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Tinkering with Transporters: Periplasmic Binding Protein-Dependent Maltose Transport in *E. coli*

Howard A. Shuman[1,2] and Cynthia H. Panagiotidis[1]

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Periplasmic binding protein-dependent transport systems represent a common mechanism for nutrient and ion uptake in bacteria. As a group, these systems are related to one another and to other transporters of both prokaryotes and eukaryotes, based on sequence similarity within an ATP-binding subunit and overall structural organization. These transporters probably all use energy derived from ATP to pump substrates across membranes. Although there is considerable information about the sequences and identity of the transporters, there is little information about how they work. That is, where do ligands bind? Where do the subunits or domains interact with one another? How is the energy of nucleotide binding and/or hydrolysis converted to conformational changes? In order to address these questions we have taken a genetic approach that involves studying mutant forms of a transporter. Rather than study mutations that result in complete loss of function, the study of mutations which perturb or alter the normal function of the transporter in a defined manner has provided a limited insight into how the answers to these questions may be obtained.

KEY WORDS: Maltose-binding protein; periplasmic binding protein; ATP-binding cassette; traffic ATPase; nucleotide-binding fold; suppressor mutations; dominant mutations.

TELEOLOGY

Selective permeability is essential to all forms of life; what comes in and goes out is of capital importance. Membrane proteins have evolved to make these critical decisions and mediate either the efficient uptake of nutrients, the appropriate balance of ions across the membrane, or the expulsion of unwanted or exported materials. Depending on the substance and its concentration, different mechanisms may be better or less well suited to a particular task. Apparently, many important growth substrates and ions can be moved across membranes efficiently by single polypeptides energized by electrochemical gradients of protons or sodium ions. The lactose permease of *Escherichia coli* is a well-studied example of this class of transporters. Nevertheless, other substances cannot, or are not, transported efficiently enough by this type of system to suit the driving force of evolution. Hence the existence of multicomponent transporters comprised of three or four subunits which can operate at exceedingly low substrate concentrations, increase the intracellular concentration of a substrate 10^5-fold higher than its extracellular concentration, and export macromolecular substrates.

HISTORY

Once it was established that in gram-negative bacteria there was an aqueous compartment separate from the cytoplasm (Neu and Heppel, 1965), the obvious question was, why? The identification of water-soluble proteins with the ability to bind specific ligands such as ions (Pardee, 1966), sugars, and amino acids (Anraku, 1968) led some to speculate that the binding proteins were somehow involved in transport of, or chemotaxis toward, the specific ligand. The

[1] Department of Microbiology, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, New York, New York 10032.

[2] To whom correspondence should be addressed.

