ATP INDUCES CONFORMATIONAL CHANGES OF PERIPLASMIC LOOP REGIONS OF THE MALTOSE ATP-BINDING CASSETTE (ABC) TRANSPORTER*

Martin L. Daus**, Heidi Landmesser**, Andreas Schlosser, Peter Müller, Andreas Herrmann, and Erwin Schneider

1Institut für Biologie/Bakterienphysiologie, Humboldt Universität zu Berlin, Chausseest. 117, D-10115 Berlin, Germany
2Institut für Medizinische Immunologie, Charité-Universitätsmedizin Berlin, Hessische Str. 3-4, D-10115 Berlin, Germany
3Institut für Biologie/Biophysik, Humboldt Universität zu Berlin, Invalidenstr. 42, D-10115 Berlin, Germany

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**These authors contributed equally to this work.

§Address correspondence to:
Erwin Schneider, Institut für Biologie/Bakterienphysiologie, Humboldt Universität zu Berlin, Chausseest. 117, D-10115 Berlin, Germany, Tel: ++49-030-2093-8121; Fax: ++49-030-2093-8126; E-mail:erwin.schneider@rz.hu-berlin.de

We have studied co-factor induced conformational changes of the maltose ATP-binding cassette transporter by employing limited proteolysis in detergent solution. The transport complex consists of one copy each of the transmembrane subunits, MalF and MalG, and of two copies of the nucleotide binding subunit, MalK. Transport activity further requires the periplasmic maltose binding protein, MalE. Binding of ATP to the MalK subunits increased the susceptibility of two tryptic cleavage sites in the periplasmic loops P2 of MalF and P1 of MalG, respectively. Lys-262 of MalF and Arg-73 of MalG were identified as probable cleavage sites, resulting in two N-terminal peptide fragments of 29 and 8 kDa, respectively. Trapping the complex in the transition state by vanadate further stabilized the fragments. In contrast, the tryptic cleavage profile of MalK remained largely unchanged. ATP-induced conformational changes of MalF-P2 and MalG-P1 were supported by fluorescence spectroscopy of complex variants labeled with 2-(4'-maleimidoanilino)naphthalene-6-sulfonic acid. Limited proteolysis was subsequently used to study the consequences of mutations on the transport cycle. The results suggest that complex variants exhibiting a binding protein-independent phenotype (MalF500) or containing a mutation that affects the catalytic carboxylate (MalKE159Q) reside in a transition state-like conformation. A similar conclusion was drawn for a complex containing a replacement of MalKQ140 in the signature sequence by leucine, while substitution of lysine for Q140 appears to lock the transport complex in the ground state. Together, our data provide first evidence for conformational changes of the transmembrane subunits of an ATP-binding cassette import system upon binding of ATP.

ATP-binding cassette (ABC) proteins exist in all living organisms and form one of the largest superfamilies. They are integral to almost every biological process and physiological system. Most are involved in the uptake or export of an enormous variety of substances across cell membranes, from small ions to large polypeptides. Many of these proteins are of considerable medical importance. Mutations in several 'ABC genes' lead to genetic diseases, such as cystic fibrosis, Tangier disease and adrenoleukodystrophy or confer resistance to antibiotics and chemotherapeutic agents (1).

ABC transporters share a common architectural organization comprising two hydrophobic transmembrane domains (TMDs) that form the translocation pathway and two hydrophilic nucleotide binding (ABC) domains (NBDs) that hydrolyze ATP. In prokaryotes,
these domains are mostly expressed as separate protein subunits, whereas in eukaryotes, especially in mammalian cells, they are usually fused into a single polypeptide chain.

The ABC domains are characterized by a set of Walker A and B motifs that are involved in nucleotide binding and by the unique 'LSGGQ' signature sequence (2). To date, the crystal structures of several mostly prokaryotic ABC domains have been reported (reviewed in 3-5). These structures largely agree on the overall fold. Accordingly, the cassette can be subdivided in an F1-type ATP-binding domain, encompassing both nucleotide binding motifs, a specific α-helical subdomain, encompassing the LSGGQ motif and a specific antiparallel β-subdomain. The configuration of the NBD dimer is less clear. However, the crystal structures of Rad50 (6), a variant of MJ0796 (7) and of MalK (8) as well as biochemical evidence (9,10) are strongly in favor of a model in which the nucleotide binding site of one NBD is completed by the LSGGQ motif of the opposing NBD.

