ATP Alone Triggers the Outward Facing Conformation of the Maltose ATP-binding Cassette Transporter*

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Huan Bao1 and Franck Duong2

From the Department of Biochemistry & Molecular Biology, Life Sciences Institute, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

Background: The conformational cycle of MalFGK2 is stimulated by ATP, MalE, and maltose.

Results: The transporter outward facing conformation depends on ATP only.

Conclusion: MalE and maltose must therefore stimulate the return of the transporter to the inward facing state.

Significance: Understanding the dynamics of the transporter is important to interpret the crystal structures.

The maltose transporter MalFGK2 is a study prototype for ABC importers. During catalysis, the MalFG membrane domain alternates between inward and outward facing conformations when the MalK dimer closes and hydrolyzes ATP. Because a rapid ATP hydrolysis depends on MalE and maltose, it has been proposed that closed liganded MalE facilitates the transition to the outward facing conformation. Here we find that, in contrast to the expected, ATP is sufficient for the closure of MalK and for the conversion of MalFG to the outward facing state. The outward facing transporter binds MalE with nanomolar affinity, yet neither MalE nor maltose is necessary or facilitates the transition. Thus, the rapid hydrolysis of ATP observed in the presence of MalE and maltose is not because closed liganded MalE accelerates the formation of the outward facing conformation. These findings have fundamental implications for the description of the transport reaction.

ATP-binding cassette (ABC)3 transporters are found both in prokaryotes and eukaryotes and transport a wide variety of substrates in and out of cells. The understanding of their mode of operation is relevant for medically important processes such as multidrug resistance, chloride conductance, cholesterol transport, and surface-antigen presentation (1). In bacteria, the maltose transporter has been studied for several decades as the prototype of ABC importers (2–4). The transporter is comprised of a cytosolic homodimeric ABC domain MalK bound to a membrane domain heterodimer, MalFG. On its periplasmic side, the transporter associates with the substrate-binding protein MalE that acts as a receptor for maltose.

Major progress in the description of ABC transporters has been achieved by x-ray crystallography (5–7). In the structure of MalFGK2 without ligands, the sites for ATP binding on the MalK dimer are separated, and the MalFG membrane domain forms a cavity that is exposed to the cytosol (inward facing state) (8). In the structure obtained in the presence of MalE and nonhydrolyzable ATP analogs, the ATP-binding sites are bound together, whereas the MalFG cavity opens toward the periplasm (outward facing state) (6, 9). Together, these snapshots of the transporter structure have provided the molecular framework to explain how ATP binding and hydrolysis is coupled to membrane transport, the so-called alternating access mechanism. However, what triggers the transport reaction, why MalE stimulates ATP hydrolysis even in the absence of maltose, and how maltose is transferred from MalE to MalFG remain unclear.

MalE consists of two lobes with the maltose-binding site located at the interface. When the sugar binds, the two lobes close and eventually capture maltose with a high affinity (conformation termed closed liganded MalE) (10, 11). Because the ATPase activity of the transporter is maximal only in the presence of MalE and maltose, it has been proposed that closed liganded MalE binds the transporter and triggers ATP hydrolysis (12, 13). The x-ray structures were interpreted with this assumption in mind, leading to the model in Fig. 1A (2). In that model, the binding of closed liganded MalE to the transporter induces the pairing of MalK. The pairing of MalK triggers the MalFG outward facing conformation, which in turn forces the opening of MalE and the release of maltose. Upon ATP hydrolysis, the transporter returns to the inward facing state, and maltose enters the cytosol. In support of the model, an EPR spectroscopy study reported that the MalK nucleotide-binding interface closes only in the presence of MalE and ATP (14). A partial closure of the MalK interface was also observed when MalFGK2 was co-crystallized with closed liganded MalE. It was concluded that MalE facilitates the pairing of MalK and therefore hydrolysis of ATP (15). However, despite the evidence and the prevalence of the model, we recently reported that closed liganded MalE does not bind the transporter but dissociates when MalE captures maltose. We also reported that open state MalE binds with nanomolar affinity to the outward facing transporter and creates a low affinity receptor for maltose (16). These data, difficult to reconcile with the former model, led