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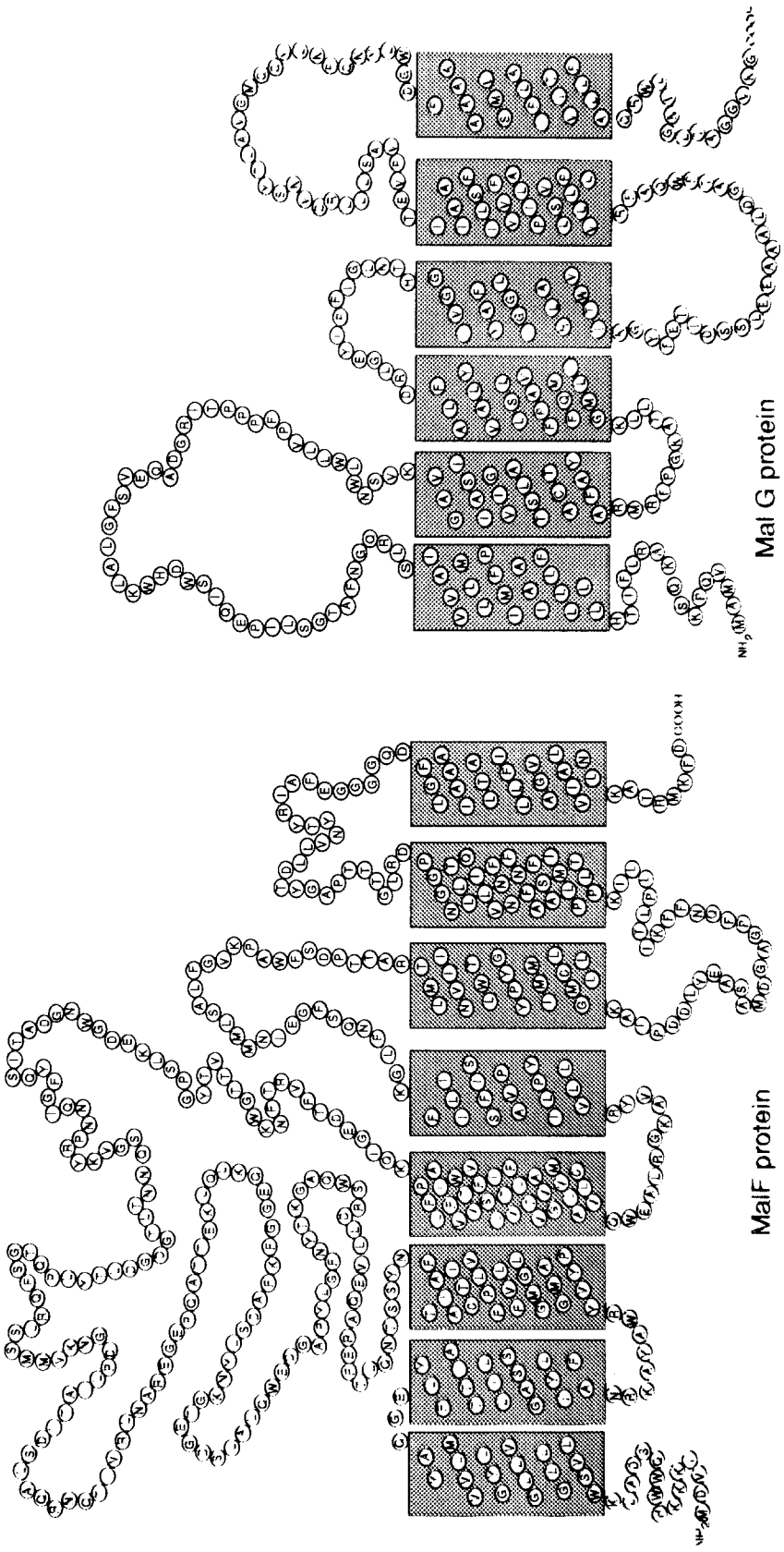

Fig. 1. Topological model for the MalF and MalG proteins. This diagram was made using the published sequences of MalF (Froshauer and Beckwith, 1984) and MalG (Dassa and Hofnung, 1985) and the topology determined by *phoA* fusions (Boyd *et al.*, 1987; Boyd and Beckwith, 1989; Boyd *et al.*, 1993; Dassa and Muir, 1993).

periplasm would then be a gathering place or station on the way to cytoplasm. Remarkably, both speculations turned out to be true (Boos., 1974; Hazelbauer and Adler, 1971). These suggestions posed an enigmatic question; how can water-soluble proteins either carry ligands or cause them to be carried into the cytoplasm? Or, how can these proteins signal that a particular substance is present in the periplasm? Much effort was put into searching for the accessory components that would collaborate with the periplasmic binding proteins in substrate transport or chemotaxis. These proteins have now been identified either by dint of sheer labor (Ames and Nikaido, 1978; Bavoil *et al.*, 1980; Dassa, 1990; Shuman *et al.*, 1980) or more often, lately, by cloning and sequencing. The current challenge is to discern how the binding proteins and their collaborators mediate the active accumulation of substrates at the expense of ATP.

THE PLAYERS

Initial genetic and physiologic studies on the uptake system for maltose and longer $\alpha(1\rightarrow4)$ glucose oligosaccharides revealed that five components were required (Silhavy *et al.*, 1979), transport was unidirectional, and that high-energy phosphate rather than $\Delta\mu_{H^+}$ provided the energy (Wiesmeyer and Cohn, 1960). Remarkable was the discovery that one of the components was an outer membrane protein receptor for phage λ (Randall-Hazelbauer and Schwartz, 1973) and a specific pore that increases the diffusion rate of maltose and longer $\alpha(1\rightarrow4)$ glucose oligosaccharides at low ($< 10^{-4}$ M) external concentrations (Szmelcman and Hofnung, 1975; Szmelcman and Schwartz, 1976; Freundlieb *et al.*, 1988; Luckey and Nikaido, 1980).

