Evidence for Multiple Pathways in the Assembly of the *Escherichia coli* Maltose Transport Complex

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The biogenesis of membrane protein complexes is a critical cellular activity that is difficult to study and poorly understood. Because of the technical challenges, the assembly of multimeric membrane protein structures has been extensively characterized in only a few cases. From studies on models such as the acetylcholine receptor, the T-cell antigen receptor, and the major histocompatibility complex Class I loading complex, it has been concluded and is generally accepted that membrane complexes have an ordered assembly pathway (1–5).

Here we study the assembly of the maltose transport complex, a simple hetero-oligomeric membrane protein complex in the cytoplasmic membrane of *Escherichia coli* (6, 7) (Fig. 1). This is a powerful model because the biochemistry and genetics of the system are highly developed, and numerous mutants are available. This complex consists of the integral membrane proteins MalF and MalG and the peripheral cytoplasmic membrane ATP-binding protein MalK, which form a 1:1:2 complex that transports maltose into the cell. MalFGK₂ is a part of the ATP-binding cassette transporter superfamily in which members share a common domain structure with high sequence similarity in the ATP-binding domain (8, 9). The domains can be linked into a single polypeptide or be separated into different proteins that assemble into multimeric complexes.

Previously, we demonstrated that MalK forms a dimer in the absence of the MalF/MalG membrane proteins and suggested that this interaction may represent an initial step in the assembly of this complex (10). This dimer is the dominant species in the population of MalK molecules found in soluble cellular extracts. We want to identify other interactions among MalF, MalG, and MalK as part of our analysis of the *in vivo* folding pathway for this complex. Assuming the participation of the MalK dimer, the possible pathways for tetramer formation are: 1) F + G → FG + K₂ → FGK₂, 2) F + G + K₂ → FK₂ + G → FGK₂, and 3) F + G + K₂ → F + GK₂ → FGK₂.

The analysis of membrane protein complex formation has generally relied on cellular co-fractionation studies or identification of stable subcomplexes of various subunit combinations to define intermediates in an assembly pathway. Previous experiments in other laboratories examining the MalFGK₂ assembly pathway have produced varied and even contradictory results. One study suggests that MalK associates with MalF in the absence of MalG but not with MalG in the absence of MalF (consistent with pathway 2) (11). Earlier work provides evidence consistent with pathway 3 but not pathway 2 (12). Subsequently, Moreau *et al.* (13) found that both MalF and MalG are required for MalK to fractionate with the membrane, consistent with pathway 1 (13). They propose that the EAA loop, a highly conserved hydrophilic motif present in a cytoplasmic domain of MalF and of MalG, might act as a primary contact point with MalK. Recently, the crystal structure of the homologous BtuCD ATP-binding cassette transporter of *E. coli* also revealed primary contacts between EAA-related cytoplasmic loops of the transmembrane subunit BtuC and the BtuD nucleotide-binding domain, consistent with the importance of the MalF/MalG EAA loops for associating with MalK (14). The current availability of structural data on ATP-binding cassette transporters and of a diverse collection of Mal mutants suggests that the biogenesis of this transporter now may be productively re-evaluated.

In the following experiments, we examined the intermolecular associations among all three subunits of the MalFGK₂ complex using immunoprecipitation techniques. Evidence for an ordered pathway in the T-cell antigen receptor assembly includes the ability of certain combinations of subunits (but not others) to co-immunoprecipitate when expressed pairwise in cultured cells (1, 2). Similarly, finding that certain combinations of Mal subunits can associate, whereas others cannot (or

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that the subunits associate with substantially different affinities, would suggest a particular order for the assembly of this complex. However, we found that each component of the maltose transporter efficiently formed an immunoprecipitable complex with each of the others when they are expressed pairwise in a cell, consistent with a non-ordered or “promiscuous” assembly pathway. Furthermore, we expressed mutant Mal proteins, previously characterized for their ability to oligomerize into the MalFGK$_2$ complex, in both two- and three-protein combinations. The behavior of the mutant and wild type proteins was evidence for a novel assembly strategy employing multiple pathways for this heteromeric complex.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—Various compatible plasmids (Table I) were combined in strain BT6 (MC1000 $\Delta$malB101::yhiTn5 (ΔmaIE,F,G,K,lain,B,malM) $\Delta_{\text{mal}}$ (lacP)). When co-expressed, MalF and MalG alleles were carried by the same plasmid. Insertion mutations in malI and malG were described previously (17, 20). malFI178 and malFI410 contain a 31-amino insertion (i31) in codons 178 and 410, respectively, of the malF gene. The $\text{ht}_{\text{MalK}}$ gene of plasmid PSS733 expresses an N-terminal-tagged form of MalK (HT-MalK) with six His residues and a thrombin cleavage site (19). Experiments done with chromosomal expression of the mal genes were performed with strains BT8, BT45, and HS169 (respectively, mal$,^+$, malG$,^+$, and $\Delta$malG, as described in Refs. 11 and 21).

