Demonstration of Conformational Changes Associated with Activation of the Maltose Transport Complex

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Dayene E. Mannerings, Susan Sharma, and Amy L. Davidson§

From the Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030

In Escherichia coli, interaction of a periplasmic maltose-binding protein with a membrane-associated ATP-binding cassette transporter stimulates ATP hydrolysis, resulting in translocation of maltose into the cell. The maltose transporter contains two transmembrane subunits, MalF and MalG, and two copies of a nucleotide-hydrolyzing subunit, MalK. Mutant transport complexes that function in the absence of binding protein are thought to be stabilized in an ATPase-active conformation. To probe the conformation of the nucleotide-binding site and to gain an understanding of the nature of the conformational changes that lead to activation, cysteine 40 within the Walker A motif of the MalK subunit was modified by the fluorophore 2-(4’-maleimidoanilino)naphthalene-6-sulfonic acid. Fluorescence differences indicated that residues involved in nucleotide binding were less accessible to aqueous solvent in the binding protein independent transporter than in the wild-type transporter. Similar differences in fluorescence were seen when a vanadate-trapped transition state conformation was compared with the ground state in the wild-type transporter. Our results and recent crystal structures are consistent with a model in which activation of ATPase activity is associated with conformational changes that bring the two MalK subunits closer together, completing the nucleotide-binding sites and burying ATP in the interface.

The periplasmic binding protein-dependent maltose transport system of Escherichia coli is a member of the ATP-binding cassette (ABC) superfamily of proteins. Members of this protein family are responsible for the transport of a variety of substrates across membranes in both prokaryotic and eukaryotic organisms (1, 2). Medically important members of the superfamily include the multidrug-resistant P-glycoprotein and the ABC 1 superfamily of proteins. Members of this superfamily are responsible for the transport of a variety of substrates across membranes in both prokaryotic and eukaryotic organisms (1, 2). In this study, we used MIANS, 2-(4’-naphthalene-6-sulfonic acid; dodecyl maltoside, n-dodecyl β-maltoside; DTT, dithiothreitol; β-ME, β-mercaptoethanol; DF, p-xylene-bispyridinium bromide; PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide.

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§ To whom correspondence and reprint requests 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.

The abbreviations used are: ABC, ATP-binding cassette; MBP, maltose-binding protein; BPI, binding protein-independent; WT, wild-type; MIANS, 2-(4’-maleimidoanilino)naphthalene-6-sulfonic acid; dodecyl maltoside, n-dodecyl β-maltoside; DTT, dithiothreitol; β-ME, β-mercaptoethanol; DF, p-xylene-bispyridinium bromide; PAGE, polyacrylamide gel electrophoresis; NEM, N-ethylmaleimide.

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The fluorescence of the sulfhydryl-specific reagent MIANS is sensitive to the polarity of its environment (15, 16) and has been used to detect conformational changes in the Na+K+-ATPase (15), the myosin ATPase (17), the P-glycoprotein (18, 19), and the lactose permease (20, 21). In this study, we used MIANS to detect conformational differences in the nucleotide-binding sites of WT and BPI complexes in the ground state and in the transition state to gain further insight into the mechanism of activation of MalFGK2 ATPase activity. We found that the conformation of the nucleotide-binding sites in the BPI complex more closely resembles the transition state than the ground state of the WT complex. Furthermore, the conformational change associated with activation of the ATPase activity relocates residues involved in nucleotide binding to a less surface-exposed environment.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Strain HN741 (Escherichia coli K-12 argH his rpsL1 malT (Con) malBΔ13 satBC ile::Tn10F lacP Tn5) (14) was transformed with the plasmid pMS421.
Conformational Change in the Maltose Transport Complex

Table 1

| Protein          | Percent remaining ATPase activity |
|------------------|-----------------------------------|
| Methanol*        | 90 ± 2.1                          |
| BPI Cys-40       | 72 ± 2.4                          |
| BPI C40G         | 63 ± 2.0                          |
| WT Cys-40        | 75 ± 2.3                          |
| WT C40G          | 67 ± 2.1                          |