A maltose-inducible periplasmic protein was identified and shown to bind 1 mol maltose/molecule with a K_D of approximately 10^{-6} M (Kellermann and Szmelcman, 1974). This protein, maltose-binding protein (MBP), has been studied by many groups for a variety of reasons. Its three-dimensional structure both in the liganded and unliganded forms has been determined (Spurlino *et al.*, 1991; Scharff *et al.*, 1992), its peregrinations through the secretory system of *E. coli* to the periplasm have been examined in minute detail (see Schatz and Beckwith, 1990 for a review), and its role as the chemoreceptor for maltose through the Tar signal transducer has been established through genetic and biochemical means (Manson *et al.*, 1985; Manson and Kossmann, 1986; Zhang *et al.*, 1992). Indeed, MBP has realized that highest degree of popularity any protein can achieve, as a protein tag for creating hybrid molecules which can be purified on the basis of its high affinity for crosslinked amylose (New England Biolabs, 1992).

As mentioned above, a periplasmic binding protein does not a transport system make. A variety of approaches led to the identification of the MalF, MalG, and MalK polypeptides. Recently, the purified $MalFGK_2$ complex has been shown to mediate MBP-dependent maltose transport in proteoliposomes at the expense of ATP (Davidson and Nikaido, 1991). MalF and MalG are polytopic membrane proteins which span the bilayer eight and six times, respectively (Fig. 1). MalK is a hydrophilic protein that is intimately associated with MalF and MalG (Bavoil *et al.*, 1980; Shuman and Silhavy, 1981; Panagiotidis *et al.*, 1993), and contains two regions (the "Walker A and B" nucleotide-binding fold) found in a variety of purine nucleotide-binding proteins (Fig. 2) (Walker *et al.*, 1982). A subset of the proteins which contain the Walker A and B regions forms a group based on sequence similarities in a region connecting the A and B sequences. These are referred to as the ATP-binding cassette (ABC)-transporters (Hyde *et al.*, 1990) or traffic ATPases (Shyamala *et al.*, 1991) and include other periplasmic binding protein-dependent transporters; export systems for hemolysins, toxins, and surface appendages; eukaryotic transporters such as the MDR-1 P-glycoprotein, the CFTR chloride channel, the peptide transporters presumably used during antigen processing in immune cells; and others. For all of these systems the central questions are similar and remain largely unanswered. Structural analysis of purified transporters will someday undoubtedly clarify the situation, but cannot be relied upon to resolve entirely all of the issues.

THE FOUR QUESTIONS

It is helpful to break down the problem of "How does it work?" into manageable specific questions. First, what is the role of maltose-binding protein? Is it essential for substrate translocation across the membrane and, if it is, why? Second, where are the sites for ligand interactions? Is there a substrate recognition site in $MalFGK_2$ in addition to that in

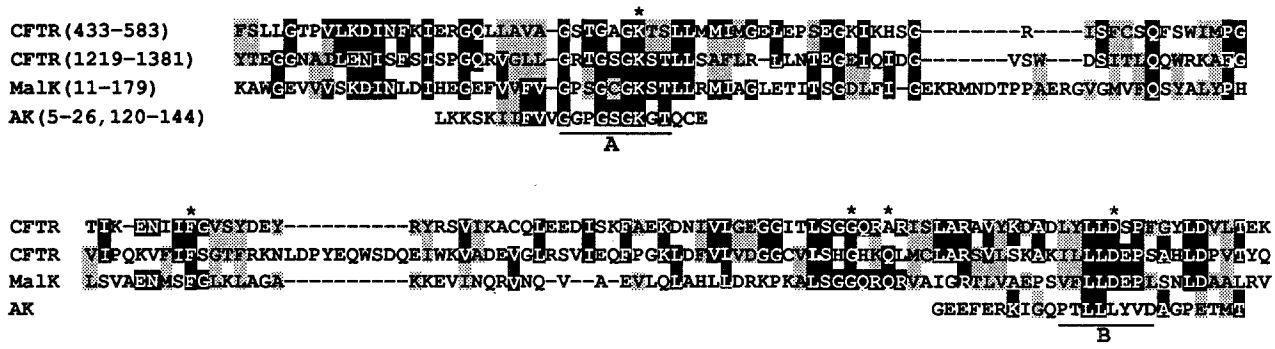

Fig. 2 Alignment of the MalK sequence with parts of the sequences of adenylate kinase and CFTR. The positions of residues altered by *malK* mutations discussed in the text are indicated by a star (★). Areas presented in reverse are identical; shaded areas represent conservative differences.