**Media**—M63 minimal medium was supplemented with glycerol, thiamine, and all amino acids except cysteine and methionine with the appropriate antibiotics, as described previously (10, 21). Isopropyl-1-thio-β-galactopyranoside was used at 1 m.

**Preparation of Labeled Whole Cell Extracts**—Cultures were grown with aeration in M63 medium at 37 °C to an $A_{600}$ of 0.3. For experiments with plasmid-borne mal alleles, 1 ml of culture was induced with 1 mM isopropyl-1-thio-β-galactopyranoside at 4 °C. For experiments with chromosomal mal alleles, uninduced cells were labeled for 5 min. Labeling was stopped by the addition of 0.05% cold Met, and the cultures were immediately placed on ice. For pulse-chase experiments, 2 ml of culture were induced with 1 mM isopropyl-1-thio-β-galactopyranoside for 5 min, and then 158 μCi of $[^35]$Met was added. After 5 min, 0.05% cold Met was added, and 1 ml of culture was removed and placed on ice (pulse sample). After 15 min of additional incubation at 37 °C, a 1-ml chase sample was placed on ice. The cells were harvested, washed one time with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA (buffer A), and then resuspended in 0.5 ml of buffer A. The resuspended cells were lysed with one 15-s burst of a probe sonicator. The lysates were stored at −80 °C.

**Immunoprecipitation of Protein Complexes**—Sonicated whole cell lysates were solubilized with 1% dodecyl maltoside for 20 min on ice. Unlysled cells and insoluble material (less than 5–10% of the total labeled signal) were removed by centrifugation for 10 min at 16,000 × g at 4 °C. The soluble fraction was diluted 1:10 in buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.01% dodecyl maltoside). Antiserum was added and incubated on ice for 1 h. Immune complexes were recovered with protein A-Sepharose CL-4B beads (Amersham Biosciences; 50% in buffer B) after incubation at 4 °C for 30 min, as described by Kennedy and Traxler (10). Immunoprecipitated samples were heated to 65 °C for 20 min in gel-loading buffer before the proteins were separated by SDS-PAGE. Antisera used included a polyclonal antiserum specific for MalF (21), a polyclonal antiserum specific for the 31-residue insertion sequence (22), and a monoclonal antiserum specific for the His$_6$ thrombin tag (Katze Laboratory, University of Washington). Control experiments showed that 70–80% of the labeled protein recognized by the antibody was recovered in the primary immunoprecipitations. Co-immunoprecipitations done with supernatant fractions after a primary immunoprecipitation showed the same relative proportions of proteins recovered in the secondary immunoprecipitation step.

For the in vitro mixing studies, the lysates of cells expressing different combinations of Mal subunits were solubilized with dodecyl maltoside as above and then mixed. The extracts were incubated at 37 °C for 3 h, and then the appropriate antiserum was added. The samples were processed for immunoprecipitation as described above.

**Quantitation**—Phosphorimages were scanned using a Molecular Dynamics phosphorimaging device (SF or Storm), and the data were analyzed with the IQMac software version 1.2. Because the intensity of each protein on a phosphorimage is directly proportional to the number of $^{35}$S-containing residues in each molecule, the relative proportions of the proteins (data reported in the text and in the tables) were calculated by dividing the intensity of the band with the number of cysteines and methionines in each different protein; thus normalizing the values for the purpose of quantitative comparison. The quantitations of co-immunoprecipitations were averaged from independently labeled and immunoprecipitated extracts. Differences in association between Mal subunits of ±0.02–0.03 are not considered significant in our analysis, consistent with the background present in control studies between MalF and TsrR47 (see “Results” below).

**RESULTS**

**Immunoprecipitation of the MalFGK$_2$ Complex**—To study the biogenesis of the integral membrane maltose transporter, we characterized proteins from pulse-labeled cell extracts that should contain fully and partially assembled complexes. In the majority of our experiments, the Mal subunits were expressed from plasmids in a strain with a deletion at the chromosomal malFGK locus so that the proteins of interest were labeled at high specific activity. MalF, MalG, and one of two functional epitope-tagged MalK derivatives, either His$_6$-tagged MalK (HT-MalK) or MalK containing a 31-amino acid insertion (MalK550), were co-expressed (10, 19). The proteins were immunoprecipitated using an antiserum that recognizes one of the maltose transporter subunits, either MalF-specific or HT-specific (for HT-MalK or i31-specific for (MalK550 or other i31 mutants).