*ATPase activity of complexes incubated with methanol has been set at 100% for each preparation, and the other values were scaled accordingly. BPI complexes were assayed in solubilized form, and WT complexes were assayed in reconstituted form as described under “Experimental Procedures.” Values for complexes incubated with methanol are as follows: BPI Cys-40, 290 ± 10 nmol P/min/mg; BPI C40G, 170 ± 20 nmol P/min/mg; WT Cys-40, 820 ± 30 nmol P/min/mg; WT C40G, 1220 ± 40 nmol P/min/mg.

Results

Specific Modification of MalK Residue Cys-40 by MIANS—In an effort to detect conformational changes associated with activation of the ATPase activity of MalFGK2, we wished to modify specifically a cysteine residue in the MalK subunit(s) with a fluorescent reporter group. The MalFGK2 complex contains a total of 10 cysteine residues (three in each MalK, three in MalF, and one in MalG (31–33)), including a reactive cysteine close to the nucleotide-binding site. This residue is located at position 40 (Cys-40) within the Walker A consensus nucleotide-binding motif (also known as the phosphate binding loop, or P-loop) of MalK (34). Modification of this residue by the sulfhydryl-specific reagent N-ethylmaleimide (NEM) inhibits the ATPase activity of the maltose transport complex (35, 36); ATP protects against inhibition (35), and replacement of this cysteine residue imparts inhibition without affecting ATPase activity (37). Based on these findings, we predicted that it would be possible to label covalently Cys-40 with MIANS, a fluorescent maleimide probe that could be used to monitor conformational changes in the MalK subunit. Like NEM, MIANS also inhibited the ATPase activity of both WT and BPI transport systems (Table 1). MIANS did not inhibit the ATPase activity of complexes containing a nondeleterious substitution...
of cysteine with glycine at position 40 in MalK (C40G) (24). These results demonstrate that MIANS reacts with Cys-40 and that modification at this site is responsible for the inhibition of ATPase activity.

To interpret possible conformational differences detected by MIANS as being associated with Cys-40 in the nucleotide-binding site(s), we assessed the extent of labeling of the other cysteines in the transport complex both by SDS-PAGE and by comparison of the intensity of the fluorescence emission spectra of complexes containing either Cys-40 or a C40G substitution. As seen in SDS-PAGE analysis of MIANS-labeled complexes, very little fluorescence is associated with the MalF and MalG proteins, and substitution of a glycine for the cysteine at position 40 greatly reduces the fluorescence associated with MalK (Fig. 1). The fluorescence emission spectra of the MIANS-labeled WT and BPI complexes revealed that the fluorescence of MIANS associated with C40G complexes was only 10–35% that seen for Cys-40 complexes (Fig. 2). Thus, although residues other than Cys-40 may be modified to some extent, the bulk of the fluorescence is associated with MIANS bound at Cys-40. Variation of buffer, pH, MIANS concentration, and length of incubation did not improve the specificity of the modification reaction (data not shown). Quantitation of MIANS incorporation into MalFGK2 through absorbance measurements at 322 nm (38) failed to address the question of whether residue Cys-40 was modified in one or both MalK subunits (data not shown), in part because of the variability in the background (non-Cys-40) labeling by MIANS. However, based on other observations (see "Discussion"), we hypothesize that only one of the two MalK subunits is modified.

