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Genetics

Assembly of a hetero-oligomeric membrane protein complex

(membrane protein assembly/protein folding/ATP-binding cassette superfamily transporter/fusion protein purification)

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ABSTRACT **The maltose transporter of *Escherichia coli* is a hetero-oligomeric complex located in the cytoplasmic membrane of the cell. The *in vivo* assembly of this complex has been examined by using an assay based on the proteolytic sensitivity of one of its components, MalF. Immediately after synthesis and insertion into the membrane, MalF is sensitive to exogenously added proteases. In a time- and complex assembly-dependent fashion, MalF becomes protease resistant. Using this assay, we show that MalF is inserted into the membrane independently of other components of the transport complex. The assembly of the maltose transport complex occurs subsequently from a pool of freely diffusing protein in the membrane. This assembly process is efficient and occurs with rapid kinetics.**

The folding of integral membrane proteins is a complicated and poorly understood process. These proteins generally have domains that are localized to different cellular compartments (the hydrophobic membrane, the aqueous cytoplasm, and the aqueous extra-cytoplasmic space) and that each have distinct structural features, dictated by their environments and amino acid sequences. Furthermore, understanding the folding of such proteins is complicated by the fact that many membrane proteins interact with other proteins to form homo- or hetero-oligomeric complexes.

Popot and Engelman (1) have proposed that the folding of integral membrane proteins can be considered as a two-stage process. (*i*) Protein topology is established as independently stable hydrophobic α-helices form membrane-spanning segments (MSSs) across the lipid bilayer. As part of this process, hydrophilic regions of the protein are retained on the cytoplasmic face of the membrane or are translocated to the extra-cytoplasmic space. (*ii*) Tertiary and quarternary structures are acquired through interactions between MSSs and/or hydrophilic domains of the protein(s). Although a number of methods exist to examine the topology of a membrane protein as defined in the first stages of folding, the later stages remain harder to study and have been examined in only a few cases. Our studies aim at understanding this second phase of folding for MalF, an integral membrane protein required for transport of maltose across the cytoplasmic membrane of *Escherichia coli*.

The signals directing the initial folding of MalF have been examined in several ways. A model of its two-dimensional arrangement in the membrane was first suggested by hydropathy analysis (2), which later was supported by fusion protein analyses (3–5). In this model, MalF crosses the membrane eight times, with cytoplasmically localized amino and carboxyl termini. In addition, our work suggests that (*i*) the protein integrates into the cytoplasmic membrane independently of the normal secretion machinery of *E. coli* (6); (*ii*) MalF has topogenic information distributed throughout its primary sequence, with the dominant topological signals being in the cytoplasmic domains (7, 8); and (*iii*) the topogenic signals within MalF act rapidly, as most domains of the protein are correctly localized to their final cellular compartment—cytoplasm, membrane, or periplasm—immediately after synthesis of the protein (9).

In contrast, we know little about the three-dimensional structure of MalF or how it is acquired. To study this, one must consider possible interactions of MalF with other proteins, especially those involved in maltose transport. In addition to MalF, maltose transport across the cytoplasmic membrane depends on the periplasmic maltose-binding protein (MBP) (or MalE protein), the integral cytoplasmic membrane protein MalG, and the peripheral cytoplasmic membrane protein MalK (10). Recently, Davidson and Nikaido (11) purified a complex containing one MalF, one MalG, and two MalK molecules from the *E. coli* membrane fraction. This purified complex can be reconstituted into proteoliposomes and can catalyze the ATP-dependent accumulation of maltose in the presence of MBP. MalK, which associates with the membrane from the cytoplasmic side, is probably responsible for the ATP hydrolysis that energizes the transport. It has a Walker consensus ATP-binding site (12), and mutations in or near this site prevent maltose transport (13).

