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Binding protein-dependent uptake of maltose into cells via an ATP-binding cassette transporter

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Maltose and maltodextrins are actively transported across the cytoplasmic membrane of *E. coli* and *Salmonella* by a periplasmic binding protein (BP)-dependent transport system. In addition to the soluble maltose binding protein (MBP or MalE), there are two transmembrane (TM) subunits, the MalF and MalG proteins, and two copies of MalK, an ATPase that is peripherally associated with the TMs at the cytoplasmic surface of the membrane. Early research on the maltose system dates back to the 1960s and 1970s (43, 108) when the genes and proteins required for growth on maltose were first identified. Comparisons of primary sequences revealed an evolutionary relationship between different BP-dependent transport systems in bacteria (31) which was most apparent in homologues of MalK. Deletion of the gene encoding MBP demonstrated that it was essential for maltose uptake, and allowed for the isolation of mutant transporters capable of recognizing and transporting maltose in the absence of MBP (94). Homologous counterparts have now been identified in all other kingdoms of life. This group of related proteins is known as the ATP-binding cassette (ABC) superfamily, where the term “cassette” refers to the nucleotide-binding subunit or domain (NBD), of which there are two copies. Transport activity has been demonstrated for many members of the superfamily, mediating both import and export from cells and organelles.

In the 1996 edition of this text, the article by Boos and Lucht highlighted many major advances in the study of the binding-protein dependent ABC transporters, including the high resolution structure and the dynamics of the binding proteins, the reconstitution of transport activity in proteoliposome vesicles, confirmation that ATP hydrolysis provides the driving force for transport and insight into the nature of interactions between the binding protein and the transporter that helped to form the foundation of a model for translocation (5). MBP and other binding proteins consist of two separate domains or lobes connected by a hinge, which form a cleft where substrate binds (90, 96). In the absence of substrate the lobes are well separated, in an “open” conformation, and upon substrate binding the lobes “close”, in most cases burying the substrate in the interior of the protein (75, 76, 110). The membrane-associated proteins form a complex that can be solubilized from the membrane and purified without dissociating into subunits. The reconstituted MalFGK₂ complex displays little or no ATPase activity in the absence of MBP, low ATPase activity in the presence of maltose-free MBP and high ATPase activity with maltose-loaded MBP, highlighting the important role of MBP in coupling maltose transport to ATP hydrolysis (16, 18). The pattern of stimulation

suggests first, that it is the closed (ligand bound) conformation of MBP that interacts most productively with the transporter and second, that binding of maltose, which triggers conformational change in MBP, is a key step in the process of coupling of transport to hydrolysis. In fact, binding protein independent mutant transporters display elevated levels of ATPase, in the absence of MBP, unaffected by the presence or absence of maltose (18).

Since 1996, there have been many advances, both in our understanding of the structure and mechanism of the maltose transporter, in the assembly of the membrane-associated transporter complex and in the mechanism of regulation of transport both at the DNA and the protein level. The transporter has been studied both in detergent and reconstituted in liposome vesicles and while many features, including the ability of MBP to stimulate ATPase activity, are retained in detergent, it has been noted that the basal ATPase activity of the transporter is elevated in detergent, as compared to liposomes (79). In this chapter, we focus on these recent developments, which have culminated in a high resolution structure of MBP in a complex with the MalFGK₂ transporter. While this review focuses on the maltose system, complementary work has been carried out on many different ABC transporters all of which has contributed in important ways to our understanding of the maltose transport system (14, 35, 73).

Regulation of Maltose Transport

The maltose transport genes are present in two divergently oriented operons: *malKlamB* and *malEFG*, separated by an array of binding sites for the transcriptional activator, MalT, and the global regulator, cyclicAMP/catabolite activation protein (cAMP/CAP) complex (reviewed in (6)). The regulation of the maltose transport system, at the DNA level, is implemented by the synergistic action of MalT and cAMP/CAP complex and, at the protein level, by interactions of MalK with unphosphorylated EIIA^{glc}, a signal-transducing component of the phosphoenolpyruvate-glucose phosphotransferase system (PTS).

DNA Level Regulation

MalT is the key regulatory element in the maltose system that integrates signals from positive and negative effectors in the cell to bring forth an appropriate transcriptional response (see reviews (4, 86)). The interaction of MalT with its effectors appears to be modulated by the presence of either ATP or ADP, suggesting that MalT's intrinsic ATPase activity may play a role in its biological function. Indeed, a recent discovery in Richet's laboratory identified two forms of MalT: the ATP-bound active form and the ADP-bound resting form, which are preferentially bound by positive and negative effectors, respectively (58). Using a MalT-D129A variant that binds but does not hydrolyze ATP, it was shown that conversion of MalT to the active form is facilitated by an exchange of ADP for ATP that is triggered by the binding of the inducer, maltotriose, and that resetting to the inactive form requires ATP hydrolysis by MalT.

