Mutation of a Single MalK Subunit Severely Impairs Maltose Transport Activity in *Escherichia coli*

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The maltose transport system of *Escherichia coli*, a member of the ABC transport superfamily of proteins, consists of a periplasmic maltose binding protein and a membrane-associated translocation complex that contains two copies of the ATP-binding protein MalK. To examine the need for two nucleotide-binding domains in this transport complex, one of the two MalK subunits was inactivated by site-directed mutagenesis. Complexes with mutations in a single subunit were obtained by attaching a polyhistidine tag to the mutagenized version of MalK and by coexpressing both wild-type MalK and mutant (His)₆MalK in the same cell. Hybrid complexes containing one mutant (His)₆MalK subunit and one wild-type MalK subunit were separated from those containing two mutant (His)₆MalK proteins based on differential affinities for a metal chelate column. Purified transport complexes were reconstituted into proteoliposome vesicles and assayed for maltose transport and ATPase activities. When a conserved lysine residue at position 42 that is involved in ATP binding was replaced with asparagine in both MalK subunits, maltose transport and ATPase activities were reduced to 1% of those of the wild type. When the mutation was present in only one of the two subunits, the complex had 6% of the wild-type activities. Replacement of a conserved histidine residue at position 192 in MalK with arginine generated similar results. It is clear from these results that two functional MalK proteins are required for transport activity and that the two nucleotide-binding domains do not function independently to catalyze transport.

Members of the ATP-binding cassette, or ABC, family of proteins function in the transport of nutrients, proteins, peptides, polysaccharides, ions, and drugs across membranes in both prokaryotes and eukaryotes (19). They have a characteristic structure that includes two hydrophobic domains that form a putative channel across the membrane and two nucleotide-binding domains (NBDs). This domain structure can be formed from one or more polypeptide chains. All four domains reside on a single polypeptide in several of the eukaryotic ABC proteins, including the multidrug resistance protein (MDR), the cystic fibrosis transmembrane regulator (CFTR), and the *Saccharomyces cerevisiae* a-factor exporter (STE6). In the periplasmic binding protein-dependent maltose transport system of *Escherichia coli*, the characteristic structure is achieved with three proteins: MalF and MalG, two hydrophobic proteins, form a stable complex with two copies of MalK, a nucleotide-binding protein (13). ATP hydrolysis by MalK provides the energy for active transport. The periplasmic maltose binding protein (MBP) functions as a high-affinity receptor for maltose in the periplasm and stimulates ATP hydrolysis by MalK via a transmembrane signalling event when maltose is present (14).

The conservation of two NBDs in the ABC family suggests that both are important for function. ATP is hydrolyzed with positive cooperativity by the maltose transport complex, demonstrating that the two subunits interact (11). In the eukaryotic ABC proteins, where both nucleotide-binding proteins reside on a single polypeptide, site-directed mutagenesis of conserved residues within a single nucleotide-binding site has been used to assess the relative importance of the two nucleotide-binding

sites in translocation activity. In CFTR, which functions as a chloride channel, ATP hydrolysis by a single NBD may be sufficient for channel opening (1) while the second NBD appears to fulfill a regulatory function in channel closure (7). In contrast, a single mutation in either NBD of MDR or STE6 results in the loss of activity, demonstrating that two functional binding sites are essential for transport in these systems (2, 3, 25). A potential problem with these experiments is illustrated by the STE6 protein (3), where substitution of arginine for a conserved lysine in the N-terminal NBD eliminated 99% of mating activity while the same substitution at the corresponding lysine residue in the C-terminal domain resulted in only 85% inhibition. Because the two NBDs are not identical, differences in the context of the conserved lysine in each NBD may result in different degrees of inhibition or no inhibition of the associated ATPase activity. Interpretation of results should be simpler in a system with two identical NBDs, such as the maltose transport system. The maltose system offers several other advantages as well. The system is well characterized genetically and has been purified and reconstituted into proteoliposome vesicles in a functional form (5, 13). It is possible to examine separately the effects of a given mutation on transport activity, nucleotide-binding activity, ATPase activity, or complex assembly (13, 27, 35). Nucleotide binding and hydrolysis by the isolated MalK protein can also be assessed (38).