Further progress in understanding the structural organization of ABC transporters was achieved with the high resolution structures of BtuCD, mediating the uptake of vitamin B₁₂ in Escherichia coli (11) and of MsbA, involved in the export of lipid A in gramnegative bacteria (12-14). These structures have provided insight into the interactions between the NBDs and the TMDs. Notwithstanding these advances, the conformational changes in the NBDs induced by ATP binding/hydrolysis (reviewed in 3, 4, 15), and the means by which they are transmitted to the TMDs to effect substrate translocation, remain largely to be elucidated.

The maltose ABC transporter of E. coli/S. typhimurium is one of the best characterized systems that can serve as a general model for ABC importers (5,16). The transporter is composed of the extracellular (periplasmic) receptor, the maltose binding protein (MBP or MalE), and the membrane-bound complex comprising the hydrophobic subunits, MalF and MalG, and two copies of the ATPase (ABC) subunit, MalK (17).

MalK and closely related ABC subunits belonging to the CUT1 and MOI subfamilies of ABC importers (18-20) contain a unique C-terminal extension which, in the crystal structure of the MalK dimer contributes substantially to monomer-monomer contacts (8). The observed conformational changes between ATP-free and ATP-bound MalKs confirm previous conclusions from protease susceptibility studies (21) and fluorescence spectroscopy (22). Association of the MalK subunits with MalF and MalG requires the so-called EAA sequence motifs that are conserved in the last cytoplasmic loop regions of TMDs of ABC import systems (23,24).

Transport of maltose is assumed to be initiated by interaction of substrate-loaded MalE with periplasmic loops of MalFG, thereby triggering conformational changes that result in ATP hydrolysis at the MalK subunits and eventually in substrate translocation (25). Experiments employing the transition state analogue vanadate further suggested that binding of liganded MalE and ATP is occurring rather simultaneously and that a transiently stable complex of MalFGK₂ with MalE is formed (26). Vanadate traps ADP in one of the two nucleotide-binding sites locking the transport complex in the transition state conformation, thereby inhibiting subsequent ATP hydrolysis (27,28). Recent spin labeling electron paramagnetic resonance spectroscopy provided evidence that closure of the MalK dimer interface coincides with opening of MalE and maltose release to the transporter (29).

The transition state for ATP hydrolysis is also proposed to be represented by mutations in MalF and MalG that allow transport of maltose in the absence of MalE albeit with lower efficiency (22,25,30). Other candidate residues for which conformational changes have been demonstrated are located in the helical subdomain of MalK (24,31) and in the LSGGGQ motif (32).

While ATP-induced conformational changes of the MalK subunits in solution (8,21) as well as in the assembled complex (22,24) have been well documented, only little is known on concomitant conformational changes of the membrane-integral subunits MalFG that are supposed to form the translocation pore. In this communication we have addressed this question by employing limited proteolysis to demonstrate conformational changes in the MalFGK₂ complex at different stages of the transport cycle. We found that tryptic cleavage sites in the second periplasmic loop of MalF and in the first periplasmic loop of MalG became more accessible to the protease in the presence of ATP, suggesting conformational changes of both subunits. This notion was strengthened by
measuring the fluorescence of the transporter labeled with an extrinsic probe in MalF and MalG, respectively. Similar proteolytic fragments as found for the ATP-bound wild type complex were obtained with a binding protein-independent (BPI) mutant and with transport complexes containing the MalK variants Q140L and E159Q, respectively, but in the absence of ATP or other cofactors. In contrast, the tryptic digestion profiles of a variant containing MalKQ140K resembled those of wild type in the absence of co-factors but did not change in the presence of ATP. These findings support the view that each of these mutations affect the conformational status of the transporter albeit with different consequences on functionality.

Materials and methods

Bacterial strains and plasmids – E. coli strain JM109 (Stratagene) was used as a general host for the plasmids listed in Table 1. The plasmid-borne mal alleles originate from Salmonella typhimurium except the malF malG alleles on pAW3, pMM38 and pDE17 which are from E. coli. The mal genes from both organisms are functionally fully exchangeable (24).