MBP? Third, where do the subunits contact one another? Where are the sites on MBP for interacting with the other subunits, where do the membrane proteins contact MBP, where do the membrane proteins contact one another? Fourth, how does energy from ATP binding and hydrolysis result in conformational changes that mediate substrate translocation across the membrane?

THE MODEL

In order to answer some of these questions we adapted a simple model used for the Na-K ATPase, to the maltose system (Fig. 3). The essential aspects of this model are: (i) the FGK_2 complex contains a substrate recognition site, (ii) the complex exists in two forms, one $[FGK_2]_O$, which is "open" to the periplasm and the other, $[FGK_2]_C$, which is "closed" to the periplasm but facing the cytoplasm, (iii) energy derived from ATP binding and/or hydrolysis is used to interconvert the two forms so that a cycle occurs.

THE ANSWERS

MBP

In order to find out if MBP was essential for transport, an *E. coli* strain was constructed that contained a large deletion within the MBP structural gene, *malE*, and that constitutively expressed the other genes for the FGK_2 complex and LamB. This strain cannot utilize maltose as a sole carbon and energy source, even if the external concentration is as high as 50 mM (Shuman, 1982). Because the LamB protein is sufficient for the efficient diffusion of maltose into the periplasm (Freundlieb *et al.*, 1988), it can be safely inferred that MBP is required for detectable substrate translocation across the cytoplasmic membrane. In order to have the bacteria provide information about the block in the transport cycle when MBP is absent, revertants which regained the ability to transport maltose without MBP were isolated. The properties of these "MBP-independent" mutants have provided a rich insight into the questions posed above.

The MBP-independent mutants exhibited variable growth rates in liquid medium containing 5.5 mM maltose as sole carbon and energy source; some grew as well as wild-type *E. coli* while others exhibited growth rates that were barely measurable (Treptow and Shuman, 1986). This suggested that the kinetics of transport among the mutants were variable. This was found to be the case: All of the mutants in which transport could be measured exhibited a K_M of about 2 mM, but each had a V_{max} that varied from about the same as wild-type to about 10- to 20-fold lower. A simple interpretation of these results in the context of the model (the transition theory) is that in wild-type bacteria the $[FGK_2]_C \leftrightarrow [FGK_2]_O$ transition is dependent on MBP, while in the MBP-independent strains, the transition occurs spontaneously. Other interpretations exist, however, notably the ever popular "hole" theory. Perhaps the structure of the FGK_2 complex has been rendered leaky to varying extents in the different mutants.

The Transition Theory

The hole theory (in its strictest sense) predicts that transport should not be saturable and no longer be energy-dependent. The fact that unrelated sugars do not inhibit ^{14}C-maltose uptake in the mutants but that a maltose analog, 4-nitrophenyl-α-maltoside, is a competitive inhibitor ($K_I = 40\,\mu M$) is inconsistent