In this report, we describe a strategy by which it is possible to purify MalFGK₂ complexes with mutations in a single MalK subunit. We find that mutations that inactivate the complex when present in both MalK subunits permit only low rates of transport (5% of wild-type activity) when present in a single subunit.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains were transformed with up to three compatible plasmids carrying the *mal* genes. Those transformants that overproduced the transport complexes were frozen at –70°C (prior to

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induction) in 10% glycerol stocks and used to inoculate overnight cultures for large-scale preparations. To obtain all of the desired combinations of proteins, two different expression systems were employed. When genes were under the control of the *trc* promoter (*trcp*), the host strain was HN741 [*E. coli* K-12 *argH* *his* *rpsL1* *malT*(Con) *malB*Δ13 *ΔatpBC* *ilv::Tn10/F'* *lacI*^q *Tn5*] (14), which contains chromosomal deletions of the genes encoding the maltose transport system and the F₀F₁ ATPase. Growth and induction of cells was as described previously (13). When genes were expressed from the T7 promoter (*T7p*), the host strain was K38 [HfrC (λ)] (29). K38 cells were grown as for HN741, expression was induced by adding a phage carrying the T7 RNA polymerase gene (12, 34), and cells were harvested 1 h later. Total membrane fractions were isolated as described previously (14) and stored at -70°C until use.

A version of the *malK* gene containing a 5' extension with six contiguous histidine codons was constructed with the vector pET15b (Novagen) by using standard techniques (30). A *NcoI*-*Bam*HI fragment carrying *malK* (28) was inserted into pET15b that had been digested with *XhoI* and *Bam*HI. The *NcoI* and *XhoI* ends were filled in with the Klenow reagent prior to ligation to generate the in-frame gene fusion termed *malKht*. The resulting MalK fusion protein, (His)₆MalK, contains a 23-amino-acid N-terminal extension, MGSSHHHHHHS SGLVPRGSHMLD. The *malKht* gene was transferred to the chloramphenicol resistance plasmid pACYC184 (8) and placed under the control of either *T7p* (pSL731) or *trcp* (pSS733).

In order to coexpress *malK* and *malKht*, *Bam*HI-*Hind*III fragments carrying promoter sequences and *malK* were inserted into the spectinomycin resistance plasmid pMS421 (16), which had been digested with *Bam*HI and *Hind*III, to generate pMSS920 (*trcp*) or pMSS921 (*T7p*). Ampicillin resistance plasmids expressing *malF* and *malG* from *trcp* have been described previously (12). Plasmids expressing *malF* (or *malF500*) and *malG* from *T7p* (pFG60 and pFG67, respectively) were constructed by inserting *NcoI*-*Bam*HI fragments carrying the *malF* and *malG* alleles into pET15b that had been digested with *NcoI* and *Bam*HI.

Mutagenesis of *malK*. Site-directed mutagenesis of *malK* was performed according to the method of Kunkel with plasmid pMF12 (23). pMF12 is a derivative of pBS SK+ with *trcp* and *malK* (15) inserted into the multiple cloning site on a *Bam*HI-*Hind*III fragment. Single-stranded pMF12 DNA was isolated from strain CJ236 with the helper phage VCSM13 (Stratagene). The primer 5'-GCAGTAAAGTCGASNNADDGCAGCCAG-3' (where N = A + G + C + T and S = G + C) was designed to allow for any amino acid substitution at position 42. The primer 5'-CGACCTGATCGVGGGTGACGTA-3' (where V = G + A + C) will allow the substitution of leucine, proline, or arginine at position 192. Following in vitro DNA synthesis with T7 DNA polymerase (U.S. Biochemicals), DNA was transformed into strain AD317 [*malT*(Con) *araD* *lac* *rpsL1* *malK-lacZ* Δ(*srlR-recA*)306::Tn10/*F'* *lacI*^q *Tn5*] and plated on maltose tetracycline indicator plates. Mutated plasmids were identified by their inability to complement the *malK* mutation in the chromosome, and plasmid DNA isolated from these colonies was sequenced to identify the mutation (Sequenase; U.S. Biochemicals). Restriction digestion and ligation reactions were used to move mutations from pMF12 to pSL731, pSS733, or pMR11 (28) for expression experiments.