The maltose transport system is part of the ATP-binding cassette superfamily along with other transporters, such as the multidrug resistance and cystic fibrosis gene proteins (14). A study of the assembly of the maltose transporter may provide important information on the assembly and structure of other ATP-binding cassette transporters. Here, we describe an assay that follows the *in vivo* folding of one component of the transporter, MalF, in the membrane. Our findings are consistent with the predictions of the Popot–Engelman two-stage model for membrane protein assembly.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strains BT8, BT10, JCB468, HS3018, BT23, and BT24 are all *malT*^{c} derivatives of MC4100 (F$^-$, *ΔlacU169, araD139, thiA, relA, rpsL*; laboratory collection). HS3018 carries the nonpolar *ΔmalE444* deletion (15). BT8 is a Mal$^+$ phage P1 transductant of HS3018. BT10 is a *malT*^{c} *zhe*::Tn*10* phage P1 transductant of EM6, which contains a *malG*$_{am}$ mutation (also *rif*^{r}, *metA*/KLF*10* *malG*$_{am}$; laboratory collection). JCB468 contains a *bla* cassette inserted in *malK* after nucleotide 15 of the coding sequence (J. Bardwell, personal communication). BT23 and BT24 are derivatives of BT10, transformed respectively with plasmids pBAD18 (L. Guzman and J.B., unpublished work) and pGJ50. Plasmid pGJ50 is a derivative of pBAD18 with the *supF* gene (16) under control of the *araBAD* promoter (G. Jander and J.B., unpublished work). Strain BT3 is a *ΔmalB101 zjb*::Tn*5* (*ΔmalE,F,G,K,lamB,malM*) phage P1 transductant of DHB4 that contains the plasmid pSX102.

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Abbreviations: MSS, membrane-spanning segment; AP, alkaline phosphatase; MBP, maltose-binding protein; HA, hemagglutinin.

Plasmid pSX102 codes for the MalF–alkaline phosphatase (AP) J fusion protein under control of the *tac* promoter (3).

Media, Enzymes, and Chemicals. Media were made according to Miller (17). Cells for proteolysis experiments were grown in minimal M63 medium supplemented with 0.2% glycerol, thiamine, and all amino acids except cysteine and methionine. Cells producing the MalF–AP J fusion were grown in LB medium. The enzymes trypsin, chymotrypsin, and DNase I and the soybean trypsin inhibitor were obtained from Boehringer Mannheim. Lysozyme, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, L-histidyldiazobenzylphosphonic acid-agarose, DEAE-Sephacel, and phenylmethylsulfonyl fluoride were obtained from Sigma; [^{35}S]methionine was obtained from Amersham.

Antibodies. Rabbit polyclonal antiserum against glucose-6-phosphate dehydrogenase was from D. Fraenkel, Harvard Medical School. Goat anti-rabbit IgG-AP was from Boehringer Mannheim. Polyclonal antiserum specific for MalF was elicited in a rabbit that had been immunized with purified MalF–AP J fusion protein. The MalF antiserum was characterized for its ability to immunoprecipitate various MalF–β-galactosidase fusion proteins (B.T., K. McGovern, and J.B., unpublished work). This antiserum specifically recognizes the large hydrophilic domain of MalF between MSSs III and IV.

Purification of the MalF–AP J Fusion Protein. We have developed another approach for purification of active bacterial AP fusion proteins based on an affinity-chromatography resin designed for the purification of calf intestinal AP (18). BT3 cells were grown with aeration at 37°C in LB medium plus ampicillin at 200 μg/ml. At OD_{600} of 0.7, isopropyl β-D-thiogalactoside was added to 5 mM, and cells were induced for 3 hr. Harvested cells were disrupted by incubation with 50 mM Tris·HCl, pH 8.0/20% (wt/vol) sucrose/10 mM EDTA, lysozyme at 0.1 mg/ml/1 mM phenylmethylsulfonyl fluoride followed by two cycles of freeze–thaw. Viscosity of the cell lysate was reduced by treatment with DNase I at 0.2 μg/ml and 20 mM $MgCl_2$. Lysed cells were fractionated by centrifugation at 12,000 × *g* for 20 min. The supernatant fraction was decanted and discarded. The membrane pellet was washed once with 50 mM Tris·HCl, pH 8.0/10 mM EDTA/1 M NaCl and then extracted with 50 mM Tris·HCl, pH 8.0/10 mM EDTA/1% Triton X-100. After centrifugation at 35,000 × *g* for 45 min, the fusion protein was found predominantly in the supernatant (the Triton extract).