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2 Supported by Chaires de Recherche du Canada. To whom correspondence should be addressed: Dept. of Biochemistry & Molecular Biology, Life Sciences Institute, Faculty of Medicine, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel.: 604-822-5975; Fax: 604-822-522; E-mail: fduong@mail.ubc.ca.
3 The abbreviations used are: ABC, ATP-binding cassette; DDM, n-dodecyl-β-d-maltoside; BMOE, bis-maleimidoethane; 5-IAF, 5-iodoacetamide fluorescein.
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**FIGURE 1. Two opposed models for maltose transport.** A, in the conventional model, closed liganded MalE triggers the outward facing conformation. MalE binds maltose (K_d = -2 μM) and then associates with the inward facing transporter (K_d > 45 μM). The transition to the outward facing conformation facilitates the opening of MalE and the release of maltose to the MalFG cavity. Upon ATP hydrolysis, the transporter returns to the inward facing state and maltose is released in the cytosol. In this model, MalE facilitates the pairing of MalK and the ATP hydrolysis step. B, in the proposed new model, ATP alone triggers the outward facing conformation. The outward facing transporter binds unliganded MalE with a high affinity (K_d = 50–80 nM). Maltose then binds to the MalE-MalFGK2 assembly (K_d = -120 μM). Upon ATP hydrolysis, the transporter returns to the inward facing conformation, and maltose is released in the cytosol. In this model, MalE stimulates the return of the transporter to the inward facing conformation. If ATP hydrolysis does not take place immediately, or if maltose is present in excess, MalE acquires its closed liganded conformation and dissociates from the transporter (negative autoregulation).

us to propose a new model (Fig. 1B and legend for details about the binding affinities).

Here, we further investigate the effect of MalE, maltose, and ATP on the conformation of MalFGK2. Indeed, an important and central question is the mechanism leading to the outward facing conformation. The transition depends on MalE and maltose in the former model, but not in the newer model. Using cross-linking, co-sedimentation analysis, native gels, and fluorescence assays, we show that MalE and closed liganded MalE do not facilitate the closure of MalK dimer, nor the transition of MalFG to the outward facing state. Instead, we find that ATP alone is sufficient. Because MalE does not facilitate the binding of ATP to the transporter (16, 17), nor the outward facing conformation, the results imply that MalE must stimulate a step occurring during the return of the transporter to the inward facing state.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The detergent n-dodecyl-β-D-maltoside (DDM) was purchased from Anatrace. The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) were purchased from Avanti Polar Lipids. Superdex 200 10/300 GL, Resource 15Q, Sephadex G-25, and Ni^{2+}-NTA chelating Sepharose columns were obtained from GE Healthcare. BioBeads were purchased from Bio-Rad. N-(1-Pyrene)-maleimide was obtained from AnaSpec. All other chemicals, including bis-maleimidoethane (BMOE) and N-ethylmaleimide, were obtained from Sigma. MalE and His-tagged MalFGK2 were purified as previously described (16), using *Escherichia coli* BL21 (DE3) carrying plasmids pBAD33-MalE or pBAD22-MalFGK_his.

**Reconstitution of MalFGK2 in Nanodiscs and Proteoliposomes**—The membrane scaffold protein MSP1D1 was obtained from the Sligar laboratory (18). The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) were mixed at a ratio of 7:3, dissolved in chloroform, and dried under a stream of nitrogen. The lipids were resuspended in TSG buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol) containing 0.2% DDM. The MalFGK2 complex, the membrane scaffold proteins, and the solubilized lipids were mixed together at a protein:MSP:lipid ratio of 1:3:60 in TSG buffer containing 0.08% DDM. Detergent was removed with BioBeads (one-third volume) and gentle shaking (overnight, 4 °C). BioBeads were removed by sedimentation, and the resulting nanodiscs were purified through Superdex 200 10/30 gel filtration equilibrated in TSG buffer. Proteoliposomes were prepared at a protein:MSP:lipid ratio of 1:360 in TSG buffer containing 0.08% DDM. Detergent was removed using BioBeads as for nanodiscs reconstitution. The proteoliposomes were gathered by centrifugation (100,000 × g, 1 h, 4 °C) and resuspended in 20 mM Tris-HCl, pH 8.0. The orientation of the complex reconstituted in proteoliposomes was determined by the labeling of residue MalF_{T177C} (in background MalKC_{406}) with 5-iodoacetamide fluorescein (5-IAF). Proteoliposomes (2 μM) were incubated with 5-IAF (10 μM) in 20 mM Tris-HCl, pH 8.0, 1 mM MgCl2 supplemented with or without 1% DDM. The reaction was stopped with DTT (2 mM), and unreacted 5-IAF was removed by gel filtration (Sephadex G-25). Proteoliposomes were extruded through a 100-nm polycarbonate filter before use.

