MalK Forms a Dimer Independent of Its Assembly into the MalFGK$_2$ ATP-binding Cassette Transporter of Escherichia coli*

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The maltose transport complex (MTC) is a member of the ATP-binding cassette superfamily of membrane transport proteins and is a model for understanding the folding and assembly of hetero-oligomeric membrane protein complexes. The MTC is made up of two integral membrane proteins, MalF and MalG, and a peripheral membrane protein, MalK. These proteins associate with a stoichiometry of 1:1:2 to form the complex MalFGK$_2$. In our studies of the oligomerization of this complex, we have shown that the ATP-binding component, MalK, forms a dimer in the absence of MalF and MalG. Epitope-tagged MalK coimmunoprecipitated with wild-type MalK, indicating that the MalK protein forms an oligomer. The relative amounts of tagged and wild-type MalK that were present in the whole cell extracts and in the immunoprecipitated complexes show that the MalK oligomer is a dimer. These hetero-oligomers can also be formed in vitro by mixing two extracts, each containing either tagged or wild-type MalK. The dimerization of MalK was also demonstrated in vivo using the bacteriophage $\lambda$ repressor fusion assay. The formation of a MalK dimer in the absence of MalF and MalG may represent an initial step in the assembly pathway of the MTC.

The maltose transport complex of Escherichia coli is a member of the ATP-binding cassette (ABC) transporter superfamily, which includes bacterial binding protein-dependent transporters, the cystic fibrosis transmembrane conductance regulator, and the P-glycoprotein of multidrug-resistant tumor cells. Each member of this family has two conserved nucleotide binding domains. The binding protein-dependent maltose transport complex (MTC) of E. coli comprises four protein subunits (3). One copy each of MalF and MalG probably form a channel in the cytoplasmic membrane through which maltose passes. Two copies of the peripheral membrane ATP-binding protein MalK are associated with MalF and MalG. The hydrolysis of ATP by MalK is presumed to energize the process of sugar transport. Additionally, MalK plays a role in the regulation of maltose transport through two distinct pathways. MalK regulates the expression of mal genes through an unknown mechanism that is dependent on the mal transcriptional activator MalT (4). MalK also interacts with unphosphorylated enzyme IIA$^{bc}$ (EIIA$^{bc}$) of the phosphoenolpyruvate:sugar phosphotransferase system, which lowers the activity of the MTC and decreases maltose transport (5).

Previous studies of the MTC have provided evidence that the two copies of MalK interact functionally within the complex. It has been shown that the MTC hydrolyzes ATP with positive cooperativity (6), and the mutation of the ATP-binding site in a single MalK subunit in the MTC severely impairs transport (7).

We are characterizing the in vivo tetramerization of the Mal proteins as a model for membrane protein assembly. In this paper, we provide biochemical evidence that MalK forms a dimer in the absence of the MalF and MalG proteins. We have utilized several MalK mutants carrying transposon-mediated frame-epitope insertions that were previously characterized for their ability to assemble into the complex and transport maltose (8). Dimerization of several of the mutant proteins with wild-type MalK was shown using coimmunoprecipitation techniques. Furthermore, the oligomerization of MalK was confirmed in vivo using the $\lambda$ repressor fusion assay (9). The dimerization of MalK may represent an initial step in the assembly of the maltose transport complex.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids that were used in this study are listed in Table I. Plasmid preparation, cloning, and transformation were carried out as described previously (10). To make plasmid constructs for co-immunoprecipitation, DNA fragments containing malK insertions mutants with the IPTG-inducible trc promoter and lacI$^f$ from pTrc99A-based plasmids were subcloned into pACYC184 using SphI and HindIII. To make the $\lambda$ repressor cl fusion, polymerase chain reaction-amplified wild-type malK gene was subcloned into pJH391 digested with Sall and BamHI. This construct, pKK700, produces a protein consisting of the N-terminal 132 amino acids of the $\lambda$ repressor fused to the N terminus of MalK.

Media—Rich (LB), minimal (M63), and MacConkey media have been described (11). Minimal media were supplemented with thiamine and all of the amino acids except cysteine and methionine and with glycerol at 0.2%. Antibiotics were used at final concentrations of 100 $\mu$g/ml for ampicillin and 30 $\mu$g/ml for chloramphenicol.