The Triton extract was dialyzed against 50 mM Tris·HCl, pH 8.0/10 mM EDTA/1% Triton X-100 and then loaded onto a DEAE-Sephacel column equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0–0.5 M NaCl in a buffer of 20 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1% Triton X-100. The fusion protein eluted from the column with a peak around 0.2 M NaCl, as determined by AP activity assays (19). Partially purified fusion protein from the DEAE column was dialyzed against 20 mM Tris/0.1% Triton X-100/1 mM $MgCl_2$/0.1 mM $ZnCl_2$ buffer and then loaded onto an L-histidyldiazobenzylphosphonic acid-agarose column in the same buffer. The bound protein was eluted with this buffer plus 100 mM Na_2HPO_4.

Proteolysis Experiments. Bacteria were diluted 1:100 from fresh overnight cultures into M63 medium and grown 3–4 hr at 37°C with aeration. At OD_{600} of 0.25–0.35, cells were harvested (for Fig. 2) or labeled with [^{35}S]methionine at 40–50 μCi/ml (for Figs. 1 and 3; 1 Ci = 37 GBq) and then harvested for preparation of spheroplasts as described (9). Proteolysis was done at 0°C with freshly prepared trypsin or chymotrypsin and stopped by the addition of phenylmethylsulfonyl fluoride at 0.4 mg/ml and trypsin inhibitor at 10–25 μg/ml. Samples for immunoblot analysis were prepared by collecting the spheroplasts by centrifugation and resuspending them in gel sample buffer. After SDS/PAGE, proteins were transferred to nitrocellulose, and the immunoblots were developed as described (20), with the following differences. The nitrocellulose filter was incubated first with buffer containing MalF antiserum at a 1:2000 dilution and then with a goat anti-rabbit IgG conjugated to AP (1:7500 dilution) as the secondary antibody. Antigen–antibody complexes were detected by incubation of the blot with the chromogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

For analysis of labeled proteins, 0.5-ml portions of the treated cells were mixed with an equal volume of 4% Triton X-100/100 mM Tris·HCl, pH 8.0/0.3 M NaCl/2 mM EDTA before immunoprecipitation. Samples were mixed and frozen and thawed three times and then centrifuged for 10 min at 4°C to remove insoluble material. The upper 0.8 ml of each was transferred to a fresh tube, and proteins were immunoprecipitated as described, with antisera against MalF and glucose-6-phosphate dehydrogenase and staphylococcal protein A (9). Equivalent amounts of each sample were analyzed by SDS/PAGE on 12.5% resolving gels. Quantitation of proteins was as described (9).

RESULTS

The results presented below show that a change in the periplasmic regions of MalF occurs as a consequence of assembly of the protein into the maltose transport complex. In the absence of this assembly, MalF is maintained in the cytoplasmic membrane in a form that is sensitive to cleavage by exogenous proteases and that remains competent for folding into its final state with MalG and MalK. Upon assembly of the MalFGK complex, the MalF protein becomes protease resistant. The experiments described here are done with cells producing the proteins from chromosomally encoded genes, so that possible artifacts from protein overproduction are avoided. Furthermore, the availability of mutants completely deficient in the production of various Mal proteins enables the characterization of MalF alone and in combination with the other proteins.

Protease Sensitivity of MalF. Our assay for the assembly of higher-order structures for MalF relies on a determination of the susceptibility of the protein to proteolytic degradation once the protein is properly localized in the cytoplasmic membrane. Two lines of experimentation have shown that MalF is rapidly inserted into the membrane. MalF–AP fusion proteins were rapidly incorporated into the cytoplasmic membrane of *E. coli*, as evidenced by their sensitivity to exogenously added protease in spheroplasts (where only the external face of the membrane is exposed) immediately after the completion of synthesis (9). In addition, we found that newly synthesized, intact MalF was also protease sensitive when spheroplasts of pulse-labeled cells were treated with exogenous proteases (Fig. 1; see also ref. 6). The proteolytic cleavage of MalF in spheroplasts must occur in domains of the protein exported to the periplasm. Potential targets for cleavage are the large ≈180-amino acid hydrophilic domain between MSSs III/IV and the ≈30-amino acid hydrophilic loops between MSSs V/VI and VII/VIII. The first periplasmic loop of MalF between MSSs I and II is small—approximately three amino acids—and probably not susceptible to proteolytic attack.

