Maltose-binding Protein Is Open in the Catalytic Transition State for ATP Hydrolysis during Maltose Transport*

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Mariana I. Auestermuhle‡, Jason A. Hall§, Candice S. Klug¶, and Amy L. Davidson‡‡

From the ‡Department of Molecular Virology and Microbiology and Interdepartmental Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030; §Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77555, and ¶Department of Biophysics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

The maltose transport complex of Escherichia coli, a member of the ATP-binding cassette superfamily, mediates the high affinity uptake of maltose at the expense of ATP. The membrane-associated transporter consists of two transmembrane subunits, MalF and MalG, and two copies of the cytoplasmic ATP-binding cassette subunit, MalK. Maltose-binding protein (MBP), a soluble periplasmic protein, delivers maltose to the MalFGK2 transporter and stimulates hydrolysis by the transporter. Site-directed spin labeling electron paramagnetic resonance spectroscopy is used to monitor binding of MBP to MalFGK2 and conformational changes in MBP as it interacts with MalFGK2. Cysteine residues and spin labels have been introduced into the two lobes of MBP so that spin-spin interaction will report on ligand-induced closure of the protein (Hall, J. A., Thorgeirsson, T. E., Liu, J., Shin, Y. K., and Nikaido, H. (1997) J. Biol. Chem. 272, 17610–17614). At least two different modes of interaction between MBP and MalFGK2 were detected. Binding of MBP to MalFGK2 in the absence of ATP resulted in a decrease in motion of spin label at position 41 in the C-terminal domain of MBP. In a vanadate-trapped transition state intermediate, all free MBP became tightly bound to MalFGK2, spin label in both lobes became completely immobilized, and spin-spin interactions were lost, suggesting that MBP was in an open conformation. Binding of non-hydratable MgATP analogs or ATP in the absence of Mg is sufficient to stabilize a complex of open MBP and MalFGK2. Taken together, these data suggest that closure of the MalK dimer interface coincides with opening of MBP and maltose release to the transporter.

The class of proteins termed ATP-binding cassette (ABC)1 is one of the largest found in nature. Their capacity to move a variety of substances across a membrane using energy from the cell is fundamentally important to bacterial physiology and multidrug resistance in addition to an array of human pathologies such as cystic fibrosis, hyperinsulinemia, and macular dystrophy (1). Transport is powered by the well-conserved ABC or nucleotide-binding domains (NBDs), whereas the specificity for substrate exists in the highly divergent associated membrane-spanning domains (MSDs). Uptake of nutrients into bacterial cells relies on binding protein-dependent transport systems that belong to the ABC transporter family (2, 3).

As a model for ABC transporters, we study the well-characterized maltose transport system of Escherichia coli (4). The transport complex (Fgk2) consists of two membrane-spanning subunits, MalF and MalG, likely consisting of bundles of eight and six α-helices, respectively (5, 6), and two copies of the cytoplasmic NBD subunit, MalK (7). After maltose diffuses into the periplasm through outer membrane porins (8), it is bound with high affinity by maltose-binding protein (MBP) (9). MBP, in addition to functioning as a maltose receptor, stimulates ATP hydrolysis by the transporter (10). To elucidate the mechanism of transport, intermediates in the transport pathway have been stabilized and characterized. Vanadate (V4), an inorganic phosphate analog that mimics the transition state of the γ-phosphate of ATP during hydrolysis (11), has been used to trap the transporter in the transition state conformation (12). In the vanadate-trapped transition state, MBP is tightly bound to the transport complex, and high affinity binding to maltose is lost (13). Based on these observations, we propose that MBP stimulates the ATPase activity of the transporter by stabilizing the transition state and that attainment of the transition state is coupled to the loss of high affinity for maltose, thereby coupling maltose transport to ATP hydrolysis (13). In our current model for maltose transport (Fig. 1), we suggest that maltose-bound MBP in the closed conformation interacts with the transporter in the “periplasmic-closed” (P-closed) state, where access to the translocation pathway from the periplasm is blocked. In the transition state, a concerted conformational change to a “periplasmic-open” (P-open) state occurs in which the nucleotide-binding domains of the MalK subunits have associated to complete the ATP-binding sites, membrane-spanning helices have reoriented to alternate the access to a central binding site in the translocation pathway from the cytoplasm to the periplasm, and MBP has opened to release maltose into the transporter. After ATP hydrolysis, the transporter returns to the P-closed state.