In experiments similar to that shown in Fig. 2, the MalF-specific or a MalF-specific antiserum was used to co-immunoprecipitate all three Mal proteins from cell extracts containing MalF, MalG, and MalK (Fig. 2). Quantification of the proteins co-immunoprecipitated showed that different proportions of the Mal subunits were recovered with the different antisera. The MalF-specific antiserum captured higher proportions of MalF, whereas the MalK-specific antiserum recovered higher proportions of MalI relative to the other Mal proteins (Table II), indicating the presence of unassembled Mal proteins and of partially and fully assembled Mal complexes in these extracts. These experiments are distinct from those of Davidson and Nikaido (23) who co-immunoprecipitated the subunits of the maltose transporter in proportions of 0.25 MalF:0.25 MalG:0.50 MalK using a MalF-specific antiserum. In that study, the Mal proteins were expressed from the chromosomal genes and were immunoprecipitated from steady-state labeled membrane preparations to look specifically at the stoichiometry of the fully assembled complex. The relative proportions of Mal sub-
units in our immunoprecipitations likely are because of our conditions, which are designed to examine the assembly process and to facilitate the characterization of mutants. In addition, we used whole cell extracts instead of isolated membranes, which allows us to capture soluble MalK not associated with the membrane. Even so, when HT-MalK was expressed from a lower copy number plasmid (and MalK is presumably limiting for tetramer assembly), the relative proportions of proteins recovered in our co-immunoprecipitations with the HT-specific antibody were similar to the expected ratios for the proteins present (Fig. 2 and Table III). In every combination, the co-immunoprecipitated protein constituted a significant proportion of the total protein signal recovered (Table III). These data indicate that each maltose transporter subunit is competent to associate to a substantial degree with each of the other subunits, raising the possibility that each of these combinations may represent assembly intermediates of the complex. Extracts from cells expressing two different pairwise combinations of the Mal proteins from their chromosomal genes gave similar results (Table III, lines 1 and 6). In each case, the degree of association between the pairs was less than that observed when all three components were expressed.

To evaluate the stability of the various heteromeric complexes, we compared the recovery of the Mal protein subcomplexes from extracts labeled with a 5-min pulse versus a 5-min pulse with a 15-min chase (with continued expression of unlabeled subunits). For the combinations of MalF/MalG, MalF/HT-MalK, and MalG/HT-MalK, there was no more than a 1% change in any of the proportions of species that co-immunoprecipitate (e.g., when MalF and MalG were co-expressed, MalF-specific antiserum recovered proportions of 0.84 MalF:0.16 MalG at the pulse versus 0.85 MalF:0.14 MalG after the chase; this is the averaged data from three independent experiments). (Likewise, when all three components of the maltose transporter were co-expressed in pulse-chase studies, there was only a modest change in the proportions of the proteins recovered, consistent with continuing assembly of the tetrameric complex; data not shown.) 80–100% of the MalF and/or MalK subunits present at the pulse time points persisted until the end of the chase, indicating that degradation by endogenous proteases was not occurring. These data indicate that the labeled hetero-
Three categories: EAA mutants are MalF<sup>EAA</sup>, MalG<sup>EAA</sup>, and MalG<sup>EAA</sup>; pd3 mutants are MalG<sup>pd3</sup> and MalG<sup>pd3</sup>; and the pd2 mutant is MalF<sup>pd2</sup>.

...was added. In no case did we detect a significant (greater than 2% of total signal) co-immunoprecipitation of a Mal subunit individually were mixed, and MalF- or HT-specific antiserum was added. Extracts expressing MalF, MalG, and HT-MalK were reassembled with unlabeled subunits produced during the chase. Furthermore, none of the heteromeric complexes was degraded by cellular proteases as misfolded protein.

To determine whether proteins were able to associate spuriously and co-immunoprecipitate under these experimental conditions, we performed two control studies. First, we tested if the pairwise Mal protein complexes form in vitro after protein solubilization. Extracts expressing MalF, MalG, and HT-MalK individually were mixed, and MalF- or HT-specific antiserum was added. In no case did we detect a significant (greater than 2% of total signal) co-immunoprecipitation of a Mal subunit that was not specifically recognized by the added antiserum, implying that the interactions we detected were formed in vivo. Second, we tested the effectiveness of the membrane solubilization and the nonspecific interaction between two unrelated membrane proteins. MalF<sup>F</sup> and TsrR<sup>47</sup> (the methyl-accepting chemotaxis protein with a 31-residue insertion) were co-expressed, and the proteins were co-immunoprecipitated with MalF- and i31-specific antisera. With MalF antiserum, the proportion of signal from TsrR<sup>47</sup> is about 3%. When i31 antiserum in the plasmid-containing strain expressing MalK<sup>550</sup>, the proportions were 0.15:0.13:0.72 (F:G:K550).

