical of LacY (i.e. the molecular mass of LacY is ~45 kDa, but it regularly exhibits an molecular mass of ~33 kDa on SDS/PAGE) and is detected in the membrane fraction exclusively when ISO membrane vesicles are present during transcription/translation (Fig. 1A). The band does not appear in the absence of plasmid lacY template DNA or the S30 extract alone (data not shown).

Integral membrane proteins are not extracted by 5% urea or at alkaline pH, and accordingly, LacY synthesized in vitro in the presence of ISO vesicles fractions with the membranes even after treatment with urea or alkali (Fig. 1, B and C). Thus, LacY synthesized in vitro appears to integrate into the membrane. Furthermore, when in vitro transcription/translation is carried out in the presence of liposomes or RSO vesicles rather than ISO vesicles, little or no membrane-associated LacY is...
observed (Fig. 1D). The findings confirm previous observations (64) showing that LacY insertion not only requires other membrane components (e.g., the SecYEG complex) but that insertion must occur vectorially from the cytoplasmic surface of the membrane.

To examine the topology of LacY inserted in vitro, the accessibility of engineered Factor Xa protease sites in a cytoplasmic or periplasmic loop to Factor Xa protease was tested. LacY with a cytoplasmic Factor Xa site at the N terminus of a biotin acceptor domain (BAD) (51) in loop VI/VII was transcribed/translated in vitro in the presence of ISO vesicles and digested with Xa protease in the absence or presence of DDM (Fig. 2A).

Clearly, in the absence of DDM, the band corresponding to full-length permease is completely digested, and a band corresponding to N terminus, the acceptor domain (BAD) with the BAD in the first four lanes and without in the second four lanes). B, mAb 4B11 binding. mAb 4B11 binds to a cytoplasmic epitope in LacY comprised of residues from loops VIII/IX and X/XI. ISO vesicles containing LacY synthesized and inserted in vivo were prepared as described (61) and purified as described under "Experimental Procedures." In vitro synthesis of LacY was carried out for 2 h at 30 °C in the presence of ISO membrane vesicles and purified as described. Both ISO vesicles preparations were then assayed for 4B11 binding as described under "Experimental Procedures." ~LacY, ISO vesicles from E. coli T184 expressing LacY; in vivo LacY, ISO vesicles from E. coli T184 expressing LacY; in vitro LacY 1 and 2, LacY with 2 Xa protease sites in cytoplasmic loop IV/V or single Cys-148 LacY, respectively.

LacY synthesized and inserted in vitro exhibits significant ligand protection against alkylation by NEM (Fig. 3B). Although background labeling is relatively high, NEM labeling is inhibited significantly and reproducibly by β-D-galactopyranosyl-1-thio-β-D-galactopyranoside. The data as a whole support the contention that LacY synthesized and inserted in vitro is in a native, functional conformation.

Insertion of N 6 -D-galactopyranosyl-1-thio-β-D-galactopyranoside.

FIG. 1. In vitro transcription, translation and insertion of LacY. A, LacY was synthesized in vitro in the absence or presence of ISO membrane vesicles (10 μg of protein). 35S-Labeled translation products from a 1-h incubation were carefully placed on a 50% sucrose cushion and centrifuged. The pellet and inner membrane fraction were analyzed. B, reaction components were fractionated as described. The inner membrane fraction was incubated with 5 μm urea for 20 min on ice in 50 mM KPi (pH 7.5), and the membranes were harvested by centrifugation. The supernatant fraction from the urea wash (sup) was precipitated with 10% trichloroacetic acid. C, the inner membrane fraction from the 50% sucrose cushion was subjected to alkali extraction at pH 11.3 or treatment with 5M urea. All samples were precipitated with 10% trichloroacetic acid. D, in vitro translation was carried out with liposomes, ISO membrane vesicles, or RSO membrane vesicles as indicated, and the vesicles were isolated by centrifugation on a 50% sucrose cushion and washed with 5 μM urea.