Preparation of Labeled Whole Cell Extracts—Strains were grown in supplemented M63 minimal medium at 37 °C with aeration to an $A_{600}$ of 0.3. Strains were induced with 1 mM IPTG for 5 or 15 min and then 1 ml of culture was labeled by the addition of 120 $\mu$Ci of $[^{35}S]$methionine for 10 min. Labeling was stopped by the addition of 0.05% cold methionine, and the cultures were immediately placed on ice. Cells were washed one time with and then resuspended in 0.5 ml of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA (Buffer A). The resuspended cells were lysed with two 15-s bursts of a probe sonicator. Lysates were aliquoted and stored at –80 °C.

Immunoprecipitation of MalK Species—The coimmunoprecipitation procedure was adapted from a method described by Davidson and Nikaido (3). Whole cell extracts were solubilized with 1% dodecyl maltoside for 20 min on ice. The insoluble material was removed by centrifugation for 10 min at 16,000 × g at 4 °C in a microcentrifuge. This insoluble fraction (Fig. 1A, lane 2) likely represents a mixture of unbroken cells, insoluble protein, and residual soluble material after removal of the soluble extract. The remaining soluble extracts were diluted 1:10 in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM...
NaCl, 1 mM EDTA, and 0.01% dodecyl maltoside (Buffer B). The insertion-specific antisemir was added and incubated on ice for 1–2 h or overnight. To isolate immune complexes, 25 μl of 50% protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) in Buffer B was added to the extracts and incubated on ice for 30 min, with mixing every 5 min. The protein A-Sepharose beads were sedimented in a microcentrifuge and washed three times with 0.5 ml of Buffer B. The beads were heated to 65 °C for 20 min in 25 μl of 50% protein A-Sepharose beads. The procedure to immunoprecipitate epitope-tagged MalK alone was adapted from a procedure used by Traxler and Beckwith (12). Whole cell lysates were solubilized with 2% SDS followed by heating at 65 °C for 20 min. This extract was then diluted 1:17 in Buffer C. MalF protein was immunoprecipitated with a specific antisemir, using the previously described method (12), and results were analyzed after SDS-PAGE on 12.5% resolving gels. Results presented are an average of 2–4 experiments. All samples were checked for integrity of spheroplasts during proteolysis by SDS-PAGE of extracts prior to immunoprecipitation (data not shown).

**Chemicals and Enzymes—**General chemicals and media supplies were purchased from Difco, Fisher Scientific, and Sigma. IPTG was purchased from Bachem. Maltose was bought from Pfanstiehl. Lyszyme, phenylmethylsulfonyl fluoride, and cAMP were from Sigma, and trypsin was from Worthington. Prestained protein molecular weight markers and acrylamide were obtained from Bio-Rad. Soybean trypsin inhibitor, SDS, and n-dodecylmaltoside were purchased from Boehringer Mannheim. [35S]Methionine was purchased from NEN Life Science Products (EXPRE35S35S label).

**RESULTS**

**Coimmunoprecipitation of a MalK Oligomer—**The oligomeric state of MalK was analyzed in extracts prepared from E. coli strains (containing a chromosomal deletion of the malFGK region) producing both wild-type and epitope-tagged MalK species. MalK550 contains an in-frame insertion of 31 mostly hydrophilic amino acids, retains MalK functions, and is recognized by a polyclonal antisemir specific for the insertion (8, 19). Cells expressing both malK550 and malK, each from a plasmid under the control of the trc promoter, were induced briefly with IPTG, labeled with [35S]methionine, and sonicated. Based on the observation that MalFGK2 tetramers are stable in dodecyl maltoside (3), these extracts were solubilized with this nonionic detergent. Although previous studies have dem-