### Table II

**Proportions of Mal subunits precipitated from 3-protein expressing cells**

| Mal proteins expressed<sup>a</sup> | (n)<sup>b</sup> | Relative proportions<sup>c</sup> | aFF:G:K | aHT: F:G:K |
|----------------------------------|---------------|--------------------------------|--------|----------|
| MalF<sup>F</sup>                 | MalG<sup>F</sup>                    | MalK<sup>F</sup>                    | 0.53±0.28: NA | NA<sup>d</sup> |
| MalF<sup>F</sup>                 | MalG<sup>F</sup>                    | MalK<sup>F</sup>                    | 0.19   | NA       |
| MalF<sup>F</sup>                 | MalG<sup>F</sup>                    | MalK<sup>F</sup>                    | 0.55±0.27: NA | NA<sup>d</sup> |
| MalF<sup>F</sup>                 | MalG<sup>F</sup>                    | MalK<sup>F</sup>                    | 0.18   | 0.82     |
| MalF<sup>EAA</sup>              | MalG<sup>EAA</sup>                  | MalK<sup>HT</sup>                   | 0.49±0.28: 0.22:0.20: | 0.91     |
| MalF<sup>EAA</sup>              | MalG<sup>EAA</sup>                  | MalK<sup>HT</sup>                   | 0.67±0.29: 0.03:0.05: | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.04   | 0.92     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.65±0.28: 0.07:0.07: | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.07   | 0.86     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.70±0.25: 0.03:0.06: | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.05   | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.80±0.14: 0.07:0.06: | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.06   | 0.87     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.80±0.15: 0.05:0.03: | 0.91     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.05   | 0.92     |
| MalF<sup>pd2</sup>              | MalG<sup>pd3</sup>                  | MalK<sup>HT</sup>                   | 0.45±0.27: 0.19:0.15: | 0.91     |

<sup>a</sup> All combinations in strain BT8 are expressing Mal proteins from alleles carried by plasmid vectors, except for the MalF<sup>F</sup> MalG<sup>F</sup> MalK<sup>F</sup> combination indicated by italics where proteins were expressed by chromosomal genes in strain BT8. MalF<sup>F</sup> and MalG<sup>F</sup> mutants were grouped into three categories: EAA mutants are MalF<sup>EAA</sup>, MalG<sup>EAA</sup>, and MalG<sup>EAA</sup>; pd3 mutants are MalG<sup>pd3</sup> and MalG<sup>pd3</sup>; and the pd2 mutant is MalF<sup>pd2</sup>.

<sup>b</sup> (n) is the number of independently labeled and immunoprecipitated extracts analyzed for each combination; the proportions from each experiment were averaged to give these values.

<sup>c</sup> aFF and aHT are immunoprecipitations done with antiserum specific for MalF and for His<sub>6</sub> thrombin tag.

<sup>d</sup> NA, not applicable; with the i31 antiserum in the plasmid-containing strain expressing MalK<sup>550</sup>, the proportions were 0.15:0.13:0.72 (F:G:K550).

### Table III

**Proportions of Mal Subunits precipitated from 2-protein expressing cells**

| Mal proteins expressed | (n)<sup>b</sup> | Relative proportions<sup>c</sup> | aFF | aHT | i31 |
|------------------------|---------------|--------------------------------|-----|-----|-----|
| MalF<sup>F</sup>       | MalG<sup>F</sup>      | MalK<sup>F</sup>      | 0.93±0.07 | NA  | NA  |
| MalF<sup>F</sup>       | MalG<sup>F</sup>      | MalK<sup>F</sup>      | 0.88±0.12 | NA  | NA  |
| MalF<sup>EAA</sup>     | MalG<sup>EAA</sup>    | MalK<sup>HT</sup>     | 0.82±0.18 | NA  | NA  |
| MalF<sup>EAA</sup>     | MalG<sup>EAA</sup>    | MalK<sup>HT</sup>     | 0.87±0.13 | NA  | NA  |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>HT</sup>     | 0.89±0.11 | NA  | NT  |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>HT</sup>     | 0.82±0.18 | NA  | NA  |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>HT</sup>     | 0.84±0.16<sup>d</sup> | NA  | NA  |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK550               | 0.94±0.06 | 0.10±0.90 | NA |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK550               | 0.95±0.05 | NA  | 0.11±0.89 |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>F</sup>      | 0.93±0.07 | NA  | NA  |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK550               | NA  | 0.08±0.92 | NA |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>HT</sup>     | NA  | 0.12±0.88 | NA |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK550               | NA  | 0.05±0.95 | 0.98±0.02 |
| MalF<sup>pd2</sup>     | MalG<sup>pd3</sup>    | MalK<sup>HT</sup>     | NA  | 0.04±0.96 | 1.00 |

<sup>a</sup> The strains expressing MalF/MalG and MalF/MalK from the normal chromosomal locus (data in italics) were H5169 and BT15, respectively.

<sup>b</sup> (n) is the number of independently labeled and immunoprecipitated extracts analyzed for each combination; the proportions from each experiment were averaged to give these values.