Site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) spectroscopy are powerful tools that can provide a great deal of information on the location and environment of an individual residue within a very large and complex protein structure (14–17). Unlike other methods that al-

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†To whom correspondence should be addressed: Dept. of Molecular Virology and Microbiology, MS: BCM 280, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-4552; Fax: 713-798-7375; E-mail: davidson@bcm.tmc.edu.

‡Abbreviations used are: ABC, ATP-binding cassette; NBD, nucleotide-binding domain; MSD, membrane-spanning domain; MBP, maltose-binding protein; V4, vanadate; SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance spectroscopy; MTS, (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate; DM, dodecyl β-maltoside; ATP-γ-S, adenosine 5'-O-(thiotriphosphate); AMP-PNP, adenylyl-imidodiphosphate; CFTR, cystic fibrosis transmembrane regulator.

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low only the monitoring of global changes in protein structure, SDSLS allows the direct probing of the local environment, structure, and proximity of individual residues. SDSLS typically involves introducing a specifically placed cysteine residue within a protein followed by a reaction with a sulfhydryl-specific nitroxide spin label. Conformational changes in protein structure can be observed through both solvent accessibility measurements and motional changes of the introduced spin label side chain. That is, conformational changes due to substrate binding; membrane binding; secondary, tertiary, and protein–protein interactions; denaturation; and other perturbations are all evident in the EPR spectra and can give key information on structural changes at specific sites. In addition, the observation of alterations in distance between sites upon ligand binding or protein–protein interaction is a powerful approach to characterizing conformational changes (18, 19). The EPR spectroscopy technique has the unique ability to address and answer questions not solvable by genetic and crystal structure analysis. In this report, we use SDSLS and EPR to study the dynamics of MBP as it interacts with the maltose transporter during nucleotide binding and ATP hydrolysis. Our findings show that MBP is open in the transition state and offer further support for the model presented in Fig. 1.

EXPERIMENTAL PROCEDURES

Mutagenesis and Cloning of MBP—Wild-type MBP with a C-terminal polyhistidine tag was generated by PCR amplification of the malE gene from the pJF2 plasmid (20) with the MalEC4His primer (5'-GGCCCCAAGCTTAGGTGTGGTTGGTGTTGGTAC-3') and the R1lacUV5 primer (5'-CCUGGAAATTGCTACTATTGACCCAC-3'). The resulting fragment was cloned into pCR 4 blunt-TOPO plasmid (which contains a tac promoter) in TOP10 E. coli cells using the Zero Blunt TOPO PCR cloning kit (Invitrogen).

MBP mutants S211C, D41C, and D41C/S211C were generated by PCR amplification of malE from pH1, pH2, and pH3, all generously given to us by the laboratory of Dr. Hiroshi Nikaido. The MalEC4His primer and the MalE reverse primer (5'-AAAGGATCTTCTTCTTCTGATTACGACACG-3') were used. The PCR product was TOPO cloned into the pCR 4 blunt-TOPO plasmid in TOP10 E. coli cells as described above.