ATP-bound MalT—In the active state, MalT forms oligomers, which cooperatively bind to multiple sites upstream of the '-35' region of the *mal* promoters (49). The induction of the maltose transport genes requires the formation of a nucleoprotein complex composed of at least five copies of MalT and three cAMP/CAP dimers (reviewed in (6). Direct contact of

the DNA-binding domain of MalT (the first domain of MalT or DT1) with the RNA polymerase (RNAP) is thought to activate open complex formation (OCF) (10). CAP, in complex with cAMP, co-activates OCF by bending the DNA to facilitate binding of MalT multimers and by making direct contacts with RNAP as well (80).

ADP-bound MalT—The monomeric resting form of MalT is independently recognized by negative effectors: MalY, a β C-S lyase; Aes, an esterase; and MalK, an ATP-hydrolyzing subunit of the maltose transporter (reviews (3, 4, 28)). Non-overlapping surface determinants on MalT were identified for distinct binding of each effector (DT1 for Aes (41) and for MalY (87, 89); DT1 (41) and DT3 for MalK (81)). In each case, binding is reversed in the presence of maltotriose suggesting that all three proteins compete with inducer binding by stabilizing the inactive form of MalT.

The physiological relevance of Aes and MalY inhibition is not yet understood. However, MalK inhibition is currently interpreted in the context of the coupling of sugar transport with induction of transcription (see reviews (4, 26, 86)). Briefly, it is thought that, in the absence of transport, MalT associates with MalK to prevent induction by endogenous maltotriose, but during transport, MalT is freed from MalK and can activate the transcription of the maltose regulon. Endogenous maltotriose, which can induce the maltose system, may come from the degradation of glycogen, the catabolism of other carbon sources or the products of gluconeogenesis (21, 22); reviewed in (6). Moreover, while it has been thought for years that maltotriose is the exclusive inducer of the maltose system (78), recent studies suggest that an alternative inducer exists. In 2005, the Boos's laboratory was able to observe induction of the maltose regulon by transport of maltodextrins in strains lacking glycogen and the metabolic enzymes for generation of internal maltotriose (26). Such low level expression of the *mal* system was observed to be unaffected in the presence of MalZ, known to degrade maltotriose and thus reduce endogenous induction, suggesting that the additional endogenous inducer is likely not maltotriose (25).

Protein Level Regulation

The transport activity of MalFGK₂ is regulated by the interaction of MalK with EIIA^{glc} as part of a global control by catabolite repression (74, 86). In the presence of glucose, EIIA^{glc} becomes largely unphosphorylated and inhibits uptake of other sugars including maltose via a process called inducer exclusion (73). In *in vitro* assays, both the transport and substrate-stimulated ATPase activities of MalFGK₂ are reduced in the presence of unphosphorylated EIIA^{glc}, suggesting direct interactions with MalK (20, 48). Consistent with the idea of an allosteric interaction between MalK and EIIA^{glc}, mutations on the C-terminal region of MalK were found that relieve inducer exclusion (3, 20, 47). Moreover, the N-terminus of EIIA^{glc} has been identified through the use of synthetic cellulose-bound peptide arrays as the region that specifically contacts complex-assembled MalK (2).

Transporter Structure and Mechanism of Action

The ATP-binding cassette: Basis of cooperativity

The presence of two ATP-binding cassettes, and therefore two ATP-binding sites, has raised interesting mechanistic questions as to how ATP is hydrolyzed and coupled to transport. ATP is hydrolyzed with positive cooperativity by the intact maltose transporter (15), indicating that the two sites interact. Mutation of conserved residues K42 or H192 in a single ATP-binding site, achieved by coexpressing regular and histidine-tagged versions of MalK, and purifying heterologous transporter complexes, greatly impaired function indicating that both sites are important for function (17). Experiments using vanadate as a transition state analogue for phosphate in the hydrolysis reaction also revealed evidence of cooperativity, as they did in P-glycoprotein (106). Vanadate completely inhibits the ATPase activity but appears to trap nucleotide (Mg^{2+} -ADP-Vi) in just one of the two sites (91).