Despite its sensitivity to externally added proteases in spheroplast preparations, MalF was resistant to endogenous cellular proteases. We measured the turnover of MalF in growing cells by comparing the amount of MalF synthesized during a short pulse-labeling with [^{35}S]methionine with the amount present after a chase with unlabeled methionine. Eighty to 100% of the labeled MalF made during a 30- to 60-sec pulse remained after a 30-min chase at 37°C. This

stability was observed in the presence or absence of the other components of the transport system.

Although newly synthesized and pulse-labeled MalF was largely sensitive to exogenous proteases in spheroplasts, 80–100% of such labeled protein in Mal$^+$ strains was protease resistant after a chase with unlabeled methionine (Fig. 1). We considered that the specific folding of higher-order structures for MalF might be responsible for the acquisition of protease resistance by the protein. As this folding could be due to either intra- or intermolecular interactions, we tested whether the assembly of MalF into a protease-resistant state was influenced by the association of MalF with other proteins—in particular, other components of the maltose transport system. MalF in cells deficient in the production of MalG exhibited high protease sensitivity immediately after synthesis (Fig. 1). However, unlike the protein in Mal$^+$ cells, the MalF protein, in the absence of MalG, did not become protease resistant after a chase. MalF also failed to acquire protease resistance in *malK*$^-$ cells (data not shown, but see Fig. 2).

We also examined the protease sensitivity of MalF under steady-state conditions by analyzing the products of proteolysis reactions on immunoblots (Fig. 2). In these experiments, MalF appeared protease-resistant in Mal$^+$ cells, whereas in *malG*$^-$ and *malK*$^-$ cells, it again was found to be protease-sensitive. In addition, we asked whether MBP (which interacts with the MalFGK complex but is not a part of it) might influence the assembly of MalF into a protease-resistant state. Cells deleted for *malE* produce protease-resistant MalF indistinguishable from that in Mal$^+$ cells on the immunoblots.

These data suggest that the resistance of MalF to exogenous proteases results from the assembly of the protein into a complex with MalG and MalK. Only the subunits directly involved in the cytoplasmic membrane maltose transport complex were required for this protease resistance. Complex assembly was very efficient, as 85–100% of the MalF protein

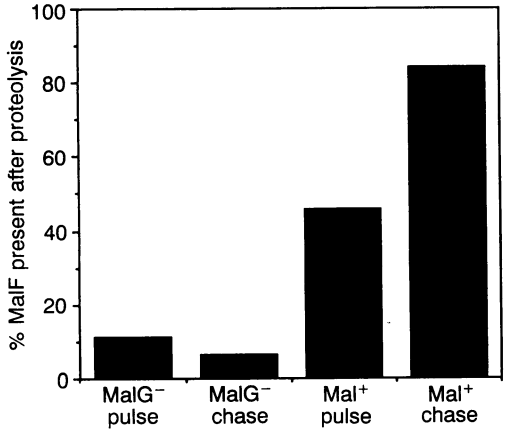

FIG. 1. Acquisition of protease resistance by MalF. Mal$^+$ and MalG$^-$ strains (BT8 and BT10, respectively) were pulse-labeled for 1 min with [^{35}S]methionine, with one-half of each culture being chased with unlabeled methionine for 30 min. Spheroplasts were treated with trypsin at 10 μg/ml, and proteins were immunoprecipitated with antiserum against MalF. Proteins were separated by SDS/PAGE, and the amount of intact, labeled MalF in the proteolyzed samples was compared with the unproteolyzed control for both the pulse and chase time points. In all samples, glucose-6-phosphate dehydrogenase was simultaneously immunoprecipitated to control for variations in sample protein concentration and integrity of spheroplasts (9). Results shown are from one typical experiment. Results from ≈10 similar experiments fall in the range noted in text for Mal$^+$ strains (similar observations were also made with a MC1000 derivative, data not shown). Protease sensitivity of MalF in BT10 typically was between 80–95% at both pulse and chase time points.