<sup>c</sup> aFF, aHT, and i31 are immunoprecipitations done with antiserum specific for MalF, for His<sub>6</sub> thrombin tag, and for the 31-residue insertion, respectively.

<sup>d</sup> The proportions given in bold indicate that the antiserum used was specific for the protein expressed from the lower copy number plasmid. MalF and MalG variants were co-expressed from the same plasmid.

<sup>e</sup> NA, not applicable.

<sup>f</sup> NT, not tested.
with the hypothesis that all of these subcomplexes represent intermediates that can lead to MalFGK₂ assembly.

The Effect of Insertion Mutants of MalF and MalG on Pairwise Association of the Mal Proteins—Previously, we isolated a number of malF and malG derivatives (17) that each expresses a mutant protein containing a similar insertion of 31 residues at different locations in the protein. We examined these for their ability to transport maltose and to tetramerize into a MalFGK₂ complex and identified several that are transport-deficient and apparently have defects in the oligomerization of the maltose transport complex (using a MalF protease sensitivity assay) (17, 21). Several of these MalF and MalG mutants (Fig. 1) were tested in the co-immunoprecipitation assay.

First, we tested MalF and MalG derivatives with disruptions in the conserved cytoplasmic EAA loop, shown previously to be important for the association of MalK with the integral membrane components (13). These MalF or MalG EAA loop mutants were expressed pairwise with MalK or HT-MalK, and immunoprecipitations were performed with MalF- or HT-specific antiserum. The MalF_EAA mutant MalF410 reduced the efficiency of co-immunoprecipitation of MalK (Table III, compare lanes 7 and 10, which have the same expression context). The MalG_EAA mutants MalG574 and MalG575, which behaved the same in these assays, also reduced the ability of the mutant to co-immunoprecipitate with HT-MalK. The association of the MalF_EAA and MalG_EAA mutants in the pairwise expression studies was not impaired (Table III).

Other i31 derivatives analyzed with these experiments were MalF and MalG mutants in insertions in a periplasmic domain of each protein (MalFpd2 and MalGpd3). Initially it seemed likely that the assembly defects of these mutants were probably because of a defect in the association of MalF and MalG with one another. Each of these periplasmic domain mutants was expressed pairwise with the wild type membrane protein partner (MalFpd2/MalG or MalF/MalGpd3) and immunoprecipitated with MalF- or i31-specific antiserum. Somewhat surprisingly, the association of these MalF and MalG mutants was not significantly different from the association of their wild type counterparts. These data imply that the assembly defects of these mutants are not manifested at the level of this pairwise association (Table III). When the MalGpd3 mutants (MalG577 and MalG579, which behaved the same in these assays) were expressed pairwise with HT-MalK, they exhibited a substantially reduced ability to interact in this assay. Interestingly, this data suggested that the folding defect of the MalGpd3 mutants with lesions in an extracytoplasmic domain is transmitted to structure(s) present on the internal surface of the membrane, where MalG presumably interacts with MalK.

Effect of Cytoplasmic Domain Mutations of MalF and MalG on MalFGK₂ Assembly—To gain further insight into the assembly of the MalFGK₂ complex, we assayed the co-immunoprecipitation of several mutants in cells expressing alleles of the malF, malG, and malK genes simultaneously. Although some of the data presented below followed a predictable pattern based on the preceding analysis, other data suggested interesting interactions among the various species. First, we examined MalF_EAA and MalG_EAA mutants with HT-MalK. When strains expressed both EAA mutants with HT-MalK, the association of HT-MalK with the mutant membrane components was significantly reduced compared with the association of HT-MalK with MalF’ MalG’ (Fig. 3 and Table II), consistent with our expectations based on the pairwise expression studies. Curiously, the quantitation of the MalF-mediated immunoprecipitations showed an elevated association between the MalF_EAA and MalG_EAA proteins in extracts also containing HT-MalK compared with that observed in the pairwise expression studies (29 versus 18% corresponding to the co-precipitated MalF_EAA signal), even though the HT-MalK was not significantly recovered in these samples.

When only one of the membrane components contained a disruption in its EAA loop, and three mal genes were co-expressed, there was also a significant but somewhat less severe reduction in the association of HT-MalK with the membrane components compared with the extracts where both EAA domains were disrupted (Fig. 3 and Table II). For example, the presence of wild type MalF with MalG_EAA mutants did not enable the recovery of significant amounts of HT-MalK in the MalF-mediated immunoprecipitations. These results indicate that an insertion in only one EAA domain can significantly affect the recovery of MalK with the membrane components by co-immunoprecipitation.

Effect of Periplasmic Domain Mutations of MalF and MalG on Complex Assembly—The MalF and MalG mutants with insertions in the periplasmic domains also were assayed by co-immunoprecipitation in three protein expression experiments. First, we tested MalF178 (MalFpd2) in these studies. This mutant did not vary significantly from wild type MalF for the co-immunoprecipitation with wild type MalG and HT-MalK (Fig. 4 and Table II). Although we previously thought that MalF178 inhibited complex oligomerization, that conclusion was not supported by this data.