**Maltose Transport Assay**—Proteoliposomes were reconstituted in R buffer (50 mM sodium phosphate, pH 6.5, 1 mM MgCl2, 5 mM ATP). The transport reaction was carried out with 10 μM MalE, 0.5 μM MalFGK2, and 20 μM [14C]maltose (57 μCi/μmol). At the indicated time, samples (50 μl) were removed and diluted into 1 ml of ice-cold 50 mM sodium phosphate buffer containing 10 mM maltose followed by filtration through a 0.22-μm nitrocellulose Millipore filter and scintillation counting. The number of cpm were converted into nmol using known amounts of [14C]maltose.

**Cysteine Cross-linking Experiments**—Cross-linking reactions were performed in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2 containing 0.5 μM MalFGK2 proteoliposomes mixed with 1 mM ATP, 10 μM MalE, and 1 mM maltose as indicated. The samples were incubated with 50 μM BMOE (10 min at room temperature) and then treated with N-ethylmaleimide (5 mM) before analysis by SDS-PAGE.

**Fluorescence Labeling and Spectroscopy**—The dye N-(1-pyrene)-maleimide was used to label the MalFGK2 cysteine mutants. Briefly, the purified MalFGK2 complex was incubated
FIGURE 2. ATP triggers the transporter outward facing conformation. A, the asterisks indicate the location of the cysteine residues introduced in MalF, MalG, and MalK and their relative positions in the inward and outward facing conformations. B, orientation of the MalFGK₂ complex in proteoliposomes. The labeling efficiency of residue MalF₄₁₇⁷C (in mutant background MalK₂₃⁹₇) was assayed using the membrane-impermeable reagent S-IAF in the presence or absence of DDM. The samples were analyzed by 15% SDS-PAGE followed by fluorescence scanning (left panel) or Coomassie Blue staining (right panel). The fluorescence of MalF₄₁₇⁷C when labeled in the presence of DDM was normalized to 100%. The labeling efficiency of MalF₄₁₇⁷C was determined by absorbance spectroscopy (498 nm), using an extinction coefficient of 75, 500 cm⁻¹ M⁻¹. C–E, the indicated transporters reconstituted in proteoliposomes were incubated with the homobifunctional cross-linker BMOE (50 μM) in the indicated conditions. The samples were treated with N-ethylmaleimide (5 mM) prior to analysis by 15% SDS-PAGE and Coomassie Blue staining. F, cross-linking results for the pairs MalF₃₉₆/MalG₁₈⁵ and MalF₄₄₆/MalG₂₃₄ in the presence of ATPₜ₅.
with 0.5 mM DTT (30 min on ice) and passed through a G25 column. The labeling reaction was then performed by mixing MalFGK₂ and N-(1-pyrene)-maleimide at a molar ratio of 1:10 in TSG buffer containing 0.01% DDM and 1 mM TCEP for 2 h at room temperature. The unreacted N-(1-pyrene)-maleimide was removed by Superdex 200 10/30 gel filtration. The purified and labeled complex was reconstituted in proteoliposomes as described above. The labeling efficiency was determined by absorbance spectroscopy (343 nm) using an extinction coefficient of 40,000 cm⁻¹ M⁻¹. A typical ratio was between 1.2 and 1.4. Because there are two cysteine substitutions per MalFGK₂ variant, this is equal to ~60–70% labeling efficiency. The pyrene-labeled MalFGK₂ variants retained ~80% ATPase activity and 70% transport activity of wild type MalFGK₂ (see Table 2).