FIG. 2. Confirmation of LacY synthesized and inserted in vitro. A, Factor Xa protease cleavage of 35S-labeled mutants with engineered Xa sites. The membrane topology of in vitro synthesized and inserted LacY was studied by introducing Factor Xa protease sites into cytoplasmic loop VII/VIII or periplasmic loop VII/VIII. These mutants were transcribed and translated for 1 h in vitro. After the isolation of ISO membrane vesicles from the reaction mixture, Factor Xa protease was added to the membrane suspension in the presence or absence of DDM. Proteolysis was carried out for 12 h at 4 °C, and the samples were subjected to SDS/PAGE. Arrows indicate the position of intact LacY (with the BAD in the first four lanes and without in the second four lanes). B, mAb 4B11 binding. mAb 4B11 binds to a cytoplasmic epitope in LacY comprised of residues from loops VIII/IX and X/XI. ISO vesicles containing LacY synthesized and inserted in vivo were prepared as described (61) and purified as described under "Experimental Procedures." In vitro synthesis of LacY was carried out for 2 h at 30 °C in the presence of ISO membrane vesicles and purified as described. Both ISO vesicles preparations were then assayed for 4B11 binding as described under "Experimental Procedures." ~LacY, ISO vesicles from E. coli T184 expressing LacY; in vivo LacY, ISO vesicles from E. coli T184 expressing LacY; in vitro LacY 1 and 2, LacY with 2 Xa protease sites in cytoplasmic loop IV/V or single Cys-148 LacY, respectively.

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The presence of H9252 single Cys-148 LacY synthesized and inserted in vitro simultaneously. However, when N6 containing a stop codon is synthesized in vitro, it is not extracted by urea (Fig. 4, lanes 1, 3, and 5). Surprisingly, N6 truncated at position 226 and lacking a stop codon so that ribosomes remain attached is partially extracted from the membrane by urea (lane 3), and urea extraction is increased in the presence of 150 mM KCl (data not shown). The data suggest that the translation intermediate, N6 with an attached ribosome, is located in a relatively hydrophilic environment. Interestingly, the quantity of urea-soluble product is decreased by the addition of puromycin, which causes release of the polypeptide from the ribosome before urea extraction (lane 4). LacY truncated at position 312 and lacking a stop codon which contains 9 transmembrane helices followed by a bound ribosome is no longer susceptible to urea extraction even before treatment with puromycin (lanes 5 and 6). Thus, ribosome binding to LacY per se does not appear to affect susceptibility to urea extraction. The data are consistent with the interpretation that the N-terminal half of LacY does not fully insert into the lipid bilayer before ribosome release or subsequent synthesis and insertion of two of the C-terminal six helices.

Ribosome Attachment and Folding of N6—Either full-length LacY or N6 with two tandem Factor Xa protease sites in cytoplasmic loop IV/V synthesized and inserted in vitro are partially proteolyzed after 2 h of digestion at 4 °C in the presence of DDM (Fig. 5, compare lanes 1 and 2 with lanes 6 and 7, respectively). In contrast, N6 truncated at position 207 or 226 and lacking a stop codon so that ribosomes remain attached is digested completely by Xa protease (compare lanes 3 and 4 with 8 and 9, respectively). However, LacY truncated at position 312 with attached ribosomes is resistant to digestion (compare lanes 5 and 10). Although it is anticipated that the tandem Factor Xa protease sites in loop IV/V of LacY synthesized and inserted in vitro should be digested in ISO vesicles by Xa protease, little or no proteolysis is observed when the construct is synthesized in vitro in ISO vesicles, and DDM solubilization is required. Possibly, loop IV/V is occluded, which accounts for this behavior. In any case, in the presence of detergent at room temperature, the constructs synthesized in vitro are completely proteolyzed (see lanes 4 and 5 in Fig. 6). The results suggest that N6 attached to the ribosome does not fold sufficiently well to protect the Factor Xa sites in loop IV/V from proteolysis. Although not shown, the addition of puromycin to N6 with attached ribosomes increases sensitivity to proteolysis by Xa protease. Thus, insertion of N6 into the bilayer leads to more efficient folding of this polypeptide fragment.