### Table I

**Bacterial strains, plasmids, and phage used in this study**

| Strain, plasmid, or phage | Relevant genotype | Source or reference |
|---------------------------|------------------|---------------------|
| Strains                    |                  |                     |
| BT6                       | MC1000 ΔmalB101 zjb::Tn5 (ΔmalE,F,G,K,lamB,malM) | Laboratory collection |
| BT8                       | MC4100malT(Con)  | 12                  |
| HS3169                    | MC4100malT(Con)ΔmalK16 zjb-729::Tn10          | 13                  |
| KTK50                     | HS3169 with pBR322 and pFL  | This study          |
| KTK51                     | HS3169 with pKK700 and pFL  | This study          |
| KTK52                     | HS3169 with pOAC100 and pFL  | This study          |
| KTK53                     | HS3169 with pBT100 and pACYC184                    | This study |
| AG1688                    | MC1061 F'128 lacIqlacZ::Tn5  | 14                  |
| Plasmids                  |                  |                     |
| pACYC184                  | pACYC 184 with lacIq       | 15                  |
| pBR322                    | pACYC 184 with lacIq       | 16                  |
| pFL1                      | pACYC 184 with lacIq       | 17                  |
| pTrc99A                   | Derivative of pKK233–2 (ColEl ori) with bla, lacIq, and trc promoter | 18                  |
| pBT100                    | pTrc99A with malK expressed from trc promoter   | 18                  |
| pKK268                    | pBT100 malK552::31 (trc promoter)                  | 8                   |
| pKK442                    | nBT100 malK554::31 (trc promoter)                  | 8                   |
| pKK634                    | pBT100 malK556::31 (trc promoter)                  | 8                   |
| pKK100                    | pACYC184 with malK and lacIq from pBT100 (trc promoter) | 8                   |
| pKK16                     | pKK100 malK550::31 (trc promoter)                  | This study |
| pKK268                    | pKK100 malK552::31 (trc promoter)                  | This study          |
| pKK442                    | pKK100 malK554::31 (trc promoter)                  | This study          |
| pKK634                    | pKK100 malK556::31 (trc promoter)                  | This study          |
| pH391                     | pZ150 with λ cl repressor containing a SaI–SacI lacZ fragment insertion | 14                  |
| pKK700                    | pH391 with malK expresses fusion of N-terminus of λ cl repressor to MalK (lac promoter) | This study |
| pOAC100                   | pH391 derivative with translational stop after 131 amino acids of λ cl repressor (lac promoter) | 14                  |
| Phage                     | AKH54 cl– phase λ | 9                   |

**Proteolysis Test of MalFGK2 Complex Assembly—**Cultures of BT8 transformed with the pLK plasmids were grown with aeration at 37 °C in supplemented M63. During the final 2 h of growth before labeling, 1 mM IPTG was added to induce expression of the malK mutations, and 2 mM CAMP was added for the last 5 min. Cells were pulse-labeled for 30 s with 40–50 μCi/ml [35S]methionine and then mixed with 0.05% cold methionine for 10 min of continued incubation. After labeling, cells were converted to spheroplasts as described previously (12). Proteolysis was done on 0.5-ml portions of spheroplasts for 20 min at 0 °C with 25 μg/ml trypsin and stopped as described previously (12). After proteolysis, spheroplasts were harvested and resuspended in 50 μl of 50 mM Tris, pH 7.6, 2% SDS, 1 mM EDTA, and heated to 65 °C for 20 min. These samples were diluted 1:17 in Buffer C. MalF protein was immunoprecipitated with a specific antisemir, using the previously described method (12), and results were analyzed after SDS-PAGE on 12.5% resolving gels. Results presented are an average of 2–4 experiments. All samples were checked for integrity of spheroplasts during proteolysis by SDS-PAGE of extracts prior to immunoprecipitation (data not shown).

**Quantitation—**Phosphorimages were scanned using a Molecular Dynamics PhosphorImager SF, and the data were analyzed with the Molecular Dynamics ImageQuant software, version 3.1. Mutant and wild-type MalK species were readily detected and quantitated in the induced whole cell extracts (Fig. 1A). These quantitations were corrected for background labeled cellular proteins.