The fluorescent measurements were carried out at 25 °C in FL buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂) on a Cary Eclipse spectrophuorometer. The fluorescence spectra were recorded from 365 to 520 nm with excitation wavelength at 345 nm (5-nm slide width). The binding of MalE to MalFGK₂ was measured by fluorescence quenching assay, using MalE-31C labeled with the maleimide derivative ATTO-655 (Atto-Tech), as previously described (16). The fluorescent measurements were carried out at 25 °C in FL buffer.

**Other Methods** — The transporter ATPase activity was determined by measuring the release of inorganic phosphate using photo colorimetric method (16). Linear gradient native gel electrophoresis, sedimentation assay, and protein iodination were performed as described (16, 19).

**RESULTS**

**ATP Controls the Outward Facing Conformation of the Transporter** — Cysteine residues were introduced at diagnostic positions on MalK, MalF, and MalG, so that sulphhydryl-based cross-linking methods could be employed to monitor the conformation of the transporter in the lipid bilayer (Fig. 2A). The cysteine residues were introduced into a complex mutated at the endogenous Cys-40 of MalK because this position naturally reacts with maleimide reagents (data not shown). According to the crystallographic data (8, 9), the distance between the periplasmic positions MalF₄₄₆/MalG₂₃₄ increases from ~9 to ~18 Å during the conformational transition from the inward facing state to the outward facing state. Meanwhile, the distance between the cytosolic positions MalK₈₃/MalK₈₃ and MalF₃₀₆/MalG₁₈₅ decreases from ~21 to ~8 Å. We thus employed the homobifunctional cross-linker BMOE with a spacer arm of 8 Å to monitor these conformational changes (Fig. 2).

We note that the C-terminal tail of MalG in the crystal structure is located near the MalK dimer interface and may potentially interfere with the formation of the cross-link MalK₈₃/MalK₈₃. However, as reported by others, an efficient ATP-dependent cross-linking occurs with this pair (and also with MalK₈₃/MalK₈₃), showing that the C-terminal tail of MalG is sufficiently mobile in solution to allow cross-link formation (20, 21). We also considered the possibility that MalFGK₂ becomes inaccessible to MalE when the transporter is reconstituted in liposomes. This was ruled out because (i) the transporter was functional (Table 1), and its activity was dependent on MalE and maltose, (ii) the sedimentation of MalE with the proteoliposome was dependent on MalFGK₂ and nucleotides (Fig. 3, A and C), and (iii) the thiol-reactive membrane-impermeable reagent 5-IAF labeled the periplasmic side of the complex with at least 50% efficiency (Fig. 2B).

We then probed what the transporter conformation is in the membrane. In the absence of ligands (apo state), there was only a little cross-link formation with the pairs MalK₈₃/MalK₈₃ and MalF₃₀₆/MalG₁₈₅ (pairs located in the cytosolic side of the membrane), whereas cross-links readily formed with the pair MalF₄₄₆/MalG₂₃₄ (located on the periplasmic side) (Fig. 2, C–E). These results were expected because the transporter resides naturally in the inward facing state in the absence of ligands (8). In the presence of ATP, however, a predominant cross-link appeared with the pairs MalK₈₃/MalK₈₃ and MalF₃₀₆/MalG₁₈₅, whereas the cross-link of MalF₄₄₆/MalG₂₃₄ was decreased by ~90%, as determined by densitometry analysis (data not shown). The binding of ATP, and not its hydrolysis, was responsible for this conformational change because ATPγS produced the same effect (Fig. 2F). Strikingly, the addition of MalE with or without maltose did not cause any differences in the cross-linking efficiencies (Fig. 2, C–F). Together, these data provided strong preliminary evidence that ATP on its own controls the transition of the transporter from inward to outward facing state.