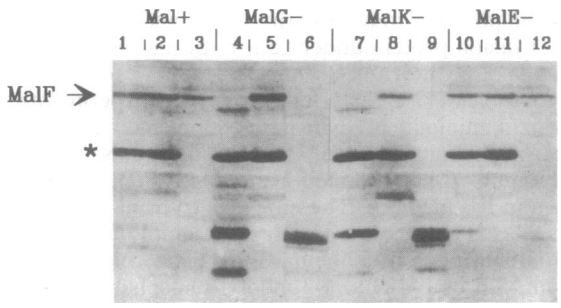

FIG. 2. Proteolytic sensitivity of MalF in different Mal strains. Spheroplasts prepared from strains BT8 (lanes 1–3), BT10 (lanes 4–6), JCB468 (lanes 7–9), and HS3018 (lanes 10–12) were treated with chymotrypsin (lanes 1, 4, 7, and 10) or trypsin (lanes 3, 6, 9, and 12) at 20 μg/ml. Portions of the unproteolyzed control (lanes 2, 5, 8, and 11) or proteolyzed reactions were separated by SDS/PAGE, and proteins were detected on the immunoblot with MalF antiserum. Position of intact MalF is indicated. A background protein band is marked with a star.

synthesized acquired protease resistance. It is possible that the small amount of protease-sensitive MalF detected in Mal$^+$ cells after the chase or under steady-state conditions reflects a slight sensitivity of the protein in transport complexes to proteases rather than a percentage of the protein that remains unassembled. Conversely, the low levels of protease-resistant MalF detected in *malG*$^-$ or *malK*$^-$ cells probably stem, at least in part, from a proportion of treated cells that had not actually formed spheroplasts, with their outer membranes permeabilized so that the proteases would have access to the periplasmic face of the cytoplasmic membrane.

Kinetics of Acquisition of Protease Resistance for MalF. We determined the rate at which MalF assumed a protease-resistant conformation in the cytoplasmic membrane. Mal$^+$ cells were pulse-labeled with [^{35}S]methionine for 30 sec and chased for various times with unlabeled methionine. Quantitation of the amount of intact MalF after proteolysis indicated that the protein acquired its protease resistance with a half-time of 30–90 sec after synthesis under these growth conditions, in which the culture had a doubling time of ≈60 min (data not shown).

Posttranslational Association of MalF and MalG. We showed that MalF synthesized in the absence of MalG or MalK was retained in the cytoplasmic membrane as a stable protein. Next, we determined whether MalF synthesized in the absence of MalG remained in an assembly-competent state. To ascertain whether the assembly of the transport complex occurred from a freely diffusing pool of proteins within the membrane or only from the association of newly synthesized proteins, we temporally separated the synthesis of MalG from that of MalF and MalK. A *malG*$_{am}$ strain, in which *malF* and *malK* were constitutively expressed, was transformed with a plasmid containing the *supF* amber suppressor gene under control of the tightly regulated *araBAD* promoter. This strain also was transformed separately with the plasmid vector as a control. Cells were pulse-labeled with [^{35}S]methionine, and the labeled MalF was incorporated into the membrane in both strains in a protease-sensitive form (Fig. 3). Approximately 30 sec after the addition of nonradioactive methionine for the chase, arabinose was added to induce expression of the amber suppressor tRNA from the *araBAD* promoter. Incubation of the culture continued for 30 min, and the protease resistance of the previously membrane-incorporated and labeled MalF then was examined. We found that a portion of the labeled MalF was chased into the protease-resistant state only after induction of the amber suppressor and, therefore, of MalG synthesis. (The fraction of MalF that acquired protease resistance was probably

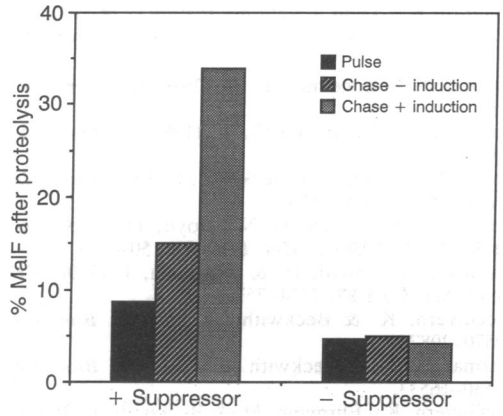

FIG. 3. Suppression of protease sensitivity of MalF in *malG*$^-$-containing strain. Cultures of the *malG*$_{am}$-containing strain with a plasmid containing *supF* and with vector plasmid (BT24 and BT23, respectively) were pulse-labeled with [^{35}S]methionine for 1 min. Chases were done with unlabeled methionine for 30 min, either with or without addition of arabinose to 0.2%. Samples were treated with chymotrypsin at 10 μg/ml and analyzed as described for Fig. 1.