Purification and reconstitution of the transport complex. Membrane vesicles were thawed, washed by dilution in 50 mM Tris-HCl (pH 8)-1 mM EDTA, and collected by centrifugation at 100,000 × *g* for 30 min. The pellet was resuspended in a solution of 20 mM Tris-HCl (pH 8), 5 mM MgCl₂, 10% glycerol, and 10 mM 2-mercaptoethanol. Proteins were solubilized by the addition of either octyl β-D-glucopyranoside (octyl glucoside) or *n*-dodecyl β-D-maltoside (dodecyl maltoside) to a final concentration of 1%. Proteins solubilized in octyl glucoside were immediately reconstituted into *E. coli* phospholipid vesicles by detergent dilution (12). Proteins solubilized in dodecyl maltoside (usually 3 mg) were mixed with 1 ml of Ni-nitrilotriacetic acid resin (Qiagen) that had been equilibrated with buffer A (50 mM KP_i [pH 8], 300 mM NaCl, 10% glycerol, 0.01% dodecyl maltoside, and 10 mM 2-mercaptoethanol) to allow binding of the polyhistidine tag to the resin (10). After 1 h of mixing at 4°C, the resin was placed into a column and washed with 15 ml of buffer A to remove unbound protein. The column was washed with 15 ml of buffer B (50 mM KP_i [pH 6], 300 mM NaCl, 10% glycerol, 0.01% dodecyl maltoside, and 10 mM 2-mercaptoethanol), and proteins were eluted in buffer B by using a step gradient with three 15-ml steps containing 25, 50, and 100 mM imidazole. Following dialysis against 20 mM Tris-HCl (pH 8)-10% glycerol-0.1 mM EDTA-10 mM 2-mercaptoethanol and concentration in a Centricon-100 microconcentrator, reconstitution of the purified protein was achieved as described previously (13).

Assays of maltose transport and ATP hydrolysis. To measure maltose accumulation, proteoliposomes were prepared in the presence of 5 mM ATP. The transport reaction was initiated by mixing proteoliposomes with 1 μM MBP and 10 μM [¹⁴C]maltose (100 mCi/mmol; ICN) and terminated by filtration of aliquots through a nitrocellulose filter (12). Values were corrected for background counts per minute on filters in the presence of MBP and maltose only. To measure ATP hydrolysis by the complex, proteoliposomes were prepared in the presence of MBP and maltose. The presence of MBP and maltose inside the vesicles stimulates ATP hydrolysis (14). Alternatively, MBP and maltose were omitted and the MalF500 protein was used to stimulate ATP hydrolysis (14). Assays were performed in 50 mM Bis-Tris (pH 6.0)-5 mM MgCl₂-1 mM dithiothreitol with [γ-³²P]ATP (ICN) as described previously (11). MBP-stimulated

ATPase activity is the difference between activity in the absence (generally 0 to 2 nmol/min/mg) and in the presence of MBP and maltose.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the procedure described by Laemmli (24) with 11% polyacrylamide gels. To improve the separation of (His)₆MalK, MalK, and MalF, the separating gel was prepared by a modification of the procedure of Lugtenberg et al. (26) by using 8.5% acrylamide and Tris-HCl at pH 8.6 rather than pH 8.8 (13). MalG migrates with the dye front and is not visible under these conditions.

Gradient centrifugation. A measure of the oligomeric state of the transport complex in detergent solution was obtained by sucrose gradient centrifugation (20). Fifty-microliter samples (containing approximately 50 μg of protein) were loaded on a 5-ml continuous 5 to 25% (wt/vol) sucrose gradient in buffer containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 10% glycerol, and 0.01% dodecyl maltoside that was layered on a 200-μl cushion of 60% sucrose. Gradients were subjected to centrifugation for 16.5 h at 27,000 rpm in a Beckman SW50.2 rotor at 4°C. Protein size standards (Pharmacia Biotech) were sedimented in parallel gradients. Fractions were collected from the top of the gradient with the Auto-densi-flow IIC (Buchler Instruments), and the refractive index of each fraction was determined. Proteins were localized within the gradient by silver staining (18) of SDS-polyacrylamide gels following electrophoresis of the fractions.