**λ cl Repressor Fusion Assay—**Strains, vector plasmids, and AKH54 were provided in the strain kit for repressor fusions courtesy of Jim Hu. The cl-malK fusion construct in strain KTK51 produces a protein that is approximately 12 kDa larger than MalK as detected by Western analysis using a MalK-specific antisemir provided by Howard Shuman (data not shown). The level of the cl-repressor–MalK fusion in uninduced KTK51 was lower than that of MalK expressed from the normal chromosomal locus in BT8. These strains were then tested for immunity to bacteriophage λ by cross-streak analysis without IPTG.
Fig. 1. Coimmunoprecipitation of MalK550 and wild-type MalK. A, BT6 containing the compatible plasmids pBT100 and pKK16 was labeled with [35S]methionine. The sonicated whole cell extract was solubilized with 1% dodecyl maltoside. Any insoluble material was removed by centrifugation and then resuspended in a volume equivalent to the original sample. Proteins were separated by SDS-PAGE and visualized with a phosphorimager. Lane 1, sonicated whole cell extract; lane 2, insoluble fraction; lane 3, soluble fraction. B, dodecyl maltoside-solubilized extracts of BT6 with plasmids pBT100 or pTrc99A and pKK16 or pACYC184 were incubated with insertionspecific antiserum against MalK550, and immune complexes were recovered by incubation with protein A-Sepharose. Lane 1, extract with both MalK550 and wild-type MalK; lane 2, extract with MalK550 only; lane 3, extract with wild-type MalK only. C, same as B except that extracts were solubilized with 1% SDS and diluted in 2% Triton X-100. D, control immunoprecipitations of dodecyl maltoside solubilized extract containing both MalK550 and wild-type MalK. Lane 1, with insertion-specific antiserum; lane 2, with plain protein A-Sepharose beads; lane 3, immunoprecipitation with anti-human Hck (N-30) (Santa Cruz Biotechnology). The positions of migration of molecular mass markers (in kDa) are indicated.

It was demonstrated that MalK tends to form inclusion bodies when highly expressed in E. coli (4, 20), more than 90% of the MalK species in our extracts was present in the soluble fraction (Fig. 1A), likely due to the short induction time used. These solubilized extracts were used for coimmunoprecipitation studies.

The soluble fractions were incubated with the insertionspecific antiserum. Under these conditions, both MalK550 and wild-type MalK were immunoprecipitated from strains expressing both proteins (Fig. 1B, lane 1). MalK550 was easily distinguished from wild-type MalK due to its reduced electrophoretic mobility on SDS-PAGE gels. Only MalK550 was immunoprecipitated from a strain expressing MalK550 alone, and no proteins were immunoprecipitated from a strain expressing only wild-type MalK (Fig. 1B, lanes 2 and 3). These results show that wild-type MalK is associated with MalK550 in these solubilized whole cell extracts. Similar results were obtained with a related MalK insertion mutant, MalK556, which has similar phenotypes to MalK550 (data not shown).

Immunoprecipitations also were carried out on extracts solubilized with 2% SDS and diluted in 2% Triton X-100. Only MalK550 was recovered in these reactions (Fig. 1C), showing that the interaction between MalK species can be disrupted by denaturation in ionic detergent and that the antiserum is specific for MalK550. The heteromeric complex also does not reform in vitro after denaturation and partial renaturation. In order to show that the observed MalK-MalK550 oligomer is due to specific interaction rather than nonspecific aggregation during sample preparation, the procedure was carried out using an equivalent amount of an irrelevant affinity purified antiserum, anti-human Hck (N-30) (Santa Cruz Biotechnology) and using protein A-Sepharose beads alone (Fig. 1D, lanes 2 and 3). The hetero-oligomeric complex was recovered only in the immunoprecipitation containing the insertion-specific antiserum (Fig. 1D, lane 1).

The immunoprecipitation also was carried out with extracts solubilized with 1% Triton X-100 instead of dodecyl maltoside. Again, we observed more than 90% of MalK species in the soluble fraction and the coimmunoprecipitation of the heterooligomeric complex (data not shown), indicating that the solubility of MalK and its oligomeric association are maintained in both nonionic detergents.