The basis for cooperativity was revealed only following the determination of the structure of the isolated ABC, and in particular, the true nature of the ABC dimer, which remained elusive for several years. Two structures of a MalK dimer have been reported, one from *Thermococcus litoralis* (23) and the second from *E. coli* (7), however, only the latter, which reports an ATP-bound dimer, reveals how the subunits interact. Two ATPs are bound along the dimer interface, each interacting with residues in the Walker A motif of one subunit and the family signature (LSGGQ) of the second subunit. This MalK structure recapitulates the dimer interface seen first in the catalytic subunit of Rad50, which undergoes an ATP-dependent dimerization (37) and the dimer of the ABC subunit MJ0796, which was stabilized by a Glu to Gln mutagenesis that prevents ATP hydrolysis (95). In common with other ABC cassettes, the N-terminal domain of MalK contains a nucleotide-binding subdomain, common to many ATPases, including RecA and the F_1 -ATPase and a helical subdomain, unique to ABC transporters, that contains the LSGGQ motif. MalK also has a C-terminal regulatory domain, present in a subset of bacterial ATP-binding cassettes, that functions both in inducer exclusion and transcriptional regulation in the maltose system, as discussed above. In addition to the Walker A and LSGGQ motifs, essentially all of the well conserved residues, including Asp158 in the Walker B motif, lie in close proximity to the nucleotide (Fig. 1). An aromatic residue (Trp13) stacks against the adenine ring of the nucleotide. The conserved residues Glu159, His192 and Gln82 lie close to the gamma phosphate of ATP. Gln82 lies in the center of the Q-loop, a flexible region that joins the nucleotide-binding and helical subdomains (112). Glu159 and His192 have both been implicated in catalysis in different systems and substitution of one or the other can eliminate ATPase activity, promoting the tight binding of ATP along the dimer interface (63, 113). Mutation of these residues has been valuable in obtaining crystal structures of isolated ATP-bound ABC dimers as well as the intact maltose transporter (70, 95, 113). The D-loop follows the Walker B motif in the linear sequence and is centrally located at the dimer interface, mediating contacts across the dimer in the closed conformation (7, 37).

The C-terminal regulatory domain contributes substantially to the dimer interface in MalK, maintaining isolated MalK as a dimer even in the absence of ATP (7, 45), conditions where other isolated cassettes behaved as monomers. Crystallization under different conditions

revealed that, in the absence of ATP, though the regulatory domains remain in contact, the nucleotide-binding domains are separated from each other; only in the presence of ATP do they came together to form the closed ATP-bound dimer (Fig. 2). The physiologic significance of this observation is strengthened by the structure of the first intact BP-dependent ATP transporter, the vitamin B₁₂ transporter, in which the NBDs are in an open conformation very similar to the nucleotide-free MalK dimer (51). The MalK dimer is also seen in an open conformation with MgADP bound to the Walker A motif, suggesting that ATP and not ADP is capable of supporting the closure of the nucleotide-binding interface (52). Several lines of investigation support the idea that ATP hydrolysis occurs in the closed conformation. First, mutations in the LSGGQ motif prevent ATP hydrolysis (88) and second, UV-irradiation of the transporter trapped with the transition state analogue vanadate induces photocleavage of the MalK peptide backbone at both the Walker A and LSGGQ motifs, indicating that both are in close proximity to the gamma phosphate during ATP hydrolysis (30).

Interpreting these results in the context of other supporting data, it appears that the NBDs of the maltose transporter undergo cyclic conformational changes, closing when ATP binds and opening following ATP hydrolysis. Insertion of a cysteine which forms a disulfide between the two regulatory domains do not interfere with transport in the intact system, indicating that similar conformational changes may occur *in vivo* (83). Molecular dynamics simulations, performed with the MalK dimer, indicate that the presence of MgATP promotes the closure of the nucleotide-binding interface, perhaps initially because of attractive forces through MgATP, although “first contacts” in the simulation are H-bonds between residues in the centrally located “D-loop” (Rad50), the Walker A and the conserved H192 (71).

Coupling of ATP hydrolysis to transport

The opening and closing of the nucleotide-binding interface during ATP hydrolysis provides a mechanism for the conversion of the chemical energy of ATP hydrolysis into the mechanical energy needed to drive maltose across the membrane and it appears likely that these motions are coupled to changes in both the TM helices and the MBP during the translocation cycle. Several changes have been noted when comparing the ATPase activities of the intact transporter with that of the isolated subunits. Mutation of residue Q140 following the LSGGQ motif prevents MBP from stimulating the ATPase activity of the intact transporter but does not affect the ATPase activity of isolated MalK, suggesting a role in communication between MBP and the ATPase (88). Suppressors of this mutation map in the second periplasmic (P2) loop of MalF, the first periplasmic (P1) loop of MalG and the EAA loop of MalG suggesting that these loops are also involved in the communication between MBP and MalK (11). In addition, ATP hydrolysis by isolated MalK is not inhibited by vanadate (39, 91). In contrast, the intact transporter is strongly inhibited by vanadate (39, 91).