Protein determinations. Protein concentrations were determined as described previously (13) by the method of Schaffner and Weissmann (31).

RESULTS

Modification of MalK. To address the question of whether a single MalK subunit is sufficient for activity, it was first necessary to identify mutations in MalK that inactivated the transport complex when present in both subunits. ABC proteins, including MalK, contain sequences, termed Walker A and B motifs, that are important for nucleotide binding and hydrolysis (37), and mutations in this region interfere with transport activity (22, 27). The conserved lysine (K42) within the Walker A motif of MalK was changed to leucine, methionine, asparagine, proline, or threonine (Fig. 1). In a second set of experiments, a conserved histidine residue outside both Walker motifs (H192) was replaced with leucine, proline, or arginine. All of these isolates were phenotypically Mal⁻ on indicator plates. The K42N and H192R substitutions in MalK have been characterized (27, 33, 39) and were selected for further analysis in this work. MalK was also modified with a 23-amino-acid N-terminal extension containing six contiguous histidine residues to allow for Ni²⁺ chelate affinity purification of the transport complex (see Materials and Methods).

The activities of the (His)₆MalK protein and of the K42N and H192R mutants in the context of the transport complex were compared with that of the wild type by overexpressing these proteins with MalF and MalG in strain HN741 with the *trcp* expression system (12). We and others have observed that the maltose transport proteins are soluble in nonionic detergents only when they are present in a translocation complex (12, 27). The presence of a polyhistidine tag or the amino acid substitutions in the MalK protein did not significantly alter the degree of overexpression or the ability to form a translocation complex, as judged by SDS-polyacrylamide gel electrophoresis of the solubilized proteins (Fig. 2, lanes 8 to 11). Transport proteins were also reconstituted into proteoliposome vesicles for activity assays (see Materials and Methods). The presence of (His)₆MalK in the complex did not significantly alter the rate of maltose accumulation or the rate of ATP hydrolysis by the transport complex (Table 1). The presence of either amino acid substitution (K42N or H192R) in MalK resulted in the loss of both the ability to transport maltose and the ability to hydrolyze ATP in these in vitro assay systems. When the modified MalK proteins were coexpressed with MalF500, a mutant protein that is capable of stimulating ATP hydrolysis in the absence of MBP (14, 36), the degree of overexpression of the transport complex was more variable (Fig. 2, lanes 2 to 6). We were unable to overexpress a complex containing both MalF500

FIG. 1. Location of mutagenized residues in MalK. The first 220 amino acids of MalK are aligned to a consensus sequence (Cons) for an ABC transporter. Identical residues are indicated by a dash. The alignment and the consensus sequence (taken from reference 5) were generated by comparison of the ABC subunits of 27 binding protein-dependent transporters, including MalK. Residues comprising three highly conserved motifs in the ABC family are underlined (5). The amino acid substitutions in MalK that were studied in detail are indicated.

and MalK (K42N) with the *trcp* expression system. However, it is clear that the presence of the amino-terminal extension on MalK does not interfere with the ability of the MalF500 protein to stimulate ATP hydrolysis and that the substitution at either K42 or H192 prevented MalF500-mediated stimulation of ATP hydrolysis by (His)₆MalK (Table 1).

Purification of transport complexes by Ni²⁺ chelate affinity chromatography. We have previously shown that the maltose transport proteins purify as a complex containing one MalF, one MalG, and two MalK proteins (13). As shown in Fig. 3A, addition of the polyhistidine sequence to MalK allowed for the purification of the entire transport complex by affinity chromatography on Ni-nitrilotriacetic acid resin. The column was washed with steps of increasing imidazole concentrations, and the transport complex eluted primarily in the highest (100 mM) imidazole fraction.

When MalK and (His)₆MalK were coexpressed with MalF and MalG, transport complexes eluted at 25, 50, and 100 mM imidazole (Fig. 3B). Since MalF and MalK comigrate under normal electrophoresis conditions (Fig. 3B, upper gel), a second gel was run to resolve these bands (Fig. 3B, lower gel).