The MalGpd3 mutants had a different phenotype from both the MalFpd2 mutant and the EAA mutants. When MalG577 or MalG579 was expressed with wild type MalF and HT-MalK, the co-immunoprecipitation of the whole complex was reduced (Fig. 4 and Table II). When MalF-specific antiserum was used, the co-immunoprecipitation of the MalG mutant with MalF was substantially less than that seen with wild type MalG or with the MalG_EAA mutants (e.g. 0.80 MalF:0.15 MalG_EAA versus 0.49 MalF:0.28 MalG’). The level of MalGpd3 protein in the MalF-mediated immunoprecipitations was comparable with that seen in the pairwise expression studies with MalF-MalG. The co-immunoprecipitation of HT-MalK with MalF dropped to near background levels for extracts containing either MalG577 or MalG579 despite the presence of substantial amounts of apparently monomeric wild type MalF. Using HT antiserum, the co-immunoprecipitation of MalF and MalGpd3 with HT-MalK dropped to near background levels. These results showed

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1 E. G. Gachelet and B. Traxler, unpublished data.
that insertions in the third periplasmic domain of MalG reduce
the overall association between the Mal subunits. In fact,
the presence of a MalGpd3 mutant largely prevents the recovery of
an expected MalF-HT-MalK complex. Strains expressing both
MalFpd2 and MalGpd3 mutants displayed the same behavior as
the strains with the MalG mutants and wild type MalF (Fig. 4
and Table II).

**DISCUSSION**

We have analyzed the assembly of a heteromeric membrane
protein by immunoprecipitation. We found that each subunit of
the maltose transporter was able to interact with each of the
other subunits when expressed together in the absence of the
third, and we isolated MalF-MalG, MalF-MalK, and MalG-
MalK complexes. For every pairwise combination, the propor-
tion of co-immunoprecipitated species represented 8–16% of
the total immunoprecipitated protein. Because of the quantity
of each pairwise complex and the similar stability of these
complexes in pulse-chase analyses, we propose that all of these
heteromeric species are intermediates that can participate in
the assembly of the maltose transport complex. The assembly
of MalFGK2 likely is achieved by any one of several possible
pathways (Fig. 5A).

We also characterized several MalF and MalG mutants in
our studies, measuring their abilities to form pairwise inter-
mediates and the whole MalFGK2 complex. A change in one of
the membrane components can affect the interactions of that
mutant with one, two, or no other Mal subunit(s). The range of
phenotypes observed for the different mutants confirms that
our co-immunoprecipitation assay is robust and reveals differ-
ent interactions in which the various domains of the Mal sub-
units participate. In the interpretation of our data from the
mutant analysis that follows, the direct quantitation of the
extent of association between proteins in immunoprecipitations
is critical for our conclusions. We propose that the behaviors of
several mutants support our hypothesis of a promiscuous as-
sembly pathway for MalFGK2.

Insertions into the conserved cytoplasmic EAA loops of MalF
and MalG reduced the amount of MalK that co-immunoprecipi-
tated with the membrane proteins but did not reduce the level
of MalF-MalG co-immunoprecipitation in both the three-pro-
tein and the pairwise expression studies. This indicates that
the EAA loops have determinants that are specific for interac-
tions with the MalK components of the transporter and that
disruption of this motif affects only the stable association of MalK
with MalF and MalG. Furthermore, both EAA domains in the
MalF-MalG membrane complex are important for the efficient
c-o-immunoprecipitation of HT-MalK. This may be because a
MalK dimer is incorporated into the complex, and each copy
needs to associate with an EAA loop in order for MalK to remain
associated as measured in this assay. Our assays are consistent
with the phenotypes of the most severe mutants characterized by
Mourez et al. (13), which disrupt the association of MalK with
solute-ligated MalF-MalG when only one was an EAA mutant.

When MalF/MalG/MalK extracts with one or two EAA mu-
tants were immunoprecipitated with MalF antibodies, the asso-
ciation of MalF with MalG was higher than the association observed when mutant or wild type MalF and MalG were
expressed pairwise (30% versus 15% for the intensity of the
MalG signal) and was similar to the association observed with
the immunoprecipitation of a wild type MalFGK2 complex. This
suggests that MalK plays a role in increasing the level of asso-
ciation between MalF and MalG EAA mutants even though the
immunoprecipitated complex did not contain MalK.