The MalK Oligomer Is a Dimer—In order to determine the oligomeric state of MalK, the amount of immunoprecipitated MalK550 and wild-type MalK was quantitated and analyzed using an equation describing the distribution of random pairs. If MalK is a dimer, cells expressing a mixture of MalK550 and wild-type MalK would contain three species of dimer: MalK550 homodimer, wild-type MalK homodimer, and MalK550/wild-type MalK heterodimer. The insertion-specific antiserum would immunoprecipitate MalK550 homodimers and MalK550/wild-type MalK heterodimers only. Assuming random mixing of MalK550 and wild-type MalK molecules, the distribution of heterodimer and both homodimers in the extract is described by the binomial expansion $p^2 + 2pq + q^2 = 1$, where $p$ and $q$ represent the proportions of MalK550 and wild-type MalK, respectively. The $pq$ term represents the proportion of heterodimer, and $p^2$ and $q^2$ represent the proportion of each homodimer. Using this equation, the relative amounts of each molecule immunoprecipitated by the insertion-specific antisera can be predicted based on the relative amounts of MalK550 and wild-type MalK present in the whole cell extracts. These amounts were determined, and the above equations was used to predict the relative amounts of MalK550 and wild-type MalK that should be immunoprecipitated (Table II). Similar binomial expansions were used to predict the relative proportions that would be present in the immunoprecipitations if the oligomer was a trimer or a tetramer. The relative amounts of the two MalK species actually present in the immunoprecipitations were found to agree with values predicted for a dimeric species (two independent analyses are shown).
populations of MalK molecules. Strains were cross-streaked against expressing this cI repressor-MalK fusion and various control proteins to confer immunity to l, active dimeric repressor protein (9). Although the control was altered by switching the vectors carrying the insertion mutants, MalK552 and MalK554, were characterized, and both were found to be deficient in maltose transport and in MTC assembly (8). These mutants were tested with the coimmunoprecipitation assay in order to determine whether their assembly defects are due to an inability to dimerize. MalK552 and MalK554 were each expressed with wild-type MalK and subjected to immunoprecipitation with the insertion-specific antiserum. Both assembly incompetent mutants form hetero-oligomers (Fig. 4). This result suggests that although both of these MalK mutants are unable to assemble into a functional transport complex, these phenotypes are not due to cI-malK. The ability of a fusion plasmid to restore a Mal phenotype (using the equation \( p^2 + 2pq + q^2 = 1 \)). Relative proportion of MalK species present in whole cell extracts. Relative proportion of MalK species predicted to be isolated by immunoprecipitation with insertion-specific antiserum if MalK is a dimer (using the equation \( p^3 + 3pq^2 + 3p^2q + q^3 = 1 \)). Relative proportions of MalK species predicted to be isolated by immunoprecipitation with insertion-specific antiserum if MalK is a trimer (using the equation \( p^4 + 6pq^3 + 4p^3q + 4pq^2 + q^4 = 1 \)). The phosphorimage for Experiment 1 is shown in Figure 1. The phosphorimage for Experiment 2 is not shown.

**Dimerization of MalK**

| MalK species \(^a\) | Whole cell signal intensity \(^b\) | IP signal intensity \(^c\) | Whole cell extract \(^d\) | IP \(^e\) | Predicted dimer IP \(^f\) | Predicted trimer IP \(^g\) | Predicted tetramer IP \(^h\) |
|-----------------|-----------------|-----------------|-----------------|--------|---------------------|---------------------|---------------------|
| Experiment 1'   |                 |                 |                 |        |                     |                     |                     |
| MalK550         | 145991          | 41366           | 0.336           | 0.627  | 0.601               | 0.475               | 0.417               |
| wtMalK          | 288044          | 24527           | 0.664           | 0.372  | 0.399               | 0.525               | 0.583               |
| Experiment 2'   |                 |                 |                 |        |                     |                     |                     |
| MalK550         | 499451          | 130638          | 0.297           | 0.602  | 0.587               | 0.455               | 0.393               |
| wtMalK          | 1183532         | 86262           | 0.703           | 0.398  | 0.413               | 0.545               | 0.606               |

\(^a\) MalK molecules present in whole cell extracts. MalK550 is the epitope-tagged MalK, wtMalK is wild-type MalK.

\(^b\) Signal intensity quantitation of the MalK species present in whole cell extracts following SDS-PAGE using ImageQuant software.

\(^c\) Signal intensity quantitation of the MalK species immunoprecipitated from dodecyl maltoside solubilized extracts with an insertion-specific antiserum following SDS-PAGE analysis (ImageQuant software).

\(^d\) Relative proportion of MalK species present in whole cell extracts.

\(^e\) Relative proportions of MalK species present in the immunoprecipitation.

\(^f\) Relative proportion of MalK species predicted to be isolated by immunoprecipitation with insertion-specific antiserum if MalK is a dimer (using the equation \( p^2 + 2pq + q^2 = 1 \)).

\(^g\) Relative proportions of MalK species predicted to be isolated by immunoprecipitation with insertion-specific antiserum if MalK is a trimer (using the equation \( p^3 + 3pq^2 + 3p^2q + q^3 = 1 \)).

\(^h\) Relative proportions of MalK species predicted to be isolated by immunoprecipitation with insertion-specific antiserum if MalK is a tetramer (using the equation \( p^4 + 6pq^3 + 4p^3q + 4pq^2 + q^4 = 1 \)).