FIG. 2. SDS-polyacrylamide gel electrophoresis of membrane proteins. Total membrane preparations were treated with octyl glucoside as described in Materials and Methods, and proteins remaining in solution following centrifugation were separated by SDS-polyacrylamide gel electrophoresis (8.5% acrylamide, pH 8.6) and visualized by silver staining (18). Arrows indicate the positions of migration of (His)₆MalK, MalK, and MalF and, in lane 1, of molecular weight markers (in thousands). Samples are from strains containing MalF500, MalG, and either MalK (wild type) (lane 2), MalK (H192R) (lane 3), (His)₆MalK (wild type) (lane 4), (His)₆MalK (K42N) (lane 5), or (His)₆MalK (H192R) (lane 6). A sample from the strain in the absence of expression plasmids is shown in lane 7. Samples are from strains containing MalF, MalG, and either MalK (wild type) (lane 8), MalK (K42N) (lane 9), MalK (H192R) (lane 10), or (His)₆MalK (wild type) (lane 11). Each lane contains 1 µg of protein.

Complexes eluting in the 25 and 50 mM fractions contained both MalK and (His)₆MalK, while complexes eluting in the 100 mM fractions usually contained (His)₆MalK only. The identity of (His)₆MalK and MalK in these gels was confirmed by Western analysis with antibody to MalK (not shown). We interpret this result to mean that complexes containing just one polyhistidine sequence bind to the nickel resin less tightly than those containing two polyhistidine sequences.

Mutant versions of (His)₆MalK (K42N or H192R) were coexpressed with MalK to generate complexes with mutations in a single NBD. As predicted, purified complexes containing one mutant (His)₆MalK subunit and one wild-type MalK subunit eluted from the column at lower imidazole concentrations than complexes containing two mutant (His)₆MalK subunits (Fig. 4). Because the 50 mM fraction generally contained more highly purified material than the 25 mM fraction, the 50 mM fractions were used to determine the activity of complexes containing a mutation in only one MalK subunit and the 100 mM imidazole fractions were used to determine the activity of complexes containing mutations in both MalK subunits.

Maltose transport and ATPase activity of purified transport complexes. Following reconstitution into proteoliposome vesicles, transport complexes from the 50 mM fractions that con-

TABLE 1. Activities of MalFGK₂ complexes with identical substitutions in both MalK subunits^a

Protein	Maltose uptake (nmol/min/mg)	ATP hydrolysis (nmol/min/mg) stimulated by:	
		MBP-maltose	MalF500
MalK (wt)	0.85	90	440
MalK (K42N)	0.048	1.9	NS ^b
MalK (H192R)	0.021	0.4	0
(His) ₆ MalK (wt)	0.52	74	370
(His) ₆ MalK (K42N)	ND ^c	ND	4.9
(His) ₆ MalK (H192R)	ND	ND	3.7
Deletion strain	0.013	0	3.3

^a Assays were performed with proteoliposomes prepared following solubilization of membrane proteins with octyl glucoside from strains overproducing MalF or MalF500, MalG, and either wild-type (wt) or modified MalK, as indicated. Preparations are the same as those shown in Fig. 2. Specific modifications to the reconstitution procedure to allow for the different assays are described in Materials and Methods. Maltose transport was assayed by using 10 µM maltose and 1 µM MBP. ATPase assays contained 100 µM ATP.

^b NS, transport complex not successfully overproduced.

^c ND, not determined.

FIG. 3. Purification of MalFGK₂ by Ni affinity chromatography. The protocol used for these purifications is described in detail in Materials and Methods. Silver-stained SDS-polyacrylamide gels display molecular weight markers (lanes 1); 0.1 µg of detergent-solubilized membrane protein prior to purification (lanes 2); 0.1 µg of protein that did not bind to column (lanes 3); and 10-µl samples of peak fractions eluting with buffer B (lanes 4), 25 mM imidazole (lanes 5), 50 mM imidazole (lanes 6), and 100 mM imidazole (lanes 7). Arrows indicate the positions of migration of (His)₆MalK, MalK, MalF, and MalG and molecular weight markers (in thousands). (A) Purification from a strain overproducing MalF, MalG, and (His)₆MalK. (B) Purification from a strain overproducing MalF, MalG, (His)₆MalK, and MalK. Upper gels are separating gels prepared with Tris-HCl (pH 8.8) and 11% acrylamide. Lower gels are separating gels prepared with Tris-HCl (pH 8.6) and 8.5% acrylamide. In the lower gels, only the region near *M*_r 45,000 is shown.