Emission Maximum of BPI Is Blue-shifted Relative to WT—The fluorescence characteristics of MIANS are sensitive to the hydrophobicity of its environment; specifically, emission maxima are blue-shifted to shorter wavelengths, and the quantum yield increased as the solvent polarity decreases (16, 17). The emission spectra in Fig. 2 were therefore used to compare the local environment of MIANS at Cys-40 in the WT and BPI complexes. For both complexes, MIANS exhibited a greater fluorescence quantum yield when bound to protein than when reacted with β-mercaptoethanol in solution. The emission maximum of MIANS attached to the WT complex was blue-shifted 12 nm from that of free MIANS-β-ME, whereas the emission maximum of the BPI complex was blue-shifted 22 nm. These results suggest that the probe is in a more hydrophobic environment when bound to either protein, and the larger blueshift associated with the BPI complex may indicate that the environment surrounding residue Cys-40 in the nucleotide-binding site is more hydrophobic in the BPI than in the WT.

Solvent Accessibility of Cys-40 in BPI Is Reduced Compared with WT—To probe further the nature of the conformational difference suggested by the blue shift in the fluorescence emission of the BPI transport complex, collisional quenching experiments were performed to assess the solvent accessibility of MIANS. Specifically, the ability of water-soluble reagents to collide with MIANS and quench its fluorescence was measured as a function of quencher concentration to compare the relative exposure of MIANS in WT and BPI complexes to the aqueous environment. The solvent accessibility of MIANS complexes was assessed using either acrylamide (Fig. 3, A and B) or DPX (Fig. 3, C and D) as a quenching agent. As anticipated, MIANS bound to the complexes was less accessible to the quenching reagents than MIANS free in solution ($K_{sv} = 3.18 \text{ M}^{-1}$ for free...
Transport complexes containing MalK Cys-40 or C40G subunits were labeled with MIANS in detergent solution and titrated with either acrylamide (A and B) or DPX (C and D) as described under “Experimental Procedures.” ATP was present at 1 mM where indicated. The Stern-Volmer plots are as follows: A, 1 μM MIANS-β-ME; B, Cys-40 BPI-MIANS; C, 40G BPI-MIANS; Cys-40 WT-MIANS; D, Cys-40 WT-MIANS with ATP; E, Cys-40 WT-MIANS; F, Cys-40 WT-MIANS with BPI-MIANS; G, Cys-40 WT-MIANS; H, Cys-40 BPI-MIANS; I, Cys-40 WT-MIANS with ATP; J, 1 μM MIANS-β-ME; K, Cys-40 BPI-MIANS; L, Cys-40 BPI-MIANS with ATP; M, Cys-40 WT-MIANS; N, Cys-40 WT-MIANS with ATP. Points are presented as mean values ± S.D. of titrations performed in triplicate.

MIANS-β-ME in the acrylamide experiments), as judged by the decreased slopes of the lines in the Stern-Volmer plot. In acrylamide quenching experiments, MIANS bound to the WT complex is significantly more exposed to solvent than in the BPI complex, with $K_{SV}$ values of 1.94 and 1.41 M$^{-1}$, respectively (Fig. 3A). Removal of Cys-40 from both complexes negates this difference, confirming that the assay is reporting on conformational changes located at the nucleotide-binding site. MIANS bound to these C40G complexes was even less exposed to solvent than MIANS at Cys-40 in the BPI transport complex (Fig. 3A). In experiments using DPX, a larger quenching reagent than acrylamide, MIANS bound to the WT complex was also significantly more exposed to solvent than in the BPI complex, with $K_{SV}$ values of 41.5 and 27.3 M$^{-1}$, respectively. In this case, the exposure of MIANS on the BPI complex to DPX was the same as that seen for the complexes containing the C40G substitution (Fig. 3C). These data provide further evidence of a conformational difference between the WT and BPI complexes which appears to affect the exposure of residues in the nucleotide-binding pocket to the aqueous milieu.

In experiments similar to those presented here, the fluorescence of MIANS positioned in the P-loop of P-glycoprotein was quenched by ATP in a concentration-dependent manner (18). We therefore added ATP to the labeled MalFGK$_2$ complexes to determine whether nucleotide binding could be detected as a change in the fluorescence properties of MIANS at Cys-40. The presence of 1 mM ATP had no effect on the fluorescence emission spectrum of either transport complex (data not shown) and did not significantly alter solvent accessibility (Fig. 3, B and D) other than a small effect on exposure of the WT to acrylamide. Because ATP protects against modification of residue Cys-40 (35), MIANS might prevent nucleotide binding in the MalFGK$_2$ system via a steric effect. If MIANS modifies only one nucleotide-binding site, it is also possible that ATP binds to one site without significantly altering the fluorescence characteristics of MIANS at the second site.