Further insight into the mechanism of coupling was gained from the observation that MBP is trapped in complex with MalFGK₂ in the presence of vanadate (8). The affinity between MBP and MalFGK₂, as judged by the concentration of MBP required for half maximal transport, has been estimated to be rather low, at 25 to 100 μ M (19, 57). These results

suggest that tight binding of MBP to the transporter may stimulate ATP hydrolysis by stabilizing the transition state conformation of the transporter. Maltose is not bound tightly by MBP in the vanadate-trapped species suggesting that MBP is open when trapped in the high affinity complex with MalFGK₂ (8). Based on these and other data, we proposed a concerted model for transport, shown in Fig. 3, in which the transport process is initiated through a lower affinity interaction between closed MBP and MalFGK₂ in a resting state. If ATP is also bound, then conformational changes resulting in the opening of MBP to release maltose, the reorientation of TM helices to receive maltose and the closure of the nucleotide-binding interface to hydrolyze ATP, all occur simultaneously (8). Following ATP hydrolysis, which would destabilize the closed nucleotide-binding interface, the transporter would return to the resting state concomitant with the release of maltose inside the cell.

Site-directed spin labeling electron paramagnetic resonance spectroscopy (SDSL EPR) can be used to monitor both the mobility of amino acid residues at a given location and the distance between two spin labels in the range of ~8 - 20 Å (77). By placing a single cysteine in each lobe of MBP and attaching a nitroxide spin label, it was possible to confirm that MBP was open in the vanadate-trapped complex (1). Furthermore, the addition of ATP in the presence of EDTA or a nonhydrolyzable analogue such as MgAMP-PNP also stabilized a complex with MBP bound in an open conformation suggesting that this global conformational intermediate is stable as long as ATP hydrolysis is prevented. Mutation of Glu159 in the ATP-binding site of MalK to prevent ATP hydrolysis also trapped a complex of MalFGK₂ and open MBP (70). The similarity between the Glu159 mutant and the vanadate-trapped species was also detected in patterns of protease sensitivity and fluorescence of the periplasmic loops of MalF and MalG (13).

Several variations of the model shown in Fig. 3 have been proposed. In a recent review (93), Shilton emphasized the importance of MBP in stabilizing a high energy conformation of MalFGK₂ that allows ATP to drive the conformational changes, ie the opening of MBP and closure of the nucleotide-binding interface, that lead to the transition state for ATP hydrolysis. In his model, he suggests that interconversion of the inward-facing and outward-facing (transition state) conformations is prevented by a high energy intermediate and that MBP binding stabilizes this intermediate. In a first step, binding of liganded MBP promotes a closer association of NBDs so that ATP can drive formation of the closed dimer and open MBP in a concerted second step. Hydrolysis of ATP then returns the transporter to the intermediate conformation with MBP bound, and finally to the resting state. We would like to emphasize that while MBP is likely to be required to overcome an energy barrier in the progression from inward to outward facing conformations, MBP is also likely to be essential in stabilizing the outward facing conformation as evidenced by the high affinity it displays for the vanadate-trapped species.

Schneider and colleagues pose another variation in which it is suggested that ATP binding can induce the closure of the nucleotide-binding interface and perhaps also the reorientation of the transporter to the outward-facing conformation in the absence of MBP (or of maltose-bound MBP) and that binding of maltose to MBP, inducing closure of MBP, would initiate translocation by triggering changes, likely transmitted through the LSGGQ motif of MalK, to hydrolyze ATP (11). This model is based on their observations of (a) enhanced