Purification of MBP and Spin Labeling—His-tagged MBP D41C, S211C, and D41C/S211C were overexpressed in TOP10 E. coli cells as described previously (21). After overnight growth, cells were spun down and resuspended in 10 ml of 200 mM Tris, pH 8. Then, 10 ml of Tris-sucrose (200 mM Tris, pH 8, and 34% sucrose) were added, followed by 200 μl of a 10 mM luciferin and 50 μl of 0.5 mM EDTA. Water (20 ml) was added, and the mixture was allowed to sit for 15 min. The reaction was terminated by addition of 800 μl of 1 M MgSO4. The periplasmic contents were collected by spinning down the spheroplasts at 8000 g. EDTA was removed by dialysis, and the proteins were bound to the cobalt resin in 20 mM Hepes, pH 8, 150 mM NaCl, and 5 mM β-mercaptoethanol buffer. The affinity resin was washed with Buffer A (20 mM Hepes and 150 mM NaCl) and eluted with Buffer B (20 mM Hepes, 150 mM NaCl, and 150 mM imidazole). Immediately after elution, the proteins were reacted with a 10-fold (for the single mutants) or 20-fold (for the double mutant) molar excess of the spin label (1-oxyl-2,2,5,5-tetramethyl-33-pyrroline-3-methyl)-methanethiosulfonate (MTSL) (Toronto Research Chemicals) overnight with rotation at 4 °C. Extensive dialysis was used to remove unreacted spin label. Protein concentrations were determined spectrophotometrically by using an extinction coefficient of 1.7 (ε282) (22).

Purification of FGK—Polyhistidine-tagged maltose transport complex (FGK) was overexpressed and purified in E. coli as described previously (12). Briefly, membranes solubilized by addition of 1% n-dodecyl β-maltoside (DM) were bound to cobalt affinity resin pre-equilibrated in Buffer C (0.01% DM, 20 mM Hepes, pH 8, 10% glycerol, and 150 mM NaCl). The resin was washed with Buffer C, and protein was eluted with Buffer D (150 mM imidazole, 0.01% DM, 20 mM Hepes, pH 8, 10% glycerol, and 150 mM NaCl). Protein concentrations were determined by the method of Schaffner and Weissmann (23).

Sample Conditions for EPR Spectroscopy—The following conditions were used to obtain EPR spectra of the spin-labeled MBP mutants: (a) MBP alone, 100 μM MBP; (b) MBP + maltose, 90 μM MBP and 1 mM maltose; (c) MBP + FGK + maltose, 50 μM MBP, 100 μM FGKc, and 1 mM maltose; (d) MBP + FGK + maltose + ATP + EDTA, 43 μM MBP, 86 μM FGKc, 0.86 mM maltose, 20 mM ATP, and 1 mM EDTA; (e) MBP + FGK + maltose + ATP + S (or AMP-PNP; both from Roche Diagnostics); Mg2+, 43 μM MBP, 86 μM FGKc, 0.86 mM maltose, 20 mM ATP + S (or AMP-PNP), and 20 mM MgC12; (f) MBP + FGK + maltose + ATP + Mg2+; 42 μM MBP, 42 μM FGKc, 0.84 mM maltose, 20 mM ATP, 1 mM EDTA, and 20 mM MgC12; and (g) MBP + FGK + maltose + ATP + Mg2+ + NaVc, 42 μM MBP, 84 μM FGKc, 0.84 mM maltose, 20 mM ATP, 20 mM MgC12, and 1 mM NaVc.

Reactions with MBP alone were done in 20 mM Hepes buffer, pH 8. Those that included the transport complex were done in 0.01% DM, 20 mM Hepes, pH 8, and 10% glycerol buffer.

EPR Spectroscopy—X-band EPR spectroscopy was carried out on a Bruker ELEXSYS E500 fitted with a Super High Q cavity. Samples were typically 25 μl and contained in a glass capillary. The spectra were normally signal averaged nine times with a scan time of 42 s and recorded at 10 mW microwave power over either a 100- or 200-G scan width.

Distance measurements between the two spin label side chains were obtained using simulation software kindly provided by Dr. Christian Stöhr (Max Planck Institute) (19). Because the geometry of the single spin labels has a slightly different conformation than that seen in the doubles spectrum, the use of a 3:1 mixture of diamagnetic (N-acetylated MTSL analog) to paramagnetic spin label (MTSL) was necessary and served as the sum of the singles for distance analysis.

Size-exclusion Chromatography—Wild-type MBP (50 μM) was incubated with 100 μM FGKc and 1 mM maltose to a final volume of 55 μl. A second reaction consisting of the same components, 0.2 mM EDTA and 1 mM ATP were added. The samples were applied to a G3000 TSK-GE LC 600 size-exclusion chromatography column (TOSO) and eluted with running buffer (0.01% DM, 20 mM Hepes, pH 8, 10% glycerol, and 150 mM NaCl). For the sample with ATP + EDTA, the running buffer had 1 mM ATP and 0.2 mM EDTA. Fractions were collected and visualized by silver staining (24) of an 11% SDS-PAGE gel.