We propose that all possible Mal subcomplexes form and partic-
ipate in assembly, accounting for this phenotype (Fig. 5B). How-
ever, the reduced affinity of MalK for an EAA mutant
prevents recovery of MalK with the membrane proteins by
c-o-immunoprecipitation. The simplest explanation for this is
that there are secondary interactions between MalK and the
mutant membrane proteins in regions outside of the EAA mo-
tif. In our experiments, undetected MalF-EAA-MalK and/or
MalG-EAA-MalK complexes might form based on these sec-
ondary interactions and promote a second assembly step that
leads to a stable interaction between the two membrane components.
However, EAA lesions disrupt critical stabilizing contacts bet-
ween MalF-MalG and MalK, leading to the loss of MalK in the
co-immunoprecipitations. In light of this explanation, it is
notable that the structure of the BtuCD ATP-binding cassette
transporter shows interactions between the BtuD ATP-binding
protein with the BtuC membrane domain outside of the equiv-
alent of the EAA loop of that transporter (with part of a trans-
membrane domain and two other cytoplasmic loops) (14).

The MalGpd3 mutants showed a different pattern of interac-
tions with MalF and MalK. Comparing the pairwise expression
studies of the mutants and the wild type protein, the MalGpd3
MalK interaction was significantly reduced, whereas the asso-
ciation of MalF and MalGpd3 appeared to be uncompromised
(Table II). The lesions in the third periplasmic domain of MalG
therefore have the unusual characteristic of altering structure
across the membrane in cytoplasmic domains that interact
with MalK. In three-component extracts containing MalF,
MalGpd3, and HTMalK, the association of MalF with both
MalGpd3 and MalK was reduced compared with the wild type
proteins, with substantial amounts of uncomplexed Mal pro-
tein in the extract (Table II; MalF-MalGpd3 still present at the
level seen in pairwise extracts, although MalF-MalK is not
recovered in significant amounts). We interpret our data from
cells expressing MalF, MalGpd3, and HTMalK as follows (Fig.
5C). We suggest that both the MalF-MalGpd3 and MalF-MalK
(but not MalGpd3-MalK) intermediate complexes are formed
(with the expected frequency seen in pairwise expression
strains); that the MalF-MalGpd3 heterodimer is a stable, dead-
end complex, which does not participate further in assembly;
and that MalGpd3 disrupts any MalF-MalK intermediates that
form, releasing both MalK and MalGpd3 from MalF (and pre-
venting the efficient recovery of the MalF-MalK intermediate

![Image](http://www.jbc.org/Downloaded from on July 25, 2018)
Assembly of the Maltose Transport Complex

**Assembly of the Maltose Transport Complex**

![Proposed assembly pathways of the maltose transport complex and various Mal mutants.](image)

**Fig. 5. Proposed assembly pathways of the maltose transport complex and various Mal mutants.** The various Mal subunits present in different assembly reactions are indicated here as: MalF, (F); MalK<sub>2</sub>, (K<sub>2</sub>); MalG, (G); MalF410, (F<sup>410</sup>); MalG577 or MalG579 (G<sup>577</sup> or G<sup>579</sup>). In each panel, the *circled* complexes represent the major species recovered in immunoprecipitations (and possibly the final assembly state that can be achieved with the different mutants). A, MalFGK<sub>2</sub> assembly. We propose that there are multiple assembly pathways for the wild type transport complex. MalK forms a dimer very efficiently. MalF, MalG, and MalK<sub>2</sub> then associate to form three different heteromeric intermediates. These intermediates all can proceed to form the final tetramer by the addition of the third component. B, effect of EAA cytoplasmic mutants on assembly. (In this example, we show only one EAA mutant for simplicity; however, the immunoprecipitation results are similar with one or two EAA mutants present in the extract, thereby suggesting this model.) The recovery of MalK with MalF<sup>EA</sup> and MalG is significantly reduced; however, the association of MalF<sup>EA</sup>-MalG with MalK is equivalent to that observed with wild type proteins. This indicates that the presence of MalK enhances the assembly of the membrane components without being immunoprecipitated with the final complex. We suggest that this is because of the formation of transient, unrecovered complexes (such as MalF<sup>EA</sup>-MalK<sub>2</sub>) that still participate in assembly and lead to the formation of a stable MalF<sup>EA</sup>-MalG complex. C, effect of MalG<sup>pd3</sup> periplasmic mutants on assembly. Overall co-immunoprecipitation of the Mal proteins is reduced such that the amount of MalF-MalG<sup>pd3</sup>-MalK<sub>2</sub> complexes recovered is similar to that recovered from pairwise expressing cells, whereas the Mal-F-MalK and the MalG<sup>pd3</sup>-MalK complexes are significantly reduced. We suggest that the MalG<sup>pd3</sup> cannot associate with MalK, preventing the formation of the MalG<sup>pd3</sup>-MalK<sub>2</sub> intermediate and the further assembly of MalF-MalG<sup>pd3</sup>. However, we propose that the Mal-F-MalK<sub>2</sub> intermediate forms but is completely disrupted by the MalG<sup>pd3</sup> subunit.

from these extracts). The 5-min window during which the pulse labelings occur would be ample time for these interactions to take place, given the rapidity of normal MalFGK<sub>2</sub> assembly. The inability of MalG<sup>pd3</sup> to assemble with MalK<sub>2</sub> leaves only one productive (although truncated) branch of the assembly pathway and accounts for the low abundance of co-precipitating proteins in the MalF-mediated immunoprecipitations.