Plasmid constructions – Cysteine residues replacing MalF(S252C) and MalG(P78C), respectively, were introduced by Stratagene’s Quikchange kit using plasmid pTAZFQ* (malF(Cys)-malG(Cys-)) (24) as template. The resulting plasmids were designated pMM38 and pDE17. Plasmid pVE1 was constructed by replacing an EcoRV fragment from pBB1 encompassing codons 25 to 292 by the corresponding fragment from plasmid pGS97-7 (32) harboring the malK801 allele. To construct pDE08, we first introduced the E159Q mutation into the malK wild type allele on pSW7 (33) by the Quikchange method, resulting in plasmid pDE06. pDE08 was then obtained by exchanging an EcoRV fragment of pBB1 by the corresponding fragment of pDE06 as described for pVE1. Plasmid pAW3 was constructed by introducing the mutations G338R and N505I, conferring the binding protein independent phenotype (34) into the malF allele on plasmid pTAZFQ(S3C/-) (24) using the Stratagene’s Quikchange kit. Plasmid pJS08 was constructed by introducing the malKht allele encompassing the pT5 promoter region from pGS91-1 (32) as a SalI-XhoI fragment into pSU19 (35), previously digested with SalI. Plasmid pMM37 was constructed by replacing an EcoRV fragment encompassing codons 25 to 292 of malK from pJS08 by the corresponding fragment from plasmid pSH25 (36) harboring the malK796 allele (Table 1).

Purification of MalFGK₂ complexes – Polyhistidine-tagged transport complex variants were overproduced in strain JM109 harboring the following plasmids: pBB01 (wild type), pAW3/pJS08 (binding protein-independent variant), pGS98-1/pES62 (MalK-Q140K), pVE1 (MalKQ140L), pDE08 (MalKE159Q), pDE17/pMM37 (MalG(P78S)MalK(C40S)), and pMM38/pMM37 (MalF(S252C)MalK(C40S)). Purification was essentially carried out as described in (37). Briefly, membranes solubilized by addition of 1.1 % dodecyl-ß-D maltoside (DDM) were bound to Ni-NTA resin equilibrated in buffer A (50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 20 % glycerol, 0.1 mM PMSF, 0.01 % DDM). The resin was washed with buffer B (20 mM imidazole, 50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 20 % glycerol, 0.1 mM PMSF, 0.01 % DDM) and protein was eluted with buffer C (50 mM imidazole, 50 mM Tris-HCl, pH 8, 5 mM MgCl₂, 20 % glycerol, 0.1 mM PMSF, 0.01 % DDM). Peak fractions were pooled, passed through a PD10 column (Amersham) equilibrated with buffer D (50 mM Tris-HCl, pH 8, 20 % glycerol, 0.01 % DDM), concentrated by ultrafiltration, shock-frozen in liquid nitrogen and stored at -80 °C.

Purification of MalE – Maltose binding protein (tag-less) was purified as described in (37).

Preparation of vanadate-trapped complexes – WT and mutant transport complexes were treated with vanadate as described in (26). Briefly, vanadate was added to a final concentration of 1 mM to a mixture containing 6 µM MalFGK₂ complex, 12 µM MalE, 15 mM ATP, 15 mM MgCl₂ and 0.1 mM maltose in buffer D and the reaction was incubated for 20 min at 37 °C unless indicated otherwise. For fluorescence measurements the samples were then desalted by passage through PD10. Stable association of MalE with the trapped complexes was confirmed using Ni-NTA affinity chromatography.

Limited proteolysis by trypsin – Treatment of transport complexes with trypsin was carried out as described in (21) with modifications. Routinely, incubation mixtures (final volume 20 µl) contained buffer D and 6
μM of complex variants. The effects of additives (15 mM ATP, 15 mM MgCl₂, 100 μM maltose, 12 μM MalE) as indicated were studied by pre-incubation with the transport complexes for 20 min at 37 °C. Vanadate-trapped complexes were prepared as described above. The proteolytic reaction (at 25 °C) was initiated by the addition of trypsin (0.1 μg; corresponding to a complex to trypsin ratio of 31:1) and terminated at the indicated times by adding a 5-fold excess of trypsin inhibitor. Subsequently, 10x loading buffer for SDS-PAGE was added and the reaction mixtures were shock-frozen in liquid nitrogen and stored at -80 °C until further analysis. Following separation by SDS-PAGE (15 %) the polypeptides were electroblotted onto nitrocellulose and probed with specific polyclonal antisera raised against purified MalK (dilution, 1: 20,000) (38), gel-purified MalF (dilution, 1:50,000) and a synthetic peptide derived from the first periplasmic loop (residues Leu-39 to His-58) of MalG (dilution, 1:10,000), respectively. Antigen-antibody interactions were visualized using the Western Blot Chemiluminescence Reagent Plus system (NEN Life Science products).