Glu-126 (Helix IV) and Arg-144 (Helix V) are in close proximity and charged-paired (65–67), and two Cys residues at these positions spontaneously form a disulfide bond (53). N6 containing mutant E126C/R144C and two tandem Factor Xa protease sites in loop IV/V with or without a stop codon (i.e., without or with attached ribosomes) were synthesized in vitro and examined for disulfide bond formation by studying proteolysis of DDM (Fig. 5, compare lanes 1 and 2 with lanes 6 and 7, respectively).

**Fig. 3.** Function of LacY synthesized and inserted in vitro. A, lactose/Dns-β-Gal counter-flow. Experiments were carried out as described under “Experimental Procedures.” Fluorescence at 560 nm was recorded (excitation, 340 nm) as described. Reactions were initiated by diluting preloaded ISO vesicles with LacY synthesized and inserted in vitro into a cuvette with or without 20 mM lactose. B, NEM labeling of single Cys-148 LacY synthesized and inserted in vitro in the absence or presence of β-galactopyranosyl-1-thio-β-galactopyranoside (TDG). Experiments were carried out as described under “Experimental Procedures.”

**Fig. 4.** Urea extraction of in vitro synthesized and inserted [35S]N6 without or with attached ribosomes. In vitro synthesis and insertion was carried out for 30 min at 30 °C. As indicated, 1.5 mM puromycin was added to the reaction mixtures, and incubation was continued for an additional 10 min at 30 °C. Samples that were not incubated with puromycin were placed on ice. Vesicles were treated with 5 mM urea for 20 min on ice and centrifuged. The supernatants (sup) were aspirated carefully and precipitated with 10% trichloroacetic acid. Both the pellet (ppt) and supernatant fractions were solubilized in 1% DDM and subjected to SDS/PAGE and phosphorimaging. The pellet samples were exposed overnight, and the supernatant samples were exposed for 2 days. The number 226 or 312 refers to the last amino acid in the truncated LacY.

*sup* C. D. Wolin and H. R. Kaback, unpublished data.
Vitro synthesized and inserted full-length LacY, N6, or N6 without a stop codon at position 207, 226, or 312, all with 2 tandem Xa protease sites in cytoplasmic loop IVN, were treated with Factor Xa protease in the presence of DDM. In vitro synthesis and insertion was carried out at 30 °C for 1 h. Membranes with 35S-labeled LacY mutants were then purified by centrifugation as described under "Experimental Procedures." Vesicles were solubilized in 1% DDM, and Factor Xa protease was added for 2 h at 4 °C. Arrows indicate the molecular mass full-length LacY and each truncation mutant. The numbers denote the position of the last amino acid in the mutants.

When co-expressed with C6, however, N6 is unstable and degraded when expressed in the absence of C6. FtsH, a membrane-bound metalloprotease with ATPase activity, is responsible for degradation of certain integral membrane proteins (68). Therefore, expression of N6 in an FtsH temperature-sensitive mutant strain was examined (Fig. 7A) because the mutant lacks FtsH protease activity at high temperature (69). When expressed in wild-type cells (FtsH+), N6 is not observed (lane 1). However, when co-expressed with C6, bands corresponding to N6 and C6 are observed (lane 2). Interestingly, when N6 is expressed in the FtsH mutant at the non-permissive temperature, a clear band corresponding to N6 is observed (lane 3). At the non-permissive temperature, co-expression of N6 with C6 in the FtsH mutant also leads to a significant increase in the amount of each polypeptide observed. Clearly, N6 is a substrate for FtsH and requires C6 for stable expression in vitro.

By expressing either full-length single Cys-148 LacY or single Cys-148 N6 in the FtsH mutant, the reactivity of Cys-148 with NEM can be compared. Although the full-length and N6 constructs are expressed at about the same levels as judged by avidin blots (Fig. 7B, top), it is apparent that although Cys-148 labels readily with NEM as a function of time in full-length LacY, the sulphydryl group is completely unreactive in single Cys-148 N6 (Fig. 7B, bottom). These data indicate that N6 synthesized and inserted in vitro does not have the same tertiary structure as it does in native, full-length LacY.