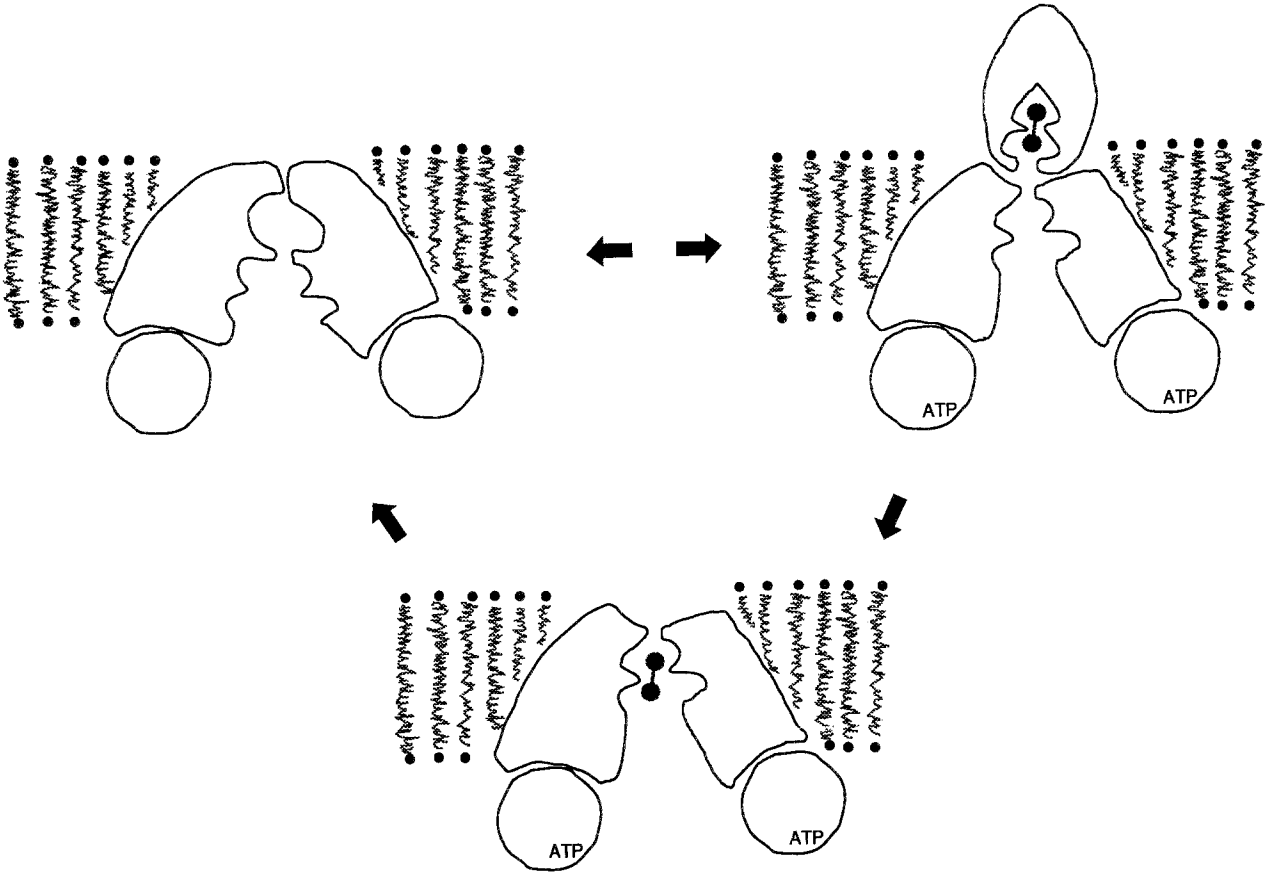

Fig. 3. A simple model for the transport cycle. The FGK_2 complex exists in two forms. One form is closed (top left) and is converted to the open form (top right) as a result of triggering by docked MBP. Maltose (the black dumbell shape) moves from MBP to a specific site within the FGK_2 complex. The open form is converted back to the closed form after maltose is deposited in the cytoplasm. ATP is shown bound to the MalK subunits; energy derived from ATP binding and hydrolysis is used to drive the cycle.

with the hole theory (Reyes, *et al.*, 1986). Importantly, other disaccharides, such as lactose and melibiose, are not transported by the mutants. Recently, it has been possible to isolate secondary mutations that alter the specificity of the MBP-independent complexes so that they can transport lactose (G. Merino, unpublished result). In addition, transport in all the mutants is dependent on MalK and, for some, it has been shown to be ATP-dependent.

From the work of Davidson and Nikaido it is known that MBP is required for the maximal rate of ATP hydrolysis by the purified FGK_2 complex *in vitro* (Davidson and Nikaido, 1990). The transition theory predicts that MBP normally interacts with the complex and subsequently triggers the transition from $[FGK_2]_C \leftrightarrow [FGK_2]_O$, and in the mutants this transition occurs spontaneously; one would expect that the mutant complexes would hydrolyze ATP in the absence of MBP. Indeed, this was found to be the case. Moreover, the rates of ATP hydrolysis correlated with the V_{max} of maltose transport (Davidson *et al.*, 1992). Therefore, we conclude that the structural alterations in the mutant complexes mimic the effects of MBP on the wild-type complex which result in the $[FGK_2]_C \leftrightarrow [FGK_2]_O$ transition and ATP hydrolysis.