Taken together, the data from the expression of various alleles of *malF*, *malG*, and *malK* in all combinations show a complex pattern of protein interactions. We propose that the analysis of the wild type proteins strongly supports the promiscuous assembly pathway model. In addition, the quantitation of the complexes recovered in the studies with the MalF and MalG mutants is consistent with this model. When initiating these studies, we did not expect to conclude that the assembly of this complex might proceed in a manner that is promiscuous rather than strictly ordered. Rather, we anticipated that pairwise expression studies would show that some combinations of proteins interacted, whereas others would not, or that differences in the frequencies of formation for some partial complexes would be as demonstrated elsewhere (1, 2, 4, 24). However, there are characteristics of the assembly of the MalFGK<sub>2</sub> complex that may favor use of a promiscuous strategy. Unlike the biogenesis of some other membrane protein complexes, the assembly of the maltose transporter is efficient and rapid (21). Under normal physiological conditions, 80–100% of the MalF protein synthesized will oligomerize into the final tetramer, with a half-time for assembly of ~60 s. A random oligomerization pattern for the various subunits might enable this expeditious formation of the MalFGK<sub>2</sub> complex (Fig. 5A). Productive intermediates could be formed with all possible combinations of Mal proteins produced by random association of components within the cell. Each of the potential subcomplexes could proceed to a final functional tetramer via the addition of the missing component with no dead-end intermediates. If this mode of assembly is used for the MalFGK<sub>2</sub> complex, it seems likely that other protein complexes also exploit this strategy.

Protein complex biogenesis via a unique, ordered assembly pathway is an appealing concept for protein folding, with sequential conformational changes progressively allowing formation of new subunit recognition sites and the orderly addition of components. Nevertheless, it seems possible that a single ordered pathway is not a universal assembly strategy. Multiple potential pathways could account for conflicting data not only for the biogenesis of MalFGK<sub>2</sub> but for other oligomers as well. For example, the acetylcholine receptor complex (a heteropentamer of similar subunits) is generally believed to oligomerize as follows (3): $\alpha\delta + \alpha\gamma \rightarrow \alpha\beta\gamma + \beta \rightarrow \alpha\beta\gamma$. However, a distinctly different pathway has also been proposed with $\alpha\beta\gamma$ as an early intermediate (5). It may be that both pathways are operational under different circumstances or cell types. Although a unique, ordered assembly pathway may be desirable or even necessary for the assembly of some complex protein structures (e.g. the bacterial flagella) (25), it might be advantageous for other complexes to exploit more than one of several possibilities or even to assemble randomly.

An alternative interpretation of our data might be that the dimerization of MalF and MalG is required for the assembly of MalFGK<sub>2</sub>, because that complex was recovered in all the mutant combinations tested. This is a simpler model and consistent with observations from recent *in vitro* reconstitution assays for maltose transport. In those experiments, a MalK dimer is likely to associate with a stable MalF-MalG complex in membrane vesicles or proteoliposomes (26). However, the reconstitution studies do not test the other pathways for MalFGK<sub>2</sub> assembly. Furthermore, this alternative model does not explain the abundance and stability of the MalF-MalK and MalG-MalK pairwise complexes in our experiments, nor the low abundance of the MalF-MalG<sup>pd3</sup> complex in the three protein-expressing extracts. As we know that the different pairwise complexes are stable during pulse-chase analysis, that at least two of the three possible pairwise subcomplexes form in cells when expressed from the chromosomal genes (Table III), and that the incorporation of MalF into the maltose transporter goes essentially to completion under normal physiological con-
ditions (21), it seems likely that all of these intermediates normally form in the cell and participate in complex assembly. It may be that the MalF-MalG heterodimer is the preferred in vivo pathway for oligomerization. However, the demonstration of the ability for these proteins to form all possible pairwise intermediates should broaden our perspective on the folding and assembly of all multisubunit complexes.

Our co-immunoprecipitation data show significant capacities of the MalF, MalG, and MalK proteins to associate with one another, consistent with a tetramerization strategy that is not strictly ordered. We suggest that the various pairwise associations consist of somewhat different interactions than those within the final tetrameric complex. The pairwise associations between the components may comprise intermediate structures that only progress to complete assembly, involving more extensive interactions among specific domains in the presence of all the components of the maltose transport complex. The effects of MalF and MalG mutants in the co-immunoprecipitation assays suggest that the different intermediate complexes that are present simultaneously in the cell interact with one another, enabling the subcomplexes to efficiently form stable heterotetramers when appropriate.

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