**DISCUSSION**

Although previous studies (32, 64) indicate that LacY translated and inserted in vitro can be inserted into ISO membrane vesicles with the correct topology (36), questions remain as to whether or not the inserted protein is in a native conformation. Because LacY, like many other membrane proteins, is resistant to traditional means of structural analysis, alternative approaches have been developed to study topology and discern the overall three-dimensional fold (see Refs. 29 and 30).
The studies presented here, which utilize some of these approaches, provide convincing evidence that LacY synthesized and inserted into the membrane in vitro is in a native conformation. (a) By using engineered Factor Xa protease sites in cytoplasmic or periplasmic loops, the topology of the polypeptide with respect to the membrane appears to be correct. (b) A mAb that binds to a discontinuous epitope comprised of residues in cytoplasmic loops VIII/IX and X/XI (61) binds to LacY synthesized and inserted in vitro as well as it binds when LacY is synthesized and inserted in vivo. (c) Cys residues at positions 126 and 144 in the in vitro system undergo spontaneous cross-linking as observed in vivo (53). (d) LacY synthesized and inserted in vitro exhibits lactose/Dns-Gal counter-flow and significant ligand protection against alkylation of Cys-148. Therefore, transcription, translation, and insertion of LacY into ISO vesicles in vitro represents a system in which the mechanism of insertion of a polytopic membrane protein and its folding into a tertiary conformation can be studied reliably.

Despite conjecture regarding co-translational insertion of polytopic membrane proteins, it is unclear how many transmembrane helices are accommodated by the translocon before insertion into the bilayer. Furthermore, little information is available regarding whether folding into a tertiary conformation begins in the translocon or only after migration into the bilayer with the assistance of chaperones such as PE (36, 37, 70). The observation that the first six helices of LacY are extracted with urea when the ribosome remains attached, whereas the first eight helices are not, suggests that the translocon. Furthermore, little information is available regarding whether folding into a tertiary conformation must occur in the bilayer after both halves of the protein have exited the translocon, a conclusion consistent with the finding that PE acts as a molecular chaperone in the folding of LacY into a native, functional conformation must occur in the bilayer after both halves of the protein have exited the translocon, a conclusion consistent with the finding that PE acts as a molecular chaperone in the folding of LacY into a native, functional conformation.

The differential sensitivity of engineered Factor Xa protease sites in cellular loop IV/V to cleavage in N6 with attached ribosomes versus fully translated N6 suggests that the conformation of the two polypeptides differs. The observation that fully translated N6 is not extracted with urea whereas a significant amount of the translocation intermediate is urea-soluble (Fig. 4) implies that a folding event occurs after N6 exits the translocon. Cross-linking data with Cys residues at positions 126 (Helix IV) and 144 (Helix V) also support the interpretation that N6 folds after insertion into the bilayer (Fig. 5). However, it seems unlikely that N6 is synthesized and inserted in vitro has the same tertiary structure as it does in vivo, full-length LacY since N6 is clearly sensitive to proteolysis by FtsH protease in the absence of C6 but stabilized in its presence (Fig. 7A). Furthermore, N6 with a single Cys residue at position 148 does not react with NEM (Fig. 7B).

Unlike the two-dimensional projection map of OxtT (75), which indicates that each helix in the two halves of the protein occupy symmetry-related positions, neither the Na+/H+ antiporter NhaA (76, 77), the Na+/sugar symporter MelB (78), nor LacY (79) exhibit such symmetry. Rather, in these transport proteins, helices from the N- and C-terminal halves of the polypeptides interdigitate. Thus, it is not surprising that N6 in LacY probably does not insert into the bilayer in a native conformation, although some of the structural features of the native, full-length protein are observed. This being the case, it seems clear that folding of LacY into a native, functional conformation must occur in the bilayer after both halves of the protein have exited the translocon, a conclusion consistent with the finding that PE acts as a molecular chaperone in the folding of LacY into its final native conformation (36, 37).

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In Vitro Synthesis of Lactose Permease to Probe the Mechanism of Membrane Insertion and Folding
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