Trapping of (τ-32P)ATP by FGK—Three reactions were set up containing 10 μM FGKc, 50 μM wild-type MBP, and 1 mM maltose. To the first reaction, [τ-32P]ATP (1 mM) and EDTA (0.2 mM) were added. To the second reaction, [τ-32P]ATP (1 mM) and Mg2+ (4 mM) were added, and to the third reaction, [τ-32P]ATP (1 mM), Mg2+ (4 mM), and NaVc (1 mM) were added. The reactions were incubated for 20 min at 25 °C and then dialyzed in 0.01% DM, 20 mM Hepes, pH 8, 10% glycerol, and 50 mM NaCl at 4 °C. Immediately after the last reaction, the samples were counted in a scintillation counter, and the ratio of nucleotide to protein was calculated. The experiment was repeated twice, and very similar results were obtained. The concentration of ATP used was determined from the optical density of the sample at 258 nm, using an extinction coefficient of 15,400 M−1 cm−1.
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RESULTS

SDSL of MBP Sites 211 and 41 Suggests an Interaction with the Transporter—MBP functions as a high affinity maltose receptor for the maltose transport system (3). Maltose binds in a cleft between the two lobes of MBP, causing them to swing together (Fig. 2). A potential interaction site of MBP with the transport complex is defined by mapping mutations that affect transport (25–29) onto the x-ray crystal structure of MBP (30, 31); they are mainly clustered on a single face, on either side of the sugar-binding cleft (Fig. 2B). Genetic suppressor experiments suggest that the lobe of MBP that contains the C terminus interacts with MalF and that the lobe of MBP that contains the N terminus interacts with MalG (25, 32, 33). Residues Asp41 and Ser211 were previously used to monitor conformational changes in MBP on binding maltose and other ligands (34). They are both located on the surface of MBP, on opposite sides of the maltose-binding cleft, within the region believed to interact with the transporter (Fig. 2). When these sites are mutated to cysteine and modified with MTSL, the capacity of MBP to bind maltose and carry out maltose transport remains intact (34). Therefore, they represent good candidates to monitor binding of MBP to the transporter.

The EPR spectra of the singly labeled MBP mutants D41C and S211C were recorded and are shown in Fig. 3. The mobility of the nitroxide spin label, arising mainly from side chain and backbone motion, can be determined from its spectrum. Fast motion of the spin label results in sharp spectral lines, whereas slow motion results in broad lines that have moved outward. The spectrum of the spin-labeled D41C mutant (Fig. 3A), consisting of three sharp peaks, revealed that the spin label is very mobile. Motion at position 211 is more restricted, as seen by the broader spectral lines (Fig. 3B). Residue 41 is located in a loop region and therefore would be predicted to have a high level of flexibility, whereas residue 211 is located within an α-helix, where its motion may be more restricted (30). The addition of maltose to either mutant results in no significant changes in mobility of the spin label side chain, indicating that these sites are not within the maltose-binding pocket and that they are sufficiently surface-exposed so that their mobility is not affected by the conformational change occurring in MBP once maltose is bound.

The first question addressed was whether an interaction between MBP and the transporter complex in the ground state (represented by ATP-free transporter) would be detected. The EPR sample concentrations of MBP and transporter were 50 and 100 μM, respectively, and because the $K_m$ for wild-type MBP in the transport reaction is between 25 and 100 μM (35, 36), it was expected that a significant percentage of MBP molecules would bind the transporter. When the spectrum of the D41C mutant with transporter was compared with the spectrum of the D41C mutant alone (Fig. 3A), it was clear that addition of the transporter results in a two-component spectrum. The more mobile component appears to correspond to the unbound MBP, whereas the less mobile component corresponds to MBP bound to the transporter. Subtraction of the unbound MBP from the composite spectrum allowed us to estimate that 60% of MBP was bound to the transporter. Interestingly, no change in mobility is observed at position 211 upon addition of the transporter (Fig. 3B). Because maltose binding significantly changes the conformation of MBP, the interaction of maltose-free (open) MBP D41C was examined in Fig. 3C. Again, ~60% of MBP was bound to the transporter, as determined by the emergence of a lower mobility component.