Detection of Conformational Changes Associated with Attainment of the Transition State for ATP Hydrolysis—We recently showed that vanadate inhibits MalFGK$_2$ by trapping the complex in its transition state conformation with ADP tightly bound in one of the two nucleotide-binding sites (11, 12). Vanadate-induced nucleotide trapping requires MBP in the WT but not in a BPI mutant (12). The ability to trap the transition state provides a second model system that can be used to study conformational changes associated with activation of ATPase activity. Because vanadate trapping of the transition state conformation requires ATP hydrolysis (12), complexes were first treated with vanadate and then labeled with MIANS. The fluorescence intensity of either the WT or the BPI complex labeled after vanadate treatment was very similar to the intensity of control preparations labeled without the treatment; however, in the WT complex, vanadate treatment resulted in a 3-nm blue shift in fluorescence emission relative to the untreated complex (data not shown). The solvent accessibility of MIANS was then assessed using acrylamide quenching. The accessibility of MIANS in the BPI complex was unchanged by vanadate treatment (Fig. 4). In the WT complexes, addition of MBP in the absence of vanadate did not alter the solvent accessibility of MIANS (Fig. 4). However, the solvent accessibility of MIANS was different when WT complexes were vanadate-trapped in the presence of MBP. The accessibility was reduced to a level resembling that of MIANS bound to BPI complexes, suggesting that the conformation of the BPI may resemble the transition state of the WT transporter.
Another feature of the WT complex trapped in the transition state species is that it binds tightly to MBP (11), whereas in the ground state it displays a relatively low affinity for MBP (39, 40). If the conformation of the BPI transporter resembles the transition state, it might display a high affinity for MBP even in the absence of vanadate, as shown indirectly for another BPI mutant, MalFG511K2 (40). As shown in Fig. 5, MBP is tightly associated with the MalF500GK2 BPI complex, both in the presence and absence of treatment with vanadate, whereas the tight binding interaction is seen for the WT only in the presence of vanadate. This result provides additional evidence that the conformation of the BPI complex resembles the transition state and may in fact represent an intermediate in the pathway from the ground state to the transition state in the WT. As such, further comparison of WT and BPI complexes might provide increased insight into the mechanism of activation of MalFGK2 ATPase activity.

WT and BPI Complexes Differ in Affinity for ATP—In preliminary experiments, we noted a difference in the ability of ATP to protect against the irreversible modification of the WT and BPI complexes by MIANS (data not shown). To pursue this observation further, the concentration dependence of ATP protection of WT and BPI complexes against modification by either 20 or 5 μM MIANS was examined (Fig. 6, A and B). The concentrations of ATP needed to protect 50% of the complexes against modification, or PC50 values, are summarized in Table II. At the 20 μM MIANS concentration, 30-fold more ATP is required to protect the WT transporters as opposed to the BPI transporters. The same results are obtained at the 5 μM MIANS concentration, although a correspondingly lower concentration of ATP is required to gain the same level of protection. These results suggest that MIANS and ATP compete for binding to MalFGK2 and that the BPI mutant may have a higher affinity for ATP than the WT transporter. In the absence of a reliable assay to measure nucleotide binding to MalFGK2, we estimated the affinity indirectly by measuring the half-saturation constant (K0.5) for ATP hydrolysis by the BPI and WT complexes (Table II). The K0.5 for the BPI transporter was 14-fold lower than the corresponding value for the WT transporter and correlated well with the ability of ATP to protect against MIANS modification. At the lower concentration of MIANS (5 μM) the PC50 values approached the K0.5, suggesting that the differential ability of ATP to protect truly reflects the differential nucleotide binding affinity of the WT and BPI complexes. These data suggest that BPI transporters have a greater affinity for ATP than do WT transporters.