crosslinking across the NBD interface upon addition of ATP with bifunctional reagents of shorter lengths (12) (40), and (b) the ability of ATP binding alone at the cytoplasmic surface to cause a change in the pattern of protease accessibility at the periplasmic surface, similar to that induced by vanadate trapping (13). These results lead the authors to suggest that, at the prevailing concentrations of ATP in the cell, the maltose transporter may actually “rest” in the outward-facing conformation with ATP bound, awaiting the binding of maltose to MBP (11). This model differs from that in Fig. 3 in the mechanism by which MBP stimulates ATP hydrolysis, either through the closure of the nucleotide-binding interface itself, or, through a different signaling mechanism that orients catalytic residues for hydrolysis within an already closed nucleotide-binding interface. To address this question, we positioned spin labels just outside the nucleotide-binding interface at positions 16 and 129 in MalK to monitor the distance between the two MalK subunits and the conditions that trigger closure of the interface (72). In the absence of nucleotide no spin-spin interactions were detected, indicating that spin labels were >20 Å apart, the limit of detection by continuous wave EPR (cwEPR). With maltose-MBP and either ATP + EDTA, or MgAMP-PNP present, conditions expected to trigger closure of the nucleotide-binding interface, the spin-labels approached to within 8 Å. Addition of nucleotide in the absence of MBP failed to induce this closure suggesting that the transporter rests in the inward-facing conformation even with ATP bound and, therefore, that MBP can stimulate ATPase activity simply by allowing the NBDs to close. A second EPR-based study employed both cwEPR and double electron electron resonance EPR (DEER), which can detect distance changes over a longer range, to examine NBD closure (33). With spin-label at position 85 in the Q-loop, a large decrease in distance was seen upon addition of ATP only (from 28 to 18 Å) with a smaller change upon vanadate-trapping (with MBP), a decrease to 15 - 17 Å. However, very different results were obtained with spin label at position 83, a residue also in the Q-loop that appears to move in concert with residue 85, as judged by the structures of MalK. At position 83, only modest changes were seen, the distance decreased from 20 to 17.5 Å with ATP only, and further decreased to 16 Å upon vanadate-trapping. Since the Q-loop is located in a sensitive region of the structure lining both the NBD-NBD and the TM-NBD interfaces (70), it is possible that positioning of either the backbone or the side chains of the Q-loop may be affected by steric constraints and/or events in addition to closure of the nucleotide-binding interface. The authors note (33) that positioning of the C-terminal tail of MalG along the Q-loop, as seen in the structure of MalFGK₂ (70) could also account for some of the results in this study. In contrast, no significant changes in the mobility of the spin-labeled residues at positions 16 and 129 in MalK were detected upon closure (72) suggesting that distance changes between these positions may better reflect the opening and closing of the interface. So, while it appears unlikely that the nucleotide-binding interface closes in the absence of MBP (72), it is possible that ATP binding induces conformational changes that affect the juxtaposition of the two NBDs and that can be sensed at the periplasmic surface of the transporter (12, 13).

Structure of the MBP-MalFGK₂ complex

The structure of the entire MalFGK₂ transporter in complex with MBP (Fig. 4) was obtained using the E159Q substitution to prevent ATP hydrolysis and lock the transporter in a conformational intermediate thought to resemble the catalytic transition state (70). In

remarkably good agreement with the model in Fig. 3, the nucleotide-binding interface of the MalK dimer is in fact closed, with two ATP bound, and the MBP is open, forming part of the boundary of an occluded, water-filled cavity that reaches halfway into the TM regions of MalF and MalG. Maltose is found at the base of this cavity, bound to residues near the center of three TM helices of MalF. The cavity appears to be large enough to accommodate longer chain maltodextrins at the base, raising the possibility that larger sugars can be transported in a single step, although different models have been proposed (26, 66). The maltose binding site in MBP forms part of the occluded cavity, however it is partially obscured by the presence of the third periplasmic (P3) loop of MalG which may function as a “scoop” to ensure that maltose is efficiently transferred from MBP to the transporter each time ATP is hydrolyzed. A two-fold symmetry is observed in the core region of the TM subunits, with TM 3-8 of MalF relating to TM 1-6 of MalG by a 180° rotation along an axis perpendicular to the membrane. The large periplasmic (P2) loop of MalF folds into a separate Ig-like domain that reaches around MBP in the complex, forming extensive contacts with MBP that may be important in both docking MBP onto the transporter and in stabilizing the transition state conformation.