This suggests that MalK550 and wild-type MalK molecules are present in the extract as randomly associated dimers.

In separate studies, the ratio of the relative proportions of MalK550 and wild-type MalK that were expressed in the cells was altered by switching the vectors carrying the malK alleles. In these experiments, the ratio of MalK550 to wild-type MalK in whole cell extracts was 4:1, in contrast to ratios close to 3:7 in the experiments above. Again, wild-type MalK communo-precipitated with MalK550 using the insertion-specific antiserum. Consistent with the prediction of the binomial expansion, the amount of wild-type MalK recovered was substantially smaller than in the above experiments and close to that expected for dimers. However, the difference between the predicted ratios for dimers, trimers and tetramers is not significant due to the small population of wild-type MalK (data not shown).

**MalK550 and Wild-type MalK Hetero-oligomer Can Be Formed in Vitro**—In order to assay for the association and dissociation of MalK in vitro, sonicated whole cell extracts were prepared from two different strains expressing either MalK550 or wild-type MalK. These extracts were mixed and incubated at 37 °C for various times and then were solubilized with dodecyl maltoside and immunoprecipitated as before, using the insertion-specific antiserum. A small amount of hetero-oligomer was recovered (Fig. 2). The proportion of wild-type MalK relative to MalK550 that was coimmunoprecipitated increased 47% over 1.5 h. The MalK oligomer can form in vitro from two separate populations of MalK molecules.

**MalK Oligomerization Assayed Using the λ Repressor Fusion System**—To assay for MalK oligomerization in vivo, full-length malK was fused to the coding region for the N-terminal DNA binding domain of the bacteriophage λ cl repressor. A strain expressing this cl repressor-MalK fusion and various control strains were cross-streaked against the λ ability of a fusion protein to confer immunity to λ indicates the formation of an active dimeric repressor protein (9). Although the control strains were λ-sensitive, the cl repressor-MalK fusion protein (expressed from pK700) conferred resistance to λ (Fig. 3), providing additional evidence for the formation of MalK dimers in vivo. The cl-MalK fusion plasmid restores a Mal phenotype to the ΔmalK strain HS3169 on maltose MacConkey plates, indicating that the fusion protein is functional for transport. There were no bands other than the cl repressor-MalK fusion protein visible on a Western blot developed with a MalK-specific antiserum (data not shown), indicating that the maltose transport function was conferred by the fusion protein and not by a MalK breakdown product.

**Insertion Mutants That Cannot Assemble into MalFGK2 Complexes Still Produce Dimers**—Previously, two other MalK insertion mutants, MalK552 and MalK554, were characterized, and both were found to be deficient in maltose transport and in MTC assembly (8). These mutants were tested with the coimmunoprecipitation assay in order to determine whether their assembly defects are due to an inability to dimerize. MalK552 and MalK554 were each expressed with wild-type MalK and subjected to immunoprecipitation with the insertion-specific antiserum. Both assembly incompetent mutants form hetero-oligomers (Fig. 4). This result suggests that although both of these MalK mutants are unable to assemble into a functional transport complex, these phenotypes are not due to...
Therefore, we tested the different dominance phenotypes of the other intermolecular interaction in the MTC assembly pathway. We have described a protease sensitivity assay to measure the oligomerization of the MTC in vivo (12). Briefly, the MalF component of the complex is initially inserted into the cytoplasmic membrane in a form that is sensitive to cleavage by trypsin in its periplasmic domains. If the protein assembles with both MalG and MalK, MalF becomes trypsin-resistant. We compared the abilities of the insertion mutant proteins expressed from the dominant malK552 and the recessive malK554 mutations to compete with wild-type MalK for complex assembly in a Mal" strain with this assay. We found that although the MalK554 mutant did not inhibit the acquisition of protease resistance of MalF relative to the positive control (the assembly and transport proficient MalK556 insertion mutant), MalK552 had a strong inhibitory effect (Table III). Although both of the assembly defective MalK insertion mutants apparently dimerize, the two mutants are different in their abilities to compete with wild-type MalK for MalFGK2 assembly. The difference in the genetic dominance of the mutations is revealed in the proteolysis assay.