**DISCUSSION**

We have used an extrinsic fluorescent probe to detect conformational changes in the nucleotide-binding site associated with activation of the MalFGK2 ATPase. In the WT system, the ATPase activity of the MalFGK2 transporter is stimulated...
through an interaction with maltose-bound MBP that stabilizes the transition state for hydrolysis (11). The need for MBP can be bypassed by mutations (BPI mutations) that result in constitutive activation of the ATPase activity of the transporter (14). A conformational difference between the WT and BPI transport complex, detected in the absence of both MBP and nucleotide, manifested itself both as a change in the position of the emission spectrum maximum and in the solvent accessibility of bound MIANS. The fluorescence of MIANS bound to residue Cys-40 of MalK was blue-shifted in the BPI complexes relative to the WT complexes, indicative of a more hydrophobic environment, and was less accessible to water-soluble quenching agents. These results suggest that residue Cys-40 in the P-loop is shifted to a more buried location in the conformation that is stabilized by the BPI mutations. In the case of the BPI MalF500GKc complex used in this study, two mutations in the transmembrane region of MalF, G338R and N505I (41), are responsible for the conformational changes in the nucleotide-binding site of the MalK subunit(s) and the activation of the ATPase activity (14).

Because vanadate can be used to lock both the WT and the BPI transporter in the transition state for ATP hydrolysis (11, 12), we also used differences in the fluorescence characteristics of MIANS to compare the ground state and transition state conformations of MalFGKc. When vanadate was used to lock the WT MalFGKc complex in the transition state, the accessibility of residue Cys-40 in the P-loop was decreased relative to the ground state. This decreased level of accessibility was very similar to the level observed for the ground state of the BPI complex, suggesting that the conformational changes that activate the ATPase activity, whether by interaction with MBP or by mutation, may be similar. In contrast, vanadate trapping of the transition state of the BPI complexes had no effect on the solvent accessibility of MIANS (Fig. 4), suggesting that the environment of residue Cys-40 does not change substantially in the transition from the ground state to the transition state of the BPI transporter. The absence of any effect in the BPI system argues that steric effects of nucleotide trapped by vanadate in one of the two nucleotide-binding sites (11) are not solely responsible for the decrease in solvent accessibility of MIANS in the WT. Since ATP protects against MIANS modification (Fig. 6), we predict that only the unoccupied nucleotide-binding site is modified after vanadate treatment. Since the fluorescence intensity of transporters was the same whether they were labeled before or after vanadate-induced nucleotide trapping, MIANS may modify only one nucleotide-binding site even in the untreated transporter. Modification of a single nucleotide-binding site by 4-chloro-7-nitrobenzofurazan-Cl has been demonstrated for P-glycoprotein, where it appears that covalent modification of one site prevents modification of the other site (42).

The similarities in the environment of the nucleotide-binding site in the BPI mutant and in the transition state suggest that the mutations in the BPI transporter have stabilized a conformation that resembles, at least superficially, the conformation of the transition state, thereby overcoming the major requirement for MBP in stabilization of the transition state and thereby activation of the ATPase activity. The observation that the BPI MalF500GKc complex binds MBP tightly in the absence of vanadate (Fig. 5) strengthens this hypothesis. An increased affinity for MBP may be a general feature of the BPI mutants, since it has been reported that the BPI MalFG511Kc complex also displays a higher affinity for MBP than the WT complex (40).