The mutations which result in this complex phenotype all reside within the MSS of MalF and MalG (Hor *et al.*, 1993). Exactly how the amino acid substitutions mimic the effect of MBP on the complex is still unclear. Nevertheless, it was important to examine the effects of normal MBP on these mutant complexes. Naively, we expected that either the complexes would have lost the ability to recognize MBP or that they would interact normally. Surprisingly, MBP *inhibited* maltose transport in essentially all of the MBP-independent mutants. Once the initial skepticism was dispelled (that is, the strains did

not get mixed up), it made perfectly good sense that amino acid substitutions in the MalF and MalG proteins which mimicked the effects of MBP on the FGK_2 complex might impair some aspects of the normal interaction of the complex with MBP. The faulty interactions between MBP and the mutant FGK_2 complexes seemed an ideal scenario to look for suppressor mutations in *malE* that would restore a productive interaction and permit high-affinity (μM) MBP-dependent maltose transport.

Sites of Contact

Mutations in *malE* that alter tyrosine-210 to other residues were able to restore high-affinity MBP-dependent transport to the *malF*506 mutant FGK_2 complexes; *malE* mutations that alter residues glycine-13 and aspartate-14 were able to restore transport to the *malG*511 mutant complexes (Hor and Shuman, 1993; Treptow and Shuman, 1988). In both cases the suppressor mutations exhibited allele specificity and had little effect on ligand binding. Tyrosine-210, glycine-13, and aspartate-14 are solvent accessible in the open and closed forms of MBP. Although aspartate-14 is thought to contribute a hydrogen bond to the sugar, mutant MBP that contains glycine at this position still bound maltose and maltohexaose sugars with a K_D value no more than 10-fold higher than the wild-type protein. A simple interpretation of these results is that the C-lobe of MBP (where tyrosine-210 is located) interacts with MalF, and the N-lobe (where glycine-13 and aspartate-14 are located) interacts with MalG. This model is attractive because the relative positions of the lobes change dramatically upon ligand binding. Changes in the relative positions of the lobes provide a direct way for MBP to communicate its state of ligand occupancy to the FGK_2 complex through interactions with different subsets of amino acid residues on the periplasmic faces of MalF and MalG.

The suppressor MBPs function only with the mutant FGK_2 complexes that were used to select them; therefore, they are nonfunctional with wild-type FGK_2. Does this mean that the mutant MBP does not interact with the wild-type complex, or does a nonfunctional interaction take place? This distinction is important because it allows one to determine whether the docking of MBP onto a complex and its triggering of subsequent steps in the transport cycle are separable. Indeed, the mutant MBPs appear to be dominant to wild-type MBP, indicating that a nonfunctional interaction takes place between the mutants and the wild-type FGK_2 complex. Therefore, we conclude that the mutants retain the ability to dock but are defective in the triggering mechanism (Hor and Shuman, 1993).

A more detailed examination of the effects of wild-type MBP on the *malG*511-encoded FGK_2 complex was performed by studying the rates of maltose transport in vesicles in the presence of different concentrations of MBP and maltose. David Dean found that at low concentrations ($\sim 10\,\mu$M), wild-type MBP dramatically increased the rate of maltose uptake by the *malG*511 mutant vesicles. Only at high concentrations of MBP and maltose ($> 200\,\mu$M) was there inhibition of transport (Dean *et al.*, 1992). An interpretation of these results is that the interaction of MBP with the mutant $F[G511]K_2$ complex has a higher affinity than the interaction between MBP and the wild-type complex. At low concentrations of MBP, dissociation of MBP from the complex is still consistent with efficient transport. In contrast, at high concentrations of MBP, transport is limited by MBP leaving the complex, presumably because completion of the transport cycle requires an unoccupied form of the FGK_2 complex.