tained a mutation (either K42N or H192R) in only one MalK subunit hydrolyzed ATP at rates that were approximately 5 to 10% of that of the wild type (Table 2). In contrast, the ATPase activity of protein purifying in the 100 mM fraction had 1 (K42N) to 2% (H192R) of the activity of the wild type. It is clear that the activity is catalyzed by the maltose transport complex and not by other contaminating ATPases, because the activity was detectable in liposome vesicles only when both MBP and the column fractions were present (data not shown). To ascertain whether the activity in the 100 mM fraction resulted from complexes with mutations in both subunits or from the presence of a small fraction of complexes with mutations in a single subunit, transport complexes were purified from strains in which only the mutant (His)₆MalK protein was expressed. The ATPase activity in the 100 mM fraction was still 1 to 2% of that of the wild type when either the K42N or the H192R mutant was used in place of the wild-type (His)₆MalK

protein (Table 2). Levels of maltose transport activity closely paralleled those of ATP hydrolysis and were similar for both the K42N and H192R substitutions (Table 2).

It should be noted that the specific activity of wild-type complexes purifying in the 50 mM fraction was roughly twofold higher than that of wild-type complexes eluting in the 100 mM fraction (Table 2). The same result was obtained in three of four trials and suggests that either the presence of a second polyhistidine tag in the complex or exposure to high imidazole concentrations may adversely affect transport activity. However, the presence of high activity in the experiment where only wild-type (His)₆MalK was expressed (Table 2) argues against this point.

Control experiments were performed to ensure that the activity in the 50 mM fractions did not result from the presence of a small fraction of complexes containing two wild-type MalK subunits. First, we attempted to purify MalFGK₂ complexes

FIG. 4. SDS-polyacrylamide gel electrophoresis of purified fractions. The protocol used for purification is described in Materials and Methods. Ten microliters of each column fraction was loaded onto SDS-polyacrylamide gels, and proteins were visualized by silver staining. Lane 1, molecular weight markers; lanes 2 to 4, 25, 50, and 100 mM imidazole fractions, respectively, from strain HN741, containing MalF, MalG, wild-type (wt) MalK, and (His)₆MalK (wt); lanes 5 to 7, 25, 50, and 100 mM imidazole fractions, respectively, from HN741, containing MalF, MalG, MalK (K42N), and (His)₆MalK (K42N); lanes 8 to 10, 25, 50, and 100 mM imidazole fractions, respectively, from K38, containing MalF, MalG, MalK (wt), and (His)₆MalK (H192R). The upper gel is 11% acrylamide, pH 8.8; the lower gel is 8.5% acrylamide, pH 8.6. Molecular weight is indicated in thousands.

TABLE 2. ATP hydrolysis and maltose transport activity of purified transport complexes containing substitutions in one or both MalK subunits^a

Protein expressed		Maltose uptake (nmol/min/mg)		ATP hydrolysis (nmol/min/mg)	
(His) ₆ MalK	MalK	50 mM imidazole	100 mM imidazole	50 mM imidazole	100 mM imidazole
WT ^b	None		1.2		93
K42N	None		ND ^c		1.1
H192R	None		ND		2.2
WT	WT	0.93	0.43	110	42
K42N	WT	0.065	0.0084	6.2	0.38
H192R	WT	0.040	0.010	13	0.79

^a Proteins eluting from the affinity column (Fig. 4) were dialyzed and concentrated and were used to prepare proteoliposomes for assays of ATP hydrolysis or maltose transport as described in Materials and Methods. The specific MalK proteins expressed during production of transport complexes are listed. The 100 mM imidazole fraction contained transport complexes with two (His)₆MalK proteins. The 50 mM imidazole fraction contained hybrid complexes with one MalK and one (His)₆MalK protein. ATPase assays contained 100 μ M ATP, and data represent the average of two experiments with two time points per assay. Maltose transport activity was assayed in the presence of 10 μ M MBP and 1 μ M maltose, and data represent the average of two experiments with four time points per assay.

^b WT, wild type.