Knowing that MBP stimulates the ATPase activity of MalFGKc, these results were originally interpreted in terms of an equilibrium between two different conformations of the transporter, an ATPase-inactive conformation that predominates in the WT and the ATPase-active conformation induced through interaction with MBP (40). The BPI mutants were believed to shift this equilibrium in favor of the active conformation, to a lesser or greater extent, accounting for the difference in maximal ATP hydrolysis rates between different BPI mutants (14, 43). Because of induced fit, the active conformation was expected to have a surface more complementary to MBP than the ATPase-inactive state and hence have a higher affinity for MBP (40). With the new knowledge that MBP stimulates ATP hydrolysis by binding tightly to the transition state conformation of the transport complex, we now suggest that the BPI conformations may resemble different intermediates in the pathway of the WT as it approaches the transition state. These conformational states may not be populated to any great extent in the cycle of ATP hydrolysis by the WT, providing an explanation for the failure of MBP to alter noticeably the conformation of MalFGKc without vanadate present (Fig. 5). Alternatively, although less likely, the inactivation caused by MIANS modification may have prevented attainment of the active conformation.

The increased ability of ATP to protect BPI complexes against MIANS modification as well as the decreased $K_{0.5}$ for ATP hydrolysis by the BPI complexes indicates that the BPI complexes exhibit an increased affinity for ATP as compared with the WT complexes. If we are correct in assuming that the BPI complex resembles an intermediate in the pathway of the WT, then incremental or ratcheted increases in the affinity for both ATP and MBP may be characteristic of the approach to the transition state for ATP hydrolysis. The affinity of MalFGKc for ATP would never exceed the affinity for the ATP in its transition state conformation, ensuring that ATP hydrolysis occurs (44). The increased affinity that the BPI complex displays for ATP could also be a by-product of the specific mutations that induced the BPI phenotype and unrelated to the normal pathway of activation of ATPase activity. Analysis of

![Fig. 7. Model of MalFGKc ATPase activation by MalK subunit dimerization.](image)
other BPI mutations may resolve this issue.

Recent structural information about ABC proteins aids in the interpretation of these findings. The crystal structures of two nucleotide-binding subunits of ABC transporters are now available, the HisP protein from *Salmonella typhimurium* (45) and the MalK protein from *Thermococcus litoralis* (46). These two proteins share a similar architecture as expected from the sequence homology, and the nucleotide-binding site is highly exposed on the surface of the monomer subunit, raising the possibility that residues from another subunit in the transport complex may contribute to nucleotide binding and/or hydrolysis (13). Jones and George (13) provide a convincing theoretical argument in support of a head to tail dimer of HisP in which the nucleotide-binding sites are buried between the monomers, and each monomer contributes residues to both nucleotide-binding sites. The proposed dimer interface, while different from the dimer seen in the crystal structure of HisP (45), is strikingly similar to a recently published dimer of the RAD50 catalytic domain (RAD50cd), an ABC protein involved in DNA double strand break repair (25). ATP promotes the association of two inactive RAD50cd proteins by binding to the signature motif (or LSGGQ) of one monomer and the P-loop of the opposite monomer to complete the active site, thereby activating the ATPase activity (25). The dimer interface seen in the crystal structure of MalK also positions the nucleotide-binding site between the two monomers, although the specific residues contributed to the nucleotide-binding site by the second subunit are not those of the LSGGQ motif (46). In either case, our data are readily interpretable by assuming that ATP is bound between the two MalK subunits in MalFGK2 and that the ATPase activity of the transport complex is regulated by MalK subunit association and dissociation. The decreased solvent accessibility of the P-loop that is associated with activation of the MalFGK2 ATPase, either by MBP or by the BPI mutations, may be the result of the nucleotide-binding site becoming more buried in the MalK dimer interface. Activation of the ATPase activity of the MalK subunit could be achieved if residues on one subunit are required to complete the nucleotide-binding site on the second subunit, and the role of the MBP is to bring the two subunits into closer proximity (Fig. 7). Likewise, the BPI mutations in MalF may activate the ATPase activity of the two subunits into closer proximity (Fig. 7). Likewise, the site on the second subunit, and the role of the MBP is to bring the buried in the MalK dimer interface. Activation of the ATPase may be the result of the nucleotide-binding site becoming more complex.

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