**DISCUSSION**

We have shown that the MalK protein forms a dimer in the absence of the two integral membrane proteins, MalF and MalG, that are associated with MalK in the maltose transport complex in E. coli. A mixed oligomer of two distinguishable forms of the MalK protein was isolated from extracts of strains that do not express MalF and MalG (Fig. 1). Quantitation of the immunoprecipitated MalK proteins showed that a dimer was isolated and not a higher order oligomer (Table II). The fact that an equation that describes the random distribution of homodimers and heterodimers accurately predicted the results of the coimmunoprecipitation assays shows that a very high proportion of the epitope-tagged MalK protein is present in the solubilized extract as a dimer. Based on these data, we suggest that MalK forms a dimer prior to assembling into the membrane-bound complex and that this dimerization may represent a step in an ordered assembly pathway of the MTC.

The formation of heterodimers by mixing whole cell extracts each containing either tagged or wild-type MalK shows that the dimerization can occur in vitro (Fig. 2). The inefficiency of heterodimer formation in this mixing experiment may indicate that the MalK dimer is relatively stable, resulting in a scarcity of free monomer and/or a slow rate of dimer dissociation. This observation also implies that the dimers observed in Fig. 1...
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were formed in vivo. The formation of dimers in vitro can be used as an assay to determine what factors may be necessary for MalK association and dissociation. It is possible that the small amount of dimer being formed in this assay is produced from MalK monomers present in the whole cell extracts. However, we consider this unlikely, as the calculations that were discussed above showed that the amount of MalK monomer present in these extracts is likely to be small.

Additional evidence for dimerization was provided by the λ ci repressor fusion assay in vivo (Fig. 3). The ci repressor-MalK fusion protein was expressed in the presence of the MalF and MalG proteins of the MTC. This shows that the dimerization that was observed in vitro occurs in vivo and therefore is likely to be biologically significant. This conclusion is further supported by the fact that the ci repressor-MalK fusion protein complements the transport defect of a small strain.

Heteromeric complexes were also identified between wild-type MalK and the MalK552 and MalK554 insertion mutants (Fig. 4). These mutant proteins are unable to assemble into MalFGK2 complexes with protease-resistant MalF in the absence of wild-type MalK (9). Therefore, the assembly defect of these MalK mutant seems to be unrelated to dimerization and likely occurs at a subsequent step in complex assembly. For transport activity, the malK552 allele is dominant to wild-type malK and MalK552 also prevents the formation of MalFGK2 complexes containing protease-resistant MalF (Table III). The insertion in MalK552 might allow stable formation of MalK/ MalK552 heterodimers that are incompetent for late stages of complex tetramerization. Paradoxically, the malK554 mutation is recessive to malK+ even though the MalK554 protein can dimerize with wild-type MalK. One possible explanation for these results is that a MalK/MalK554 complex may be destabilized at a subsequent stage of complex assembly. This situation would liberate the wild-type MalK to interact anew with the intracellular population of MalK molecules. The assembly of a stable tetramer of wild-type MalFGK proteins could then "select" for the rarer wild-type MalK homodimers (resulting in protease-resistant MalF and maltose transport). Further studies will identify the stage of assembly that is blocked with these mutants and contribute to our understanding of the assembly pathway.

The dimer form of the nucleotide binding domain protein, HisP, from the ABC transporter histidine permease in Salmonella typhimurium has also been described by Nikaido et al. (21). They found that purified HisP hydrolyzes ATP with a nonlinear dependence on protein concentration, in a manner suggesting that the active form of HisP is a dimer. In gel filtration analysis of purified HisP, only about 3% of the soluble protein was in the dimer peak, whereas the rest of the protein eluted as a monomer. In contrast to this, there appears to be a high proportion of MalK dimers in our solubilized extracts. The discrepancies between the results with the MalK and HisP proteins may be due to differences in assay conditions. Purified proteins were assayed in the HisP system, whereas complex cell extracts were assayed in the MalK experiments. Although MalK and HisP are homologues, they have distinct biological roles and may therefore have different dimerization and assembly characteristics. In addition to assembling into a membrane bound transport complex and hydrolyzing ATP as HisP does, MalK also regulates the expression of mal genes and the activity of the transporter (4, 5). All of the MalK insertion mutants examined in this study are proficient for the MalK/MalT-dependent mal gene regulation (8). It is possible that the dimerization of MalK may also be important in this process.