**ATPase assay** – Hydrolysis of ATP was assayed in microtiter plates essentially as described in (39).

**Maltose transport** - Uptake of [¹⁴C]maltose in proteoliposomes containing the MalFGK₂ variants indicated in Table 2 was essentially carried out as described in (37).

**Protein determination** - Protein concentrations were determined using the BCA kit from BioRad (Munich, Germany).

**MIANS modification of transport complexes** - 2-(4'-Maleimidylanilino)naphthalene-6-sulfonic (MIANS) was purchased from Molecular Probes (via Invitrogen, Germany). For MIANS modification studies, transport complexes were labeled in buffer D by incubation at 4 °C with 10 µM MIANS for 15 min. The reaction was terminated by adding 1 mM DTT and excess MIANS was removed from the protein sample by a PD10 desalting column (Amersham/Pharmacia) or by Ni-NTA affinity chromatography.

**Fluorescence measurements** – Fluorescence measurements were performed using an Aminco Bowman Series 2 spectrofluorometer. Fluorescence spectra for MIANS-labeled proteins in buffer D were recorded using an excitation wavelength of 340 nm (slit width 4 nm) and an emission range of 360-600 nm (slit width 4 nm) at 25 °C.

**N-terminal sequence analysis** – Determination of the amino-terminal sequence of tryptic fragments was carried out as described in (21). Briefly, 12 lanes of an SDS gel were loaded each with 20 μg of vanadate trapped WT complex, previously treated with trypsin (ratio 50:1, w/w) for 8 min. After electrophoresis, proteins were electroblotted onto a polyvinyliden difluoride membrane and further treated as described (21).

**NanoLC-MS/MS analysis** – Fragments MalF-F1, MalG-G1 and MalK-K1 were generated from vanadate-trapped complex by trypsin treatment (50:1) (see above) and separated by SDS-PAGE. After staining with Coomassie R, protein bands of the expected molecular masses were excized, destained with 30 % acetonitrile (ACN), shrunk with 100 % ACN, and dried in a Vacuum Concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with trypsin were performed overnight at 37 °C in 0.1 M NH₄HCO₃ (pH 8). About 0.1μg of protease was used for one gel band. Peptides were extracted from the gel slices with 5 % formic acid. Tryptic digests were analyzed by nanoLC-MS/MS using a nanoHPLC system (CapLC, Micromass, Manchester, UK) and a quadrupole time-of-flight tandem mass spectrometer (Q-Tof micro, Micromass, Manchester, UK) and a 50-μm-i.d. analytical column (C18 reversed phase, 5 μm particle size, 20 cm length; NanoSeparations, Nieuwkoop, The Netherlands) was used for nanoLC separations. ESI emitter (360 µm o.d., 20 μm i.d., 5 μm tip i.d., distal coated) were obtained from NewObjective (Woburn, MA, USA). A linear gradient from 0 to 40 % ACN in 30 minutes with a flow rate of 25 nL/min was applied for peptide separation. Mascot Distiller (Matrix Science, London, UK) was used to create peak lists from MS and MS/MS raw data. Mascot Server (Matrix Science, London, UK) was used for database searching. Peptides from the Mascot result file were checked manually.

**Electrophoresis** – SDS-polyacrylamide gel electrophoresis was performed with 15 % polyacrylamide gels using the procedure described by Laemmli (40). Fluorescent bands
RESULTS

The experimental system - We chose to study conformational changes of the MalFGK2 complex at different stages of the transport cycle by limited proteolysis of the purified complex in detergent solution rather than in proteoliposomes. The latter have the disadvantage that the proteins are incorporated in two different orientations hence leading to only half of the complexes with their MalK subunits being exposed to the medium (37). On the other hand, different steps in the transport cycle that were demonstrated for the complex when in a lipid environment are also intrinsic properties of the detergent solubilized complex: spontaneous ATPase activity that is (i) stimulated in the presence of liganded MalE (25,27,37) and (ii) sensitive to inhibition by vanadate (27,28,37) (see also Table 2). These observations are taken as evidence for tight coupling between hydrolysis of ATP and substrate translocation. Moreover, under vanadate-trapped conditions the binding protein forms a stable complex with MalFGK2 in its open conformation after having released bound maltose (26,29), thereby supporting the notion that completion of a transport cycle also occurs in solution. Together, we reasoned that the purified detergent-solubilized MalFGK2 complex is preferentially suited for the intended study. Nonetheless, the capability of wild type and some mutant transport complexes to transport maltose was verified in proteoliposomes (Table 2). The observed initial rates of maltose uptake were well comparable with those reported previously (39).