With a structure of the entire MBP-MalFGK₂ complex available, it is now possible to interpret a wealth of biochemical and genetic data in light of the structure and many of the predictions made from this earlier data fit nicely with the structure. Six of ten residues that bind maltose have been identified in genetic studies as being important in transport activity (27, 97). A cytoplasmic loop of MalF and MalG, predicted to have a helix-turn-helix structure (85) and containing a conserved EAA motif was identified as a site of interaction between the TM domains and the NBDs using genetics and biochemistry (64). In the structure, this loop does have a helix turn helix configuration and the first helix, containing the EAA, lies in a cleft on the surface of MalK that is lined by residues in two helices from the helical subdomain, the helix following the Walker A and residues in the Q-loop (70). The conserved Glu401 makes a salt bridge with Arg47 in MalK. While mutations in the EAA motif of either MalF or MalG had only minimal impact on function, mutations of both simultaneously eliminated transport and MalK was found in the cytosolic fraction of the cell rather than the membrane fraction, suggesting that transporter assembly had been affected. Suppressors of the phenotype were isolated in *malK* that restored function and these mapped mainly to the helical domain of MalK. Crosslinking experiments confirmed the proximity of these residues in MalK to the EAA loops of MalF and MalG (40). Fusions between MalK and LacK had also pinpointed the helical domain, from residues 89 to 140 as being important in recognition of the TM domains (109).

Superpositioning of the structure of nucleotide-bound MalEFGK₂ with nucleotide-free ModB₂C₂A (36) reveals a striking similarity in the fold of the TM subunits, with the exception that the former is in an outward-facing conformation and the latter in an inward facing conformation. Together, they provide strong evidence in support of an alternating access model for translocation. The EAA loops are further apart in ModB₂C₂ than in MalFGK₂ suggesting that they move in concert with the nucleotide-binding domains upon closure. The EAA loops are also rotated within the cleft by 20° relative to the nucleotide-binding subdomain of MalK suggestive of a flexible joint between subunits. Another point

of contact between the TM domain and MalK is between the last 7 residues of MalG and the nucleotide-binding interface of the MalK dimer. These residues pack along the Q-loop of one MalK subunit (residues 83-89), and make contact with residues Ser83, Ala85 and Leu86 in the Q-loop of the second MalK subunit (70). This placement of the MalG tail in the transition state complicates the interpretation of changes revealed by patterns of crosslinking between residues in this region of the protein during the transition from inward to outward-facing conformations (12).

Sites of interaction between MBP and MalFGK₂ have been predicted using a variety of genetic methods (59) and suppressor analysis has suggested that residue 210 in the C-terminal lobe interacts with MalF, and residues 13 and 14 in the N-terminal lobe interact with MalG (38). This is clearly seen to be true in the structure although extensive contact is also made between the N-lobe of MBP and the P2 loop of MalF. A cysteine at position 13 which readily formed a crosslink with a cys in MalG at position 78 are in close proximity in the structure (11). Spin labels attached at positions 41 and 211 were immobilized by interaction with the transporter (1), and clearly line the interface between MBP and MalFGK₂ in the structure. Residues 213 and 214 were also shown to be very important for transport (98). In agreement with earlier work (60), both the crosslinking at position 13 and spin-labeling at position 41 suggest that the N-lobe of MBP contacts MalFGK₂ similarly in the presence and absence of maltose and in the resting and transition states (1, 11). The contact at position 211 is greatly enhanced in the transition state offering insight into the motions that may occur in the interaction of MBP with MalFGK₂ in the progression from the ground state to the transition state (1).

Binding protein independent (BPI) transporters

The BPI transporters isolated by Shuman and coworkers (9, 94, 105) display elevated levels of basal ATPase activity and specificity for maltose, though with greatly reduced affinity as compared to the wild type. It has been suggested that BPI mutants may reside in an intermediate state, between the resting and transition states that can achieve the transition state in the absence of MBP (93) or that they may even rest in an outward-facing conformation (13), exposing the low affinity binding site to the periplasmic surface. A decrease in accessibility of a fluorophore attached at the ATP-binding site (54) and an increase in protease susceptibility of several periplasmic loops (13) is suggestive of a conformational shift toward the transition state. In addition, several BPI transporters display higher affinity for MBP (19, 54), a characteristic of the transition state. At the high concentrations of MBP in the periplasm (1 mM), WT (wild-type) MBP will inhibit BPI transport, perhaps because it fails to dissociate, or because the MBP initiates interaction in an open rather than a closed conformation and bound maltose is not appropriately directed to the membrane-binding site. However, in some mutants, including the BPI MalG511 MalFGK₂ transporter, MBP still interacts productively when present at lower concentrations (19). Hall and colleagues noted that while MBPs that fail to close in solution fail to stimulate ATP hydrolysis by the WT transporter, they do interact productively with the MalG511 complex to stimulate the ATPase activity (34). This may reflect a productive binding of open MBP to this transporter with an already open translocation pathway, or, the ability of this mutant, through its higher affinity to BP, to induce closure, or partial closure of MBPs