Interactions among the Membrane-Bound Subunits

A severe disadvantage to studying the FGK_2 complex is that in wild-type bacteria it is present in vanishingly small quantities that are membrane bound and difficult to purify. In order to ameliorate these conditions the three genes have been cloned on plasmids and connected to transcriptional controls that are easy to turn on and off. Once cloned, it was possible to examine all possible (8) permutations of the three genes. Two facts emerged from this study. First, MalF and MalK could form a stable complex without a functional MalG polypeptide, but the amino terminal region of the MalG protein stabilized MalF from proteolysis. In the complete absence of MalG, MalF was broken down quantitatively to a form migrating at 20,000 molecular weight rather than its normally aberrant migration at 40,000. This did not occur in preparations from strains containing a *malG* amber mutation about half way through the gene (Panagiotidis *et al.*, 1993). Another indication that the amino terminal part of MalG interacts with the carboxy-terminal part of MalF comes from the observation that precise fusion of the C-terminal residue of MalF with the amino terminal residue of MalG results in a hybrid that retains partial function as a MalF subunit, a MalG subunit, as well as both

together. If the C-terminus of MalF were normally far away from the N-terminus of MalG, such a MalF–MalG hybrid protein might not be able to achieve an active configuration (S.-F. Yan and H. A. Shuman, unpublished results).

Energy and Conformational Change

The ability of the FGK_2 complex to pump substrates against considerable gradients is the consequence of using ATP rather than $\Delta\mu_{H^+}$ as an energy source. What does energy coupling mean in terms of questions that are answerable? The MalK subunit has been assigned the role of energy coupling because it contains sites for binding ATP and indeed the isolated purified subunit can hydrolyze ATP. A clue comes from the structure of the enzyme adenylate kinase (AK) which shares sequence similarity with MalK in the regions thought to be important for ATP binding. One region is a glycine-rich loop which contains a lysine that coordinates the negative charge on the phosphate of ATP, the other region is a hydrophobic stretch that adjoins the planar part of the nucleotide. AK can exist in two conformations in the presence and absence of nucleotides. In the presence of nucleotides, the glycine-rich loop and the hydrophobic stretch form a tight pocket and, in their absence, the two regions are further apart (Tsai and Yan, 1991). A tantalizing possibility is that the corresponding regions in MalK undergo a similar alteration in conformation and these states interact differently with the MalF and MalG subunits during the transport cycle. So questions begin to emerge. Is the glycine-rich loop critical for energy coupling? Site-directed mutations in the *malK* gene which alter lysine 42 (the one that corresponds to the important AK lysine 21) to four other residues result in the production of stable polypeptides which associate with the MalF and MalG subunits but have no transport activity and little, if any, ability to bind ATP. One might imagine that these mutant MalK proteins would be dominant; they can occupy the same sites as the normal MalK protein but are inactive (spoilers). Curiously, not all are dominant as expected. Mutants in which lysine 42 has been changed to asparagine and glutamine are dominant to wild-type. However, the other mutants in which isoleucine or glutamate is present are recessive. They must be less able than wild-type MalK to interact functionally with MalF and MalG. This implies that residues in the glycine-rich loop might be critical for the pathway of communication between MalK and MalF:MalG (Panagiotidis *et al.*, 1993).

CODA

Not surprisingly, significant questions other than those discussed here remain. Implicit in the foregoing discussion is the assumption that only the liganded form of MBP interacts with and triggers the transport cycle of the FGK_2 complex. Unfortunately there are no data that contradict the theory that both liganded and unliganded forms interact with the complex and that triggering can happen *after* docking of unliganded MBP. Winfried Boos and Erich Bohl have derived a mathematical description of the individual steps of binding protein-dependent transport that is based on the known kinetic behavior of the maltose transport system. Surprisingly, these data and their mathematical model are most consistent with the idea that both forms of MBP interact with the FGK_2 complex.

Another set of fundamental questions relates to the interactions among membrane-spanning segments of integral membrane proteins. Presumably these fit together and form the path for substrate translocation, and their movement with respect to one another results in the transmembrane passage of information in addition to ligand. Even with detailed structural information about the FGK_2 complex, it will be challenging to understand the relationship between the arrangement of the MSS, "signal transduction," and substrate translocation.

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