**ATP Controls the Binding of MalE to the Transporter** — We reported recently that MalE binds with nanomolar affinity to the outward facing transporter (16). Because ATP alone appears sufficient for the transition to the outward facing conformation, we hypothesized that ATP alone should also control the binding of MalE to the transporter. Accordingly, the stabilization of MalFGK₂ in the outward facing state with ATP plus vanadate allowed an efficient co-sedimentation of MalE with the transporter (Fig. 3A, lanes 2). In the absence of vanadate, the binding of MalE was weak because the transporter hydrolyzes...
ATP and returns to the inward facing conformation, which has no affinity for MalE. Accordingly, when the mutants MalK83/MalK83 and MalF396/MalG185 were treated with ATP plus BMOE to stabilize the transporter in the outward facing state, the binding of MalE was as efficient as with ATP plus vanadate (Fig. 3, compare lanes 2 with lanes 4). In contrast, when the transporter was locked in the inward facing state with the pair MalF446/MalG234, the binding of MalE was at background levels, even in the presence of ATP and vanadate (Fig. 3A, right panel, lane 4). The results therefore confirm that ATP controls the transition from inward to outward facing state, which in turn controls the binding of MalE to the transporter.

We next employed a fluorescence-based assay to determine the binding affinity of MalE to MalFGK2 in the inward and outward facing states. We reconstituted the transporter in proteoliposomes and nanodiscs, and analyzed the binding of MalE by co-sedimentation assay and native gel electrophoresis, respectively. Maltose promotes the dissociation of MalE when MalFGK2 is stabilized in the outward facing state. The binding of MalE to MalFGK2 in proteoliposomes was analyzed by sedimentation assay. The indicated amount of [125I]MalE was incubated with MalFGK2 in the presence of ATP or ATP and BMOE (room temperature, 10 min). The samples were analyzed by native gel electrophoresis and autoradiography. Note that MalF and MalK are migrating at the same position on 12% SDS-PAGE.
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A  wild type

B  MalK$_{83}$-MalK$_{83}$

C  MalF$_{396}$-MalG$_{185}$

D  MalF$_{446}$-MalG$_{234}$
TABLE 2
ATPase activity of the pyrene-labeled transporters reconstituted in proteoliposomes

| Transporter        | ATPase activity | Transport activity |
|--------------------|-----------------|--------------------|
|                    | A               | B                  |
|                    | +MalE + maltose | +MalE + maltose    |
| Wild type          | 11 ± 4          | 892 ± 91           |
| MalK_{S_8}-MalK_{C_3} | 9 ± 2           | 734 ± 65           |
| MalF_{396}-MalG_{185} | 12 ± 3          | 792 ± 84           |
| MalF_{446}-MalG_{234} | 7 ± 2           | 704 ± 73           |

emissions but increased the excimer emissions (Fig. 5, A and B). The reverse was observed with the pair MalF_{446}-MalG_{234}. This was expected because the pair monitors the conformational change on the opposite side of the membrane (Fig. 5C). In contrast, MalE and maltose had no effect on the monomeric and excimer emissions, in agreement with the conclusion that ATP alone supports the outward facing conformation of the transporter. Furthermore, we observed that MalE and maltose had no effect on the kinetics of the conformational transition (Fig. 6). These time-resolved experiments showed that liganded MalE is unable to modify the kinetics of MalK closure, even though we employed a cysteine pair that produced the highest fluorescence signal difference (i.e., MalK_{S_8}-MalK_{S_8}). We therefore conclude that MalE is not required, nor does it facilitate the closure of the nucleotide-binding domain in the intact and functional maltose ABC transporter.

DISCUSSION

We report that ATP alone is sufficient for the closure of the MalK interface and for the conversion of MalFG to the outward facing conformation. This conclusion is based on cysteine cross-links and pyrene-based fluorescence of probes placed at diagnostic positions on the transporter. The cysteine substitutions and the pyrene label affect modestly the ATPase activity of the transporter, and do not affect its capacity to import maltose (i.e., 70–80% activity of the wild type; Table 2). The conclusion we reach is in fact not surprising but rather in agreement with other studies reporting that ATP drives the dimerization of MalK in the membrane or in solution (26–28). The conclusion is also consistent with the x-ray structures that showed the rigid body rotation of MalFG upon closure of MalK (8). That ATP alone regulates the conformation of the transporter has also been reported for other type I and type II importers (ModBC and BtuCD, respectively), as well as for ABC exporters (7, 29–32).