that would not close on their own (34). It is likely that these mutants present a low affinity membrane-binding site to the periplasm in the absence of MBP. Most often, two mutations are required for a BPI phenotype, one is located in the C-terminal transmembrane helix and a second in a periplasmic loop. Interestingly, expressed alone, one of the BPI periplasmic loop mutations, L334W, alters the specificity of the transporter, allowing for transport of lactose in addition to maltose. In this mutant, MBP is required for transport of both sugars, though lactose is not bound by MBP (62). It is suggested that lactose gains access to the low affinity membrane binding site from the periplasm in this mutant, consistent with the idea that this residue has an important role in stabilizing the inward-facing resting state of the transporter in the absence of MBP. A second lactose specificity mutant was obtained, this one a BPI mutant, through truncation of MalF after 3 TM helices, which eliminates the determinants of specificity for maltose identified in TM 5,6 and 7 of MalF (61). It remains to be seen how transport occurs in this mutant, perhaps a second MalG subunit comes into play, substituting for the missing helices of MalF, to create a cavity with decreased specificity for sugars.

Transporter Assembly in the Membrane

Membrane Targeting and Insertion

The observation by Traxler and Beckwith in 1992 that MalF is protease-resistant when it is properly folded in the context of the intact MalFGK₂ transporter complex has provided a valuable assay for transporter assembly (102). The Sec translocase constitutes the general pathway for protein export to the periplasm or outer membrane and, using either a SecE-depleted, a SecA-inhibited or a SecY mutant strain (69, 104) in conjunction with the MalF protease sensitivity assay, it was shown that proper insertion of MalF into the membrane is dependent on Sec (53). Recently, the Sec-dependence of MalF and MalFGK₂ biogenesis was readdressed following the discovery of a membrane insertase, YidC, that co-purifies with the Sec translocase (84, 111) and which assists in the co-translational folding of the lactose permease, LacY (67). Photo-crosslinking experiments put nascent MalF in close proximity to the membrane-localized YidC, but the insertion of the MalF chains into the membrane does not require YidC as shown by protease sensitivity and biotinylation assays (107). However, depletion of YidC in cells showed reduced levels of intact MalF and of the whole MalFGK₂, as monitored by pulse chase experiments, suggesting that YidC may be critical for the folding of MalF into a stable conformation before its incorporation into the maltose transport complex. Moreover, in the same study, it was shown *in vitro* that MalF was directed to the Sec translocon by the signal recognition particle (SRP), which mediates co-translational targeting of most TM proteins to the membrane by its interaction with hydrophobic sequences on the nascent chain (53). Previously, *in vivo* studies have shown that MalF localization in the membrane was affected by mutations in the components of SRP (100, 101).

Subunit Interactions

Following the independent folding and membrane insertion of the domains, the assembly of the MalFGK₂ complex is thought to be mediated by subunit interactions (29, 55, 102). A collection of mutant transporters has been generated by a transposon-mediated mutagenesis

technique that generates proteins with 31 amino acid insertions (i31) at random locations (103). These mutants have been useful in understanding the subunit interactions and transporter assembly, and in the light of the recent crystal structure of the intact transporter (70), may now be fully appreciated. Mutants were shown to be defective in either transport, regulation or assembly, as judged by MalF protease sensitivity assays. In MalK, the insertion sites of assembly-defective mutants map to the region on the Q-loop, where it contacts the transmembrane domains (TMD), as seen in the crystal structure, and near the signature motif, LSGGQ, which mediates MalK dimerization in the presence of ATP (50). In MalG, an insertion site in the periplasmic (P3) loop prevented interaction with MBP, restoring BPI transport to a BPI mutant that is normally inhibited in the presence of MBP (68). The same MalG-P3 loop (the scoop loop) was shown to reach into the MBP sugar-binding cleft in the catalytic intermediate conformation of the transporter. Moreover, MalF and MalG mutants having insertions in the conserved cytoplasmic loops containing the EAA motif were found to be deficient in MalK binding but associate efficiently with each other (44). Interestingly, certain insertions in the periplasmic loops of either MalF or MalG seem to alter the cytoplasmic domains that interact with MalK. Insertion mutants of the third periplasmic domain of MalG failed to co-immunoprecipitate with the MalK dimer (44), while insertion/deletion mutants of the second periplasmic domain of MalF failed to fractionate with MalK in the membrane pellet (99).