consistent with the amount of full-length MalG produced by the rather inefficient suppression of the *malG* amber codon by *supF*.) Because synthesis and labeling of MalF occurred before induction of MalG synthesis occurred, MalF must have remained in an assembly-proficient state in the absence of MalG. Therefore, newly synthesized MalG can randomly associate with the pool of MalF in the membrane, leading to the acquisition of protease resistance for MalF.

DISCUSSION

Our results suggest that by examining the proteolytic sensitivity of MalF, one can study the assembly process for the maltose transport complex. We propose that, in the assembled MalFGK complex, protease-sensitive regions of MalF in the periplasm become inaccessible to exogenous protease, either because they are masked or because of a conformational change in MalF. This change requires not only MalG, which resides in the membrane with MalF, but also MalK, which interacts with MalF and MalG on the cytoplasmic face of the membrane. Although it has been suggested that a homolog of MalK, HisP, of the histidine transport system of *Salmonella typhimurium* has regions that extend into the cytoplasmic membrane (21), there is no evidence that such a tight membrane association exists for MalK. MalK seems to mediate from the cytoplasmic face of the membrane (either directly or indirectly through MalG), a change in the periplasmic regions of MalF. However, the conformational shift in MalF, resulting in its protease resistance does not at any point require an interaction with MBP.

The proteolytic sensitivity of MalF in spheroplasts in the different strains shows that MalF does not *require* MalG or MalK for membrane insertion. We conclude that MalF *ordinarily* inserts into the membrane independently of MalG and MalK, based on the rapidity of its membrane insertion in the *malG*$^-$ and *malK*$^-$ strains. Therefore, the assembly of the maltose transport complex occurs within the membrane, rather than in the cytoplasm before its insertion into the membrane.

In addition, MalF does not fold into a "dead-end" conformation in the absence of complex assembly. A MalF polypeptide will associate with a MalG polypeptide that is synthesized at a later time (Fig. 3). The synthesis of MalG in the *malG*$_{am}$ strain can be induced (via the induction of an amber suppressor) after the synthesis and labeling of MalF with >30% of the labeled MalF becoming protease resistant during the 30-min chase. Similar levels of protease resistant MalF are detected when induction of amber suppressor synthesis occurs 5 min into the chase (data not shown). This level of protease-resistant MalF is lower than that seen in a true Mal$^+$ strain (as in Fig. 1). We believe this difference is due to limiting amounts of MalG available for assembly into transport complexes relative to the amount of MalF. In general, the amount of protein synthesized via amber suppression by *supF* is lower than wild-type expression. We, therefore, expect only a fraction of full-length MalG to be produced under these conditions, compared to that in a *malG*$^+$ cell. In these experiments, the expression of MalF is constitutive. Newly synthesized (and unlabeled) MalF along with all the previously synthesized (labeled and unlabeled) MalF compete for the relatively small pool of the full-length, amber-suppressed MalG (which is continually synthesized during the chase).

We conclude from the experiments of Fig. 3 that the assembly of the maltose transport complex occurs from a freely diffusing pool of unassembled MalF and MalG molecules in the membrane. One might have imagined a preference for newly synthesized MalF and MalG polypeptides to coassemble, especially as MalF and MalG are translated from the same message. The translation and membrane insertion of MalG presumably occur very near those of MalF. However, such a preference in the assembly process would have prevented suppression of the protease sensitivity of the labeled MalF polypeptides and, therefore, is inconsistent with our observations.