^c ND, not determined.

with the Ni²⁺ affinity resin in the absence of the (His)₆MalK protein and found that no ATPase activity was detected in any imidazole fraction. Second, to examine whether MalK subunits, once assembled into a complex, are freely interchangeable, complexes containing either wild-type MalK only or (His)₆MalK (K42N) only were prepared separately and incubated together prior to purification. No ATPase activity was recovered following purification, demonstrating that MalK subunits are unable to rearrange themselves to generate wild-type complexes. Finally, if transport complexes formed stable dimers or higher order oligomers, wild-type complexes could copurify with complexes containing a single mutant (His)₆MalK, providing an alternative explanation for the appearance of activity in the 50 mM fractions. We find this possibility unlikely, as we have previously shown that the wild-type transport complex in detergent solution contains one MalF, one MalG, and two MalK proteins (13). However, by examining their migration through sucrose density gradients (20), we verified that substitution of MalF500 for MalF or (His)₆MalK (K42N) for MalK does not affect the association state of the complexes. As shown in Fig. 5, the modified complexes comigrated through the gradient near aldolase (M_r , 158,000), catalase (M_r , 232,000), and the wild-type complex, as expected for a complex containing one MalF, one MalG, and two MalK proteins (M_r , 170,000).

DISCUSSION

All ABC transporters appear to have two NBDs that are either identical or highly conserved. It is not known whether both NBDs are required for function in all systems or, if both are required, whether the two NBDs function identically or have distinct roles in the translocation cycle. We have, for the first time, succeeded in generating hybrid maltose transport complexes that contain amino acid replacements in only one of the two identical MalK subunits. Single amino acid replacements in the transport complex were made by coexpressing mutant and wild-type versions of the *malK* gene. Selective use of a polyhistidine-tagged version of MalK, (His)₆MalK, per-

FIG. 5. Sucrose density gradient centrifugation. Membrane proteins were solubilized with dodecyl maltoside and subjected to sucrose density gradient centrifugation as described in Materials and Methods. Fractions were collected from the gradient and subjected to SDS-polyacrylamide gel electrophoresis to identify the fraction containing the majority of the indicated protein(s). The refractive indexes of peak fractions containing the protein standards are plotted as a function of M_r for catalase (232,000), aldolase (158,000), and bovine serum albumin (66,000). The positions of migration of the wild-type MalFGK₂ complex (WT) (predicted M_r , 170,000), of a complex containing MalF500 in place of MalF (F500), and of a complex containing (His)₆MalK with the K42N substitution in place of both MalK subunits (HTK42N) are indicated by arrows. The protein standard ferritin (M_r , 440,000) migrated to the bottom of the tube and was not included in the analysis.

mitted isolation of the hybrid complexes by affinity purification. We found that hybrid complexes containing one wild-type MalK subunit and one mutant MalK subunit had only 5% of the transport activity of the wild type.

This low level of activity in the hybrid complex suggests that both NBDs are required for activity and, furthermore, that the two NBDs do not function independently. It should be noted that the inherent asymmetry of the transmembrane MalF-MalG domain generates two nonequivalent sites for assembly of the MalK subunits. Assuming that there is no strong selection for assembly of variants of MalK in one site over the other, two distinct populations of hybrid complexes containing one mutant and one wild-type MalK subunit will be generated. Recovery of full activity following purification of hybrid complexes with mutations in a single subunit would have implied that only one NBD was required and that the functional NBD could occupy either site. If both NBDs catalyzed transport independently, the hybrid transporters would have had 50% of the activity of the wild type. The same would have been true if one population of complexes were fully active and the other were inactive. The fact that the NBDs do not function independently is consistent with the results of our previous work, where cooperative interactions between the two nucleotide-binding sites were detected (11).

The lysine 42 and histidine 192 mutations were chosen for study because they inactivate maltose transport by different mechanisms. Transport is energized by the hydrolysis of ATP, and lysine 42, an essential residue within the ATP-binding site of MalK, was mutagenized to determine whether two functional nucleotide-binding sites are required. Histidine 192 is also essential for transport, but is not within the ATP-binding motif and is not required for ATP binding or hydrolysis by the isolated MalK protein (39). This residue may act in a step subsequent to nucleotide binding that is important in transmembrane signalling or in the coupling of ATP hydrolysis to transport. Even if two functional ATP-binding sites are required for transport, the possibility remains that not all MalK

functions will be essential in both halves of the transport complex.