The low affinity interaction between MBP and the ground state of the transporter (35) contrasts sharply with the extremely high affinity binding of MBP to the transporter in the vanadate-trapped transition state (13). The spectra of the two singly labeled MBP mutants bound to the vanadate-trapped transporter are shown in Fig. 3, A and B. In contrast to the spectra of D41C and S211C with transporter in the absence of ATP, the peak corresponding to unbound MBP is gone, indicative of high affinity binding (transporter is present in 2-fold molar excess of MBP). The spin labels at positions 211 and 41 are both completely immobilized, suggesting that both residues are on located on the transporter-binding interface of the vanadate-trapped transition state intermediate.

MBP Has Opened in the Transition State—In our model (Fig. 1), we suggest that closed, maltose-bound MBP interacts with the transporter to induce ATP hydrolysis (13). Based on the tight binding of MBP to the transporter in the transition state and the loss of high affinity for maltose, we further suggest that both MBP and the periplasmic surface of the transporter (FGK2) have opened in the transition state, allowing maltose to be released directly into the transporter. To test these ideas, the doubly spin-labeled D41C/S211C mutant was used to measure changes in distance between the two lobes of MBP during the transport cycle. Comparison of the x-ray crystal structures of maltose-free and maltose-bound MBP reveals a change in distance from 24 Å (open) to 15 Å (closed) between the α carbons of these residues, which lie on opposite sides of the maltose-binding cleft of MBP (Fig. 2).
and changes in the mobility of the spin labels that were not seen in the singly labeled preparations (Fig. 4, A and B). From distance measurements, a distribution of distances between the two introduced spin labels in maltose-bound MBP was found to center around 7.5–9 Å.

Addition of the transporter to maltose-bound MBP had little effect on the spin-spin interactions observed in the spectrum (Fig. 4A), suggesting that MBP is still closed when it interacts with the transporter in the initial steps of translocation (see “Discussion”). However, a loss of spin-spin interaction is observed when MBP is tightly bound to the vanadate-trapped transporter (Fig. 4, A and C), indicating that MBP is open in the catalytic transition state, as predicted in the model (Fig. 1). As observed with the single mutants, the spin labels in the double mutant are also immobilized in the transition state conformation.

Surprisingly, using MBP D41C, we determined that whether MBP was in the open (maltose-free) or closed (maltose-bound) conformation had no effect on the affinity between MBP and FGK2 in the absence of ATP; there was ~60% bound in both cases (Fig. 3C). To determine whether MBP is always closed when bound to the ATP-free transporter, the spectrum of doubly labeled MBP with FGK2 was obtained in the absence of maltose. In Fig. 4A, the spectrum of MBP D41C/S211C reveals motional changes, suggestive of binding without the emergence of substantial spin-spin interaction, although deconvolution of the spectrum with the simulation software revealed a small component that was closed, which was not present in the spectrum of unliganded MBP. These results suggest that binding to FGK2 did not trigger closure of a small fraction of maltose-free MBP.

ATP Binding Stabilizes the Open State of MBP—In the x-ray crystal structures of the isolated MalK subunits, ATP binding promotes association of the nucleotide-binding domains (37). Hydrolysis of ATP was prevented by incubation with EDTA to chelate Mg2+, which is essential for ATPase activity. We hypothesized that ATP binding and subunit association would promote conformational changes in the intact transporter through the interaction between MalK and the MSDs. To stabilize the pre-hydrolysis ATP-bound state, doubly spin-labeled MBP D41C/S211C was incubated with transporter treated with ATP and EDTA (Fig. 5A). The spectrum revealed that the spin labels were completely immobilized and too far apart to trigger spin-spin interaction, suggesting that MBP was in the high affinity, open conformation. The enzyme appeared to be catalytically active because addition of Mg2+ to allow hydrolysis to ADP and Pi resulted in a spectrum very similar to that of transporter bound to MgADP and to the spectrum obtained in the absence of nucleotide (Fig. 5A). Given the high concentration of FGK2 in the sample, it is likely that all ATP had been hydrolyzed in the interim between addition of Mg2+ and recording of the EPR spectrum. Under these conditions, the spin-
spin interaction was again observed, and increased mobility of spin labels indicated a loss of tight binding between MBP and FGK2.