Our results, however, disagree with the accepted notion that closed liganded MalE facilitates the closure of the MalK dimer. This mechanism was proposed (and perhaps intuitively accepted) because a rapid ATP hydrolysis activity occurs only in the presence of MalE and maltose. To the best of our knowledge, this model has been directly tested by EPR spectroscopy only (14). Why the study concluded that MalK closure strictly depends on MalE is puzzling. The spin label reduced the MalK ATPase activity by 2-fold (from 2,100 to 1,150 nmol/min/mg), yet the transporter was still active. The spin labels were, however, placed at two different positions on the same MalK subunit, giving rise to four different possible ways of spin interactions. Most importantly, the EPR distance measurements were performed in detergent solution, whereas the transporter ATPase is ~100-fold higher than in the membrane and poorly dependent on MalE and maltose (16). The sensitivity of EPR in the membrane environment is indeed problematic because of avoiding nanoparticle aggregation.

FIGURE 4. Equilibrium titration of MalE binding to MalFGK_{2} in nanodiscs. The MalFGK_{2} complex in nanodiscs was incubated with ATP (1 mM), BMOE (50 μM), and vanadate (10 μM) as indicated (10 min at room temperature). The nanodiscs were repurified on a desalting G25 column before fluorescence quenching. The results shown are the mean and standard deviation results presented in Table 1. A, MalFGK_{2}, wild type. B, MalFGK_{2} with mutations MalK_{C_3}, MalK_{C_3}, (MalK_{C_3}-MalK_{C_3}). C, MalFGK_{2} with mutations MalF_{396}-MalG_{185}. D, MalFGK_{2} with mutations MalF_{446}-MalG_{234}. The left and right panels are the same curves but fitted to a different x axis.
the large spin decay inherent to intermolecular dipolar interactions (33), and accordingly, the authors could not report the interspin distances with the transporter reconstituted in liposomes. Nevertheless, this pioneering work showed that ATP is essential for the pairing of MalK, and this conclusion is consistent with our results.

Our results also contrast with the interpretation of the crystal structure that captured closed liganded MalE with the transporter in a semi-closed conformation (15). It was concluded that closed liganded MalE binds to MalFG and induces the preclosure of MalK and thus ATP hydrolysis. Our pyrene-based fluorescence experiments rather show that the kinetics of MalK pairing is the same, whether MalE and maltose are present or not (Fig. 5). In addition, we find that the binding of closed liganded MalE to the inward facing transporter is quasi-null in solution ($K_a > 45 \mu M$; Table 1). In contrast, the binding of MalE to the outward facing transporter occurs with nanomolar affinity ($K_a \approx 60 nM$; Table 1). Clearly, a far more dominant interaction is taking place between ATP-bound outward facing MalFGK$_2$ and unliganded open state MalE. Given the high concentration of ATP in the cytosol (~1 mM), we think this mode of interaction represents the resting state of the transporter. This view is supported by an in vivo cross-linking analysis, which reported that MalE is bound to the transporter even in the absence of maltose (34). This view is also consistent with our conclusion that transporter-bound MalE forms a receptor for maltose (16). In contrast, a model involving the low affinity interaction between closed liganded MalE and MalFGK$_2$ ($K_a > 45 \mu M$), as a way to deliver the sugar and activate the transporter, seems unsustainable because the concentration of
periplasmic MalE decreases further as the environmental maltose concentration decreases.

An obvious question raised by this work is how MalE stimulates the MalK ATPase. The co-crystal of closed liganded MalE with MalFGK₂ has led to the proposal that MalE lowers the energy barrier for ATP hydrolysis (15). However, it cannot be excluded that the binding of MalE to the outward facing transporter instead stimulates the release of ADP and/or Pi and thus the ATP turnover rate (and furthermore in the presence of phosphate release is coupled to transport). Earlier studies suggested that ATP product release during maltose transport (16), and reopening and conformational changes of the “EAA” motifs are crucial for the function of the maltose ATP-binding cassette transporter (17). Together, these results imply that MalE must stimulate a step that occurs when the transporter returns to the inward facing conformation. This conclusion is compatible with the autoregulation model presented in Fig. 1B.

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