Transport Complex Assembly

Ordered assembly of a multimeric complex is characterized by progressive addition of components, thus certain combinations of the subunits are formed and others are not. It was generally accepted that membrane complexes assemble in an orderly manner based on previous work with the acetylcholine receptor, the T-cell antigen receptor, and the major histocompatibility complex Class I loading complex (24, 32, 42, 56, 82). In 2004, the finding that maltose transport components associate in different combinations contradicted this notion. All combinations of MalF, MalG and MalK dimer, in pairwise expression assays and in pulse-labelled extracts, were efficiently recovered by co-immunoprecipitation (44). The different subcomplexes that were isolated may represent intermediates in the pathway of assembly of the heterotetrameric complex, MalFGK₂. Although a preferred route may exist (with the MalK dimer as platform for transport assembly (45, 92)), the identification of other possible subunit associations suggests a promiscuous assembly mechanism that favors the rapid and efficient assembly of the maltose transporter (102).

Functional reassembly of the purified individual domains of the maltose transporter has been successfully demonstrated *in vitro*. The MalFGK₂ complex can be reconstituted by incubation of purified MalK with inverted membrane vesicles containing MalFG (65). An intact transport complex can also be disassembled with urea and then reassembled into a functional transporter in proteoliposomes or in detergent solution (48, 92). This *in vitro* modular assembly provides a powerful strategy that allows differential labeling of the domains of the transport complex for biophysical studies such as electron paramagnetic resonance spectroscopy (EPR) and fluorescence transfer energy transfer (FRET).

Concluding Remarks

One of the strengths of the maltose transport system as a model for understanding the structure and function of ABC transporters lies in the fact that we now have both high resolution structural information and a wealth of biochemical, biophysical and genetic information upon which to construct a feasible model for function. The Chen lab has recently solved the structure of an inward-facing nucleotide-free MalFGK₂ (46). Comparison of the inward-facing and outward-facing conformations confirm that rigid body rotations of transmembrane helices coupled to the opening and closing of the nucleotide-binding subunits result in alternating access of the central sugar-binding pocket (Fig. 5). The availability of these two structures unlock a new set of more detailed questions about the mechanism. Another area that requires elucidation is the role of the two ATP-binding sites in the overall translocation cycle. It is not yet clear, for example, whether one or both ATPs are hydrolyzed during a single cycle of transport.

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Fig. 1. Structure of the MalK monomer highlighting conserved residues and motifs

Just one of two subunits in the ATP-bound MalK (PDB: 1Q12) is shown. The helical subdomain is colored blue, the nucleotide-binding subdomain is green and the C-terminal regulatory domain (truncated) is colored yellow. Conserved residues and motifs discussed in the text are highlighted and labeled in the figure. Two ATPs, which lie along the interface in the actual dimer structure (see Fig. 2), are colored CPK. Figure prepared with Pymol.

Fig. 2. ATP hydrolysis by the MalK dimer

In the absence of nucleotide (ground state, 1Q1B), the nucleotide-binding interface between the two NBDs (colored different shades of blue and green) of MalK are separated with dimer contacts maintained through contact of the regulatory domains (yellow). In the ATP-bound structure (1Q12), the nucleotide-binding interface is closed. In the ADP-bound structure (2AWO), the interface is again open suggestive of a cycle of ATP hydrolysis linked to conformational change. (Reprinted from (52), with permission).

Fig. 3. A model for maltose based on concerted conformational changes

In the absence of maltose, the transporter rests in a conformation in which the nucleotide-binding interface is open and the sugar-binding site in the membrane is exposed to the cytoplasm, even in the presence of ATP. When maltose binds MBP, it closes, and the closed form of MBP initiates a cycle of transport. In a concerted motion, MBP opens as it becomes more tightly bound by the transporter and the nucleotide-binding interface closes as TM helices reorient to alternate exposure of the sugar-binding site from the cytoplasm to the periplasm to receive maltose as it is released from MBP. ATP hydrolysis occurs in this conformation and following hydrolysis, the nucleotide-binding interface becomes unstable, returning the transporter to a resting state. (Adapted from (8), with permission).

Fig. 4. Structure of MBP-MalFGK₂

In the structure (2R6G), MBP is trapped in complex with ATP-bound MalFGK₂ using an E159Q substitution in MalK to prevent ATP hydrolysis. Subunits are colored as indicated in the figure. On the right, a space-filling model is cut away to reveal an outward-facing occluded pocket containing bound maltose. (Adapted from (70).

Fig. 5. Structure of MalFGK₂ in inward-facing conformation

In the structure (3FH6), MalF lacking the first two TM helices was used for crystallization. Subunits are colored as indicated. On the right, as space-filling model is cut away to reveal an inward-facing empty maltose binding pocket. (Reprinted from (46) with permission).