Non-hydrolyzable ATP analogs, criticized in some cases for not being accurate mimics of ATP, failed to stabilize a dimer of isolated NBDs (38). However, in our intact transporter, addition of either MgAMP-PNP or MgATP-S resulted in the formation of the open, tightly bound conformation of MBP, just as ATP did (Fig. 5B). The very sharp peaks that appear in the ATP-PyS spectrum result from a small fraction of the spin label reacting with the sulfur from the ATP analog. These results demonstrate that either ATP (with EDTA) or the ATP analogs were able to induce the same conformational change in MBP that was observed in the vanadate-trapped transition state intermediate.

The maltose transporter, like other ABC transporters, has a relatively high $K_m$ (≈100 μM) for ATP in the hydrolysis reaction (39). The MalK dimer crystal structure in three conformations (one ATP-bound) shows that a snug ATP-binding pocket is only formed in the presence of ATP (37). Without ATP, both nucleotide-binding sites are “open” and therefore likely to bind nucleotide initially with low affinity. However, on addition of ATP, protein-ATP contacts are established that drive a conformational change to a closed site, in which the contacts between protein and nucleotide are optimized, and ATP is likely to be bound with higher affinity. In our EPR experiments, saturating concentrations of ATP induced opening and high affinity binding of MBP to FGK2. In an attempt to observe the high affinity MBP-FGK2 interaction biochemically, the formation of a complex between MBP and the transport complex was monitored by size-exclusion chromatography (Fig. 5C). MBP and FGK2 were combined and split into two aliquots. The first half was run using an ATP-free buffer, whereas the second half was run using buffer containing EDTA and saturating ATP concentrations. In the absence of ATP, the transporter interacts to a small degree with MBP. However, when ATP is included in the running buffer, the transporter co-elutes with an equal amount of ATP bound to transporter after dialysis.

The EPR and biochemical results suggest that the P-open state can be formed by ATP- or ADP-Vi-promoted association of the MalK NBDs and high affinity binding to MBP. However, ATP, and not ADP-Vi, can be washed away from FGK2 and MBP in the P-open conformation. In Fig. 5D, MBP and FGK2 were incubated with [α-32P]ATP and EDTA or MgATP and vanadate, followed by extensive dialysis. In the reaction with vanadate, close to 1 mol nucleotide/transporter was bound, whereas in the reaction with ATP-EDTA alone, very little nucleotide (~0.1 mol/transporter) was observed. This suggests that over the time course of the dialysis, the MalK NBDs can open and allow dissociation of ATP, and as the concentration of external ATP decreases, the likelihood that the NBDs will encounter another ATP molecule to reform the P-open complex decreases. In contrast, the Vi-ADP complex, being a stable transition state analog, is bound very tightly by the MalK NBDs and effectively traps the P-open conformation (Ref. 13 and this study). These results hint at the existence of subtle differences within the NBDs that allow tighter binding to ATP in its transition state conformation. However, in terms of their overall conformations, the V trapping and ATP-bound states appear to be very similar.