One might argue that the difference in the protease sensitivity of MalF is from changes in the topology of the protein rather than from changes in the higher-order structures of the protein. That is, the protease-sensitive form of MalF may represent one in which different regions of the protein are exposed in the periplasm than are exposed in the protease-resistant form of the protein. To address this possibility we assayed the topology of MalF in Mal$^+$, *malG*$^-$, and *malK*$^-$ strains with several MalF–AP fusions. Changes in the topology of the fusion proteins are reflected in changes in AP activity (7, 8). The activities of the MalF–AP B, J, K, L, and O fusions (3) were measured in the three background strains at several time points after induction of the synthesis of the MalF–AP fusions. In this way, we could detect any dependence of AP activity of the fusion protein on the stoichiometry of the other Mal proteins. For all fusions, the amount of AP activity measured and the shape of the induction curve are similar in the three backgrounds (data not shown). These results indicate that the topology of MalF does not vary in response to interactions with either MalG or MalK. Gennis and colleagues (22) also have suggested that the membrane topologies of the *Rhodobacter sphaeroides* light-harvesting complex L subunit and cytochrome b_1 proteins are similarly independent of the other proteins with which they interact.

Approximately 90% of the MalF synthesized during a pulse-labeling is chased into the protease-resistant form in Mal$^+$ strains. There are at least two possible explanations for this observation. (*i*) MalF may be synthesized in essentially stoichiometric amounts with MalG and MalK, so that most Mal proteins made are assembled into transport complexes. Alternatively, MalF may be the limiting component for complex assembly with excess MalG and MalK components being degraded or remaining unused. The first possibility is similar to that seen with the assembly of some eukaryotic homo-oligomeric membrane proteins. The latter more closely resembles the assembly of some eukaryotic hetero-oligomeric membrane protein complexes, as discussed below.

The process of membrane protein complex assembly has been examined for proteins such as the hemagglutinin (HA) of influenza virus, and the acetylcholine receptor. Oligomerization of these proteins occurs in the endoplasmic reticulum

and is required for transport to the plasma membrane. The homotrimeric complexes of HA form with a half-time of 7–10 min after translocation into the endoplasmic reticulum (23). Similar to the assembly of MalF with MalG, the assembly of the HA complex occurs from a freely diffusing pool of HA monomers within the endoplasmic reticulum membrane (24). Although the oligomerization process for HA is of equivalent final efficiency (80–90%; ref. 23) to that of the maltose transport complex (at least from the perspective of MalF), the required time for assembling the maltose transporter is much shorter.

The assembly of the hetero-oligomeric acetylcholine receptor is somewhat different. The $\alpha_2\beta\gamma\delta$ pentamer assembles stepwise, proceeding from the formation of $\alpha\gamma$ and $\alpha\delta$ dimers (25). The subsequent oligomerization steps are less defined but probably proceed from the formation of the $\alpha\gamma\alpha\delta$ tetramer to the final addition of the β subunit (26). The β subunit may be the limiting component for complex assembly (26, 27). About 70% of the α subunit synthesized in cultured mouse muscle cells is never assembled into receptors and is rapidly degraded (28). Furthermore, all the receptor subunits when expressed individually have relatively short half-lives (27), in marked contrast to the persistence of unassembled MalF in the *E. coli* cytoplasmic membrane in both *malG*$^-$ and *malK*$^-$ strains.

The conclusion that the MalF and MalG components of the maltose transporter are independently synthesized and inserted into the membrane before complex assembly supports the notion that such proteins fold independently into assembly-proficient structures. They must then associate and fold into their final functional forms. A related phenomenon may occur with the folding of different domains of the same protein. There are now several reports of proteins, originally present in the membrane as single polypeptide chains, which can be functionally reconstituted from or synthesized as two separate polypeptides. These proteins include bacteriorhodopsin, the β_2-adrenergic receptor, lactose permease, the Fe^{3+} transport FhuB protein, and the yeast a-factor transporter STE6 (29–33). The ability of separated portions of a polypeptide to functionally associate may reflect common features in the assembly of higher-order structures of both single subunit and oligomeric membrane proteins, as predicted in the Popot–Engelman model (1).

Although membrane protein assembly has been examined in other systems, there are many advantages to studying this process in *E. coli* with the maltose transport complex. Our knowledge of the two-dimensional structure of MalF, the *in vivo* behavior of the Mal proteins, the *in vitro* biochemical characterization of the maltose transport complex, and the availability of many *mal* mutations make the maltose transport complex an attractive system for the study of membrane protein folding.

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