We have confirmed previous results (27) showing that the K42N substitution within the Walker A motif of MalK inactivates the translocase when it is present in both MalK subunits. Both the transport and ATP hydrolysis activities of the purified transport complex were reduced to 1% of that of the wild type. Since the K42N mutation interferes with labeling of MalK by 8-azido-ATP (27) and presumably also prevents nucleotide binding to MalK, the presence of this mutation in a single subunit would be expected to disrupt cooperative interactions between the two nucleotide-binding sites. The fact that the hybrid complex has significantly more transport activity than a complex with the K42N mutation in both NBDs (6 versus 1%) raises the possibility that ATP hydrolysis occurring independently at a single site can support low rates of transport activity. In this respect, the maltose transport system may be analogous to the F_1 ATPase, which has three catalytic nucleotide-binding sites. F_1 ATPase exhibits a low rate of catalysis when a single site is occupied by ATP, and binding of ATP at a second site greatly stimulates the overall rate of hydrolysis (9). Like the maltose transporter, the F_1 ATPase also exhibits positive cooperativity in the hydrolysis of ATP (6). Since the K42N mutant is somewhat leaky, further work will be required to demonstrate conclusively that hydrolysis at a single site will support low rates of maltose transport.

Like the K42N substitution, the presence of the H192R substitution in both MalK subunits reduced transport activity to 1% of that of the wild type. Despite the fact that the H192R substitution did not affect nucleotide binding or ATP hydrolysis by the isolated MalK subunit (39), the binding protein did not stimulate ATP hydrolysis by the mutant transport complex, nor did the presence of the MalF500 mutation that normally renders ATP hydrolysis constitutive. This histidine residue is not absolutely conserved in the ABC family; in two transporters, CFTR and LIV-1 (17), it is present in only one of the two NBDs. If the two MalK proteins function differently, it is conceivable that some important residues will be required for function in only one of the two subunits. However, with histidine 192 present in just one subunit, transport complexes retained only 4% of the transport and 12% of the ATPase activity of the wild type. Hence, H192, like K42, is required in both subunits for optimal transporter function. The requirement for H192 in both subunits suggests that a second, as yet undefined function separate from ATP binding must occur in both subunits before transport can occur. These results lend support to a model in which both MalK subunits function identically. Despite the lack of identity between MalF and MalG, there may be a degree of dyad symmetry in the function of the complex such that hydrolysis at either site can be coupled to translocation. The existence of HlyB, an ABC protein with one transmembrane domain and one NBD that presumably functions as a homodimer, is consistent with this hypothesis.

Senior and coworkers have proposed a model for the action of MDR in which the two NBDs function identically but alternate in catalysis. The model is based on data suggesting that both ATP-binding sites are catalytic but that only one can take on the catalytic conformation at a given time (32). It is proposed that hydrolysis at one site drives a complete cycle of drug translocation and the transporter resets itself, with the second site becoming the catalytic site. This type of interaction between NBDs, which is known as alternating site cooperativity, has been proposed for the function of F_1 ATPase and succinyl-coenzyme A synthetase (4, 21) and provides an attractive model for the function of the maltose transporter.

Our finding that mutations in a single NBD severely impair the function of the transporter is in general agreement with previous work on the eukaryotic ABC proteins MDR and STE6 (2, 3). Much of this early work on the eukaryotic systems was performed *in vivo*, where variable expression or mislocalization of the mutant proteins could complicate interpretation of the results, whereas we worked with purified protein complexes and were able to assay ATPase activity as well as transport activity with identical mutations present in one or both NBDs. In more recent work, a purified form of MDR containing substitutions in a single nucleotide-binding site was employed and no ATPase activity was detected (25). Further use of this technique will be valuable in addressing questions regarding mechanisms of translocation and assembly in the maltose transport complex. For example, after introducing changes into a single MalK subunit, it may be possible to detect and study allosteric interactions between the two NBDs. This method should be generally applicable to the study of many oligomeric proteins in nature, where it has been difficult or impossible to study the effect of mutations in a single subunit.

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