![Image](https://example.com/image.png)
DISCUSSION

In our model for transport (Fig. 1), we previously proposed a concerted conformational change in the transition state for ATP hydrolysis (13). ATP-mediated association of the MalK subunits is accompanied by a rearrangement in the MSDs that exposes a low affinity maltose-binding site in the transmembrane region to the periplasm, and simultaneously, MBP opens to release maltose into the transporter. The data presented in this article strongly support the idea that the association of the nucleotide-binding domains of the MalK subunits coincides with increased affinity of the transporter for MBP and with opening of the lobes of MBP and suggest that a switch between two different conformational states, P-closed and P-open in Fig. 1, may be sufficient to support maltose transport. SDSL and EPR of MBP labeled at D41C and S211C allowed us to monitor binding of MBP to the transporter in the ground state (ATP-free transporter), the pre-hydrolysis ATP-bound state (stabilized with ATP and EDTA or non-hydrolyzable MgATP analogs), the hydrolytic transition state (stabilized with MgADP-Vi), and the post-hydrolytic MgADP-bound state. The nucleotide-binding domains of the MalK subunits are associated in the vanadate-trapped intermediate, as demonstrated by highly specific vanadate-induced photo cleavage of the polypeptide backbone at both the Walker A and LSGGGQ motifs from opposing subunits (40), and the spin-spin interaction induced by domain closure in the doubly spin-labeled MBP is lost in this species, indicating that MBP is open. Although we have not yet detected conformational changes in the transmembrane region, the opposing motions on opposite sides of the membrane are likely to be part of a more global conformational change that includes rearrangement of the transmembrane helices. Intriguingly, both ATP in the absence of Mg2+ and non-hydrolyzable analogs of ATP also stabilized a complex of open MBP with FGK3 that is essentially indistinguishable from the vanadate-trapped species as judged by our EPR assay. Presumably, ATP binding induced nucleotide-binding domain closure in the intact FGK3 complex, which was sufficient to stabilize the conformational change to the P-open conformation in the presence of MBP. The crystal structures of the isolated MalK subunits in three conformations (37) demonstrate the role ATP has in promoting the association of the N-terminal nucleotide-binding domains. In the closed, ATP-bound structure (37), two ATP molecules are sandwiched between the nucleotide-binding domains, and they comprise a large portion of the dimer interface. The motional changes between ATP-free and ATP-bound MalKs are dramatic. In comparing these structures with previous structures (41, 42), it appears that the γ-phosphate of ATP coordinates a rotation of the helical subdomain relative to the nucleotide-binding subdomain within a single NBD that is an integral component of the overall domain closure (37). The helical subdomain interacts with the MSDs (43, 44), suggesting that motion of this domain will translate into movement of the transmembrane helices. Consistent with this hypothesis, we observed little difference between the spectra recorded in the absence of nucleotide and spectra recorded in the presence of ADP, regardless of whether the ADP-bound samples were analyzed by adding MgADP directly or by adding Mg2+ to the ATP-bound transporter to allow hydrolysis to occur. The presence of the γ-phosphate of ATP or an analog such as vanadate is essential for the formation of the P-open state of transporter; once Pi is released, the transporter returns to the resting (P-closed) conformation, and MBP is released.

A simple two-state model for transport by ABC proteins may be a common feature of the family. Results obtained with the cystic fibrosis transmembrane regulator (CFTR) appear somewhat analogous to our findings with the maltose transporter (45). CFTR is an ABC protein in which interaction of nucleotide with the ABC domains opens a chloride channel across the membrane. The CFTR channel opens in the presence of ATPγS, AMP-PNP, or ATP plus EDTA, indicating that ATP hydrolysis is not essential for channel opening (45, 46). Closing of the channel can be achieved either through dissociation of ATP or hydrolysis to ADP and Pi, which likely promotes the separation of NBDs and hence the return to the resting state (45). Under physiologic conditions (in the presence of Mg2+), it is likely that ATP hydrolysis rather than dissociation of ATP will dominate the return to the ground state in both CFTR and the maltose transporter because closure of the nucleotide-binding interface is promoted by ATP binding, and the closed dimer state stabilizes the transition state for ATP hydrolysis (40). A point that is not yet clear in the case of maltose transport is whether maltose is transiently trapped in a pocket between MBP and FGK, in the P-open conformation, awaiting the opening of the MalK dimer interface and reorientation of the transmembrane helices after ATP hydrolysis, as depicted in our model (Fig. 1), or whether the transmembrane pathway for maltose is open, forming a channel from the MBP to the cytoplasm in the P-open state. In the latter case, maltose could be released into the cytoplasm upon ATP binding when MBP is tightly bound in an open conformation, whereas in the former case, transport would coincide with the return to the ground, P-closed state after ATP hydrolysis.