ATP binding renders MalF and MalG substantially more susceptible to limited tryptic digestion - To investigate changes in the conformation of the subunits the purified and transport-competent MalFGK2 complex (Fig. 1; Table 2) was exposed to protease at a complex to trypsin ratio of 31:1 (w/w) at 25 °C for up to 8 min. Incubation with the protease was carried out in buffer D, in the absence or presence of ATP, under ATP hydrolysing conditions (e.g. supplemented with maltose-loaded MalE and MgATP), and under vanadate-trapped conditions. The reaction mixtures were subsequently analyzed by SDS-PAGE and immunoblotting using subunit specific antisera directed against MalK, MalF, and a periplasmic peptide (Leu-39 to His-58) of MalG.

As shown in Fig. 2 (top) in the control sample MalF was largely resistant to trypsin, giving rise only to two faint bands with apparent molecular masses of 29 (F1) and 26 (F2) kDa, respectively. The bands were visible already after one minute of exposure to trypsin but their intensities did not change with time. In contrast, in the presence of ATP, the intensity of F1 substantially increased, while F2 remained unchanged. The same profile was observed under ATP-hydrolyzing conditions (lanes 10-12) while vanadate-trapping increased the amount of fragment F1 somewhat further. This was accompanied by a decrease in the amount of intact MalF (lanes 14-16).

In case of MalG, limited proteolysis in plain buffer caused partial digestion resulting in a single stable cleavage product (G1) of ~ 8 kDa after incubation for 8 minutes only (Fig. 2 center, lanes 2-4). Since the antiserum recognizes a peptide fragment derived of the first periplasmic loop of MalG, the tryptic fragment is likely to encompass the N-terminal portion of MalG. In the presence of ATP the G1 peptide was detectable already after 4 minutes and its amount increased substantially after 8 minutes (lanes 6-8). Again, adding the necessary co-factors to initiate ATP hydrolysis did not change this profile (lanes 10-12) but vanadate-trapping resulted in a further increase in the amount of G1 (lanes 14-16). Quantification of the bands obtained after eight minutes of incubation with trypsin revealed that the amount of G1 was 1.7-fold higher in the vanadate-trapped sample (lane 16) than in the presence of ATP alone (lane 8). Furthermore, a slightly smaller second fragment was observed after 8 minutes (lane 16).

The MalK subunits behaved differently. Besides intact MalK, a major peptide fragment of ~ 35 kDa (K1) and three minor faster migrating fragments (K2,3; K4; please note that the previously observed fragments K2 and K3 are not separated under the conditions used here; see below) were found under all conditions tested (Fig. 2, bottom). This profile is similar to that reported for purified MalK when exposed to trypsin in the presence of MgATP only (21,32). Thus,
the assembled complex the MalK dimer apparently resides in a conformation that renders the potential cleavage sites less accessible to the protease. This conclusion is in agreement with previous findings using everted membrane vesicles (41). Moreover, the conformational changes observed upon binding of ATP to the purified complex (22) do not change the solvent exposure of these residues. The finding that the tryptic peptide profile of MalK did not change upon addition of ATP or in the presence of vanadate also argues against the notion that the observed MalF- and MalG-derived peptides result from complex disassembly due to MalK degradation.

Incubation with cofactors prior to the addition of trypsin did not affect the stability of MalFGK2 complex. This was demonstrated by re-chromatography on a Ni-NTA column and subsequent analysis of the eluted protein-containing fractions by SDS-PAGE (see Fig. 1, lane 8 for the vanadate-trapped complex).

It should also be noted that, where included in the reaction mixture, the MalE protein was not attacked by trypsin (data not shown). Furthermore, raising the trypsin to complex ratio to 1:3 (w/w) confirmed the above results but also gave rise to numerous additional peptide fragments under all conditions tested. Thus, in order to maintain the integrity of the complex a 31:1 ratio (complex to trypsin, w/w) was exclusively used in further experiments (see below).

Together, these results indicate that ATP binding at the MalK subunits causes conformational changes of MalF and MalG that result in the exposure of tryptic cleavage sites. Trapping the transporter in the transition state by vanadate enhanced this effect especially in case of MalG.

**Identification of tryptic fragments by nanoLC-MS/MS analysis reveals putative cleavage sites in periplasmic loops of MalF and MalG** - In order to identify the chemical nature of the tryptic fragments derived of MalF (fragment F1) and of MalG (fragment G1) we used mass spectrometry. To this end, the peptide fragments were prepared at a larger scale as described in 'Materials and methods'. A total of 18 peptides derived from the N-terminal part of MalF including the amino terminus were identified by nanoLC-MS/MS. (Table 3). No fragments corresponding to the MalF sequence beyond Lys-262 were found suggesting the residue being the potential cleavage site (Fig. 3A). This conclusion is further strengthened by the fact that the molecular weights of the fragments encompassing residues Met-1 to Lys-262 sum up to 29,283 which corresponds well to the apparent molecular weight of MalF-F1 obtained by SDS-PAGE (see Fig. 2, top).

In case of the MalG fragment G1 two peptide fragments that encompass the N-terminus (6AMVQPK) and a sequence of the first periplasmic loop (6LALGFSVEHADGR) (Table 3, Fig. 3B). Assuming the potential cleavage site at Arg-73, the resulting peptide would exhibit a calculated molecular weight of 8,184 which is in good agreement with the data from SDS-PAGE (see Fig. 2, center). Moreover, the conclusion that the fragment might be identical to a peptide encompassing residues 1 to 73 of MalG would also be consistent with the epitope that is recognized by the antiserum (see 'Materials and methods').

Analysis of the MalK-derived 35kDa-fragment by nanoLC-MS/MS resulted in the identification of peptides corresponding mostly to the C-terminal portion of MalK (Table 3). The peptide being closest to the amino terminus of MalK has the sequence 68MNDDIPPAER. This corresponds exactly to the N-terminal sequence of peptide MalK-T1 identified in a previous study using soluble MalK (21) and thus confirms the above notion. Since no significant changes in the digestion profile were observed for MalK under any of the conditions applied the chemical nature of the minor fragments were not further analyzed.

The N-termini of the tryptic fragments MalF-F1 and MalG-G1 were also independently confirmed by Edman degradation (not shown).

**Fluorescence spectroscopy confirms conformational changes of loop regions in MalF and MalG** - The results from limited tryptic digestion indicated that periplasmic loop regions of MalF and MalG expose cleavage sites in the ATP-bound state that are stabilized by vanadate trapping. To further confirm these conformational changes by an independent approach we employed fluorescence spectroscopy using MIANS as an extrinsic probe covalently linked to cysteine residues. We chose MalFS252C and MalGP78C, respectively, as suitable targets for MIANS modification (Fig. 3A, B). While Ser-252 in MalF is one of two serine residues close
to the potential tryptic cleavage site Lys-262, Pro-78 in MalG was recently found to functionally tolerate substitution by cysteine (Daus and Schneider, unpublished). The cysteine residues were introduced into cys-less variants of MalF and MalG, respectively, (24) and the transport complexes containing a C40S variant of MalK (36) were purified (Fig. 1) and functionally characterized (Table 2). Treatment with MIANS, separation of the subunits by SDS-PAGE and inspection of the gels under UV light clearly revealed that only the monocys variants of MalF and MalG were labeled (Fig. 4). Please note that MalKC40S became not labeled although the protein contains two additional cysteine residues in the C-terminal domain (C350, C360) which are apparently inaccessible to MIANS.

The fluorescence characteristics of MIANS are sensitive to the polarity of its environment, that is changes of solvent polarity result in a shift of the emission maximum. Comparing the fluorescence spectra of MIANS-labeled transport complexes containing MalFS252C and MalGP78C variants in plain buffer or under vanadate-trapped conditions revealed a blue shift in the emission maximum of about 6 and 4 nm, respectively (Fig. 5A,B). These findings indicate that the fluorophore is in a more hydrophobic environment when bound to the vanadate-trapped complexes. Similar blue-shifts and quantum yields were observed when labeling with MIANS had been carried out prior to or after vanadate-trapping. Thus, treatment with vanadate did not affect the accessibility of the cysteine residues for the fluorophore.

At first glance, this might appear contradictory to the increased susceptibility of the respective loop regions to trypsin and thus, to an increased exposure to the solvent. However, one has to take into account that MIANS is bound to cysteine residues in MalF and MalG, respectively, that are 10 and 5 residues apart from the tryptic cleavage sites. Therefore, opposing changes in the micro-environments of the protein-bound fluorophore and the residues recognized by trypsin cannot be excluded. Nonetheless, as intended for this study, the fluorescence spectroscopy data are in support of the above notion that the loop regions in MalF and MalG in the vicinity of the tryptic cleavage sites undergo a conformational change upon binding of ATP/vanadate.

The tryptic digestion profile of a binding protein-independent transport complex in the absence of cofactors resembles that of ATP-bound wild type MalFGK2. - Having established conditions to monitor conformational changes of the transport complex by limited proteolysis we set out to examine possible consequences of mutations affecting transport functions. Several maltose transport complex variants exist that display a binding protein-independent phenotype mostly due to the combination of two mutations in the transmembrane segments of MalF or MalG (34). These variants exhibit spontaneous ATPase activity (see also Table 2) and have been proposed to resemble the conformation of the transition state (22,25). If so, limited tryptic digestion in plain buffer would result in the formation of MalF- and MalG-derived peptide fragments similar to those observed with vanadate-trapped wild type complex. The results shown in Fig. 6 using the purified MalF500GK2 complex (Fig. 1, lane 4) support this prediction. Fragments MalF-F1 and MalG-G1 were stably formed in the absence of any cofactor (ATP, Mg²⁺, MalE, maltose or vanadate). Moreover, the profiles remained unaffected when co-factors were added. The susceptibility of MalK was comparable to wild type under all conditions although an increase in the relative amounts of the smaller fragments was observed. Thus, these findings are consistent with the notion that binding protein-independent transport complexes reside in a conformation that is normally induced upon ATP binding.

Limited tryptic digestion reveals that mutant transport complexes carrying mutations in the ABC signature (MalKQ140K, L) reside in different conformational states. - Next, we analyzed the susceptibility to trypsin of complex variants carrying mutations in MalK that were previously suspected to interfere with signaling during the transport cycle (32). Mutations affecting Gln-140 in the extended ABC signature sequence of MalK (134LSGGQRQ140) displayed different phenotypes depending on the chemical nature of the substituting amino acid. Replacement by lysine, asparagine or leucine abolished transport due to the failure of the respective complexes to hydrolyze ATP (32 and Table 2). However, the purified soluble variants MalKQ140K and MalKQ140N exhibited normal ATPase activity and a tryptic digestion
profile similar to wild type MalK. In contrast, soluble MalKQ140L did not hydrolyze ATP and displayed strong resistance to trypsin (32). Together, these results were interpreted in favor of a role of Q140 in transmitting conformational changes from the ABC domains to the transmembrane subunits. Subjecting a purified transport complex containing MalKQ140K (Fig. 1, lane 3) to tryptic digestion in buffer D resulted in profiles similar to wild type (Fig. 7, lanes 2-4). Surprisingly however, other than wild type, vanadate-trapping did not change these profiles (Fig. 7, lanes 6-8; compare to Fig. 1, lanes 6-8). Thus, the data suggest that the mutation locks the complex in the ground state, being unable to undergo an ATP-induced conformational change.

In contrast, exposure to trypsin of a complex containing the MalKQ140L variant (Fig. 1, lane 2) resulted in digestion profiles very similar to those obtained with the binding protein-independent variant and vanadate-trapped wild type (Fig. 8; compare to Figs 6 and 2). Substantial amounts of stable fragments F1 and G1 could be detected that did not change under vanadate-trapping conditions. Again, the mutation apparently causes a conformational alteration that resembles that of the ATP-bound wild type complex. Together, these data support the notion that MalKQ140L and presumably the entire ABC signature motif is involved in signaling ATP binding at MalK to the MalFG subunits.

A mutation affecting the proposed 'catalytic carboxylate' (MalKE159Q) also locks the transporter in a transition-like conformation - Another complex variant included in this study contained a glutamate to glutamine exchange at position 159 in MalK. The crystal structure of the MalK dimer (8) and of other ABC domains (7,43) gave rise to the notion that the glutamate immediately following the conserved aspartate residue in the Walker B site of the nucleotide binding fold may serve as 'catalytic carboxylate' by activating a water molecule for its attack on the γ-phosphate. Substituting glutamine for the respective glutamate in MJ0796 eliminated ATPase activity of the protein but stabilized the dimeric configuration in the presence of ATP (44). Thus, it appears not unlikely that the mutation might also cause a major conformational change in the context of the assembled transport complex. To test for this hypothesis we subjected a purified maltose transport complex containing the MalKE159Q variant (Fig. 1, lane 5) to limited proteolysis. In agreement with the above data and those from a study using the mammalian P-glycoprotein (45), the mutant complex exhibited no ATPase activity (Table 2). As shown in Fig 9, the tryptic digestion profiles of MalF and of MalG observed in the absence of any co-factor strongly resemble those obtained with the vanadate-trapped wild type complex (compare Fig. 8 with Fig. 2). These profiles did not change when the protein was exposed to trypsin in the presence of ATP and vanadate. As in case of BPI and MalKQ140L, MalK was slightly more susceptible to trypsin, resulting in increased amounts of the smaller fragments. These data suggest that the mutation irreversibly locks the complex in a transition state-like conformation.

DISCUSSION

The aim of the presented study was to demonstrate conformational changes of the transmembrane subunits MalF and MalG of the maltose ABC transport complex upon ATP-binding to the MalK dimer. While conformational changes of MalK are well documented (8,21,22,32), subsequent alterations of the pore-forming subunits that eventually lead to translocation of maltose are unknown. We employed limited proteolysis to detect such conformational changes which has proven to be a useful tool in a previous study on soluble MalK (21) as well as for a complete ABC transporter, P-glycoprotein (46,47). We found that tryptic cleavage sites in the periplasmic loops P2 of MalF and P1 of MalG become more exposed to the protease upon binding of ATP to the MalK subunits. In case of MalG the effect was further increased under vanadate-trapped conditions. These results were supported by demonstrating an ATP-induced shift in the emission fluorescence spectra of an external probe covalently attached to residues in close vicinity of the identified tryptic cleavage sites. Together, these data are consistent with the notion that ATP binding to the MalK subunits causes conformational changes in the transmembrane subunits MalF and MalG.

In the absence of co-factors the MalFGK2 complex was largely resistant to trypsin under the experimental conditions used.
This finding was somewhat surprising especially for MalF considering the large periplasmic loop (P2) encompassing about 184 amino acid residues as proposed from the topological model (Fig. 3A). Thus, P2 is likely buried within the complex structure but becomes partially exposed to solvent upon binding of ATP to the MalK subunits (Fig. 2A). The P2 loop is characteristic of MalF proteins from enterobacteria and a few other examples (48). MalF homologs in most other bacteria (48,49) and in archaea (50) lack this domain and often contain six rather than eight transmembrane domains. Nonetheless, the P2 loop in \textit{E. coli} and probably in all other enterobacterial MalF is essential for function as suggested from mutational analysis. Tapia \textit{et al} (51) found that four out of five mutations in P2 affected MalK localization. In the context of other results (52) the authors proposed that this was due to an altered interaction with MalG.

Our finding that a tryptic cleavage site in the P2 loop was stably exposed in the presence of ATP does of course not exclude the possibility that the indicated conformational change is actually affecting TMDs 3 and 4 that are connected by P2. These have been proposed based on suppressor mutational analyses to contact each other and to interact with TMD 5 of MalF and TMD 5 of MalG, respectively (53). Consequently, conformational changes of these transmembrane domains including their connecting peptide loop during the transport cycle appear not to be unlikely.

In case of the P1 loop of MalG that becomes partly exposed upon ATP binding and, even more pronounced, under vanadate-trapped conditions, Dassa (54) proposed on the basis of mutational analysis that the peptide fragment encompassing residues 50 to 75 is essential for transport function. Nelson and Traxler (55) studied the effect of peptide insertions in MalG and found that an insertion at position 68 reduced transport by 57 % as compared to wild type. Moreover, insertions at positions 84 and 90, respectively, abolished transport.

Limited proteolysis also proved to be a useful tool to analyse the effect of mutations on peptide formation thereby gaining further insight into the involvement of the respective residues in individual steps of the transport cycle.

Binding protein-independent mutations of the maltose transport complex that are located in