In P-glycoprotein, an ABC transporter that transports hydrophobic drugs, nucleotide-induced conformational changes have been observed both directly by electron microscopy (47) and indirectly by fluorescence techniques (48–50), infrared spectroscopy (51, 52), protease susceptibility (53–55), and cysteine cross-linking (56). In contrast to CFTR and the maltose transporter, some data suggest that ATP binding and ATP hydrolysis may induce different conformational states in this protein, suggestive of a more sequential mechanism of action (47, 57). A major conformational change in P-glycoprotein was observed by electron microscopy upon addition of AMP-PNP, and further conformational changes were observed in the vanadate-trapped transition state intermediate (47). Vigano et al. (51) observed intermediates in P-glycoprotein by measuring the kinetics of 2H3-H exchange of the amide H by Fourier transform attenuated total reflection infrared spectroscopy and found significant differences between the ATP-bound state (using ATPγS) and the ADP-Vi state. They suggested that ATP binding resulted in a conformational change that decreased the affinity for drug, whereas hydrolysis was required to mediate a restructuring of the MSDs that resulted in increased accessibility to the external medium (51). Differences between the ATP analog-bound state and the Vi-trapped state were also observed by trypsin susceptibility experiments (53–55) and cysteine cross-linking of residues in the MSDs (56). How can one reconcile the apparent differences between P-glycoprotein and the maltose transporter in terms of the consequences of nucleotide binding versus nucleotide hydrolysis? One of the main differences between the maltose transport system and multidrug exporters is the presence of a binding protein. In our system, MBP can contribute to the stability of the P-open state through binding interactions with the transport complex. In the multidrug exporters, there is no accessory protein to contribute as significantly to the stability of the ATP-bound state. Because enzymes are optimized to bind the transition state best, ADP-Vi (transition state ATP) might be able to stabilize conformations further along the catalytic pathway than ground state ATP can, in the absence of additional stabilizing interactions. Our results suggest that the ATP-protein contacts in MalK, as well as the binding of both lobes of MBP to the
transporter, stabilize the P-open complex, allowing it to be formed readily.

SDSL and EPR also allowed us to monitor for the first time the relatively low affinity interactions between MBP and the ground state of the transporter. Addition of FGK₂ to spin-labeled MBP with or without maltose partially decreased the mobility of spin label at position 41, indicating that both maltose-free and maltose-bound MBP bind FGK₂ in the absence of ATP. The resulting spectra appeared to be a composite of transporter-bound MBP and free MBP rather than an average of the two signals, indicating that rates of association and/or dissociation of MBP from FGK₂ are slow as compared with the EPR time scale (ns). At the protein concentrations used in this study (50–100 μM), roughly 60% of MBP was bound to FGK₂, both in the presence and absence of maltose. This value is reasonable, given previous estimates of the Kₘ of MBP in the transport reaction (25–100 μM) (35, 36). The similar affinity of transporter for ligand-free (open) versus ligand-bound (closed) is perhaps surprising, given that maltose-bound MBP is better able to stimulate ATP hydrolysis than maltose-free MBP (10) and that the conformational change is so substantial (31); however, the absence of an effect of FGK₂ on the spectrum of MBP singly labeled at position 211 in the absence of ATP may provide a clue to the basis for the similar affinity. Perhaps, whether MBP is open or closed, initial recognition between MBP and FGK₂ is mediated solely through the N-terminal lobe of MBP containing residue 41 with the transporter. It is reasonable to assume that MBP would progress into a productive engagement with FGK₂ only after its closure to ensure maximal efficiency in coupling of maltose transport to ATP hydrolysis. In this model, the C-terminal lobe would become engaged with FGK₂ only after its closure to ensure maximum mobility of spin label at position 41, indicating that both maltose-free and maltose-bound MBP remain open when bound to the transporter, stabilize the P-open complex, allowing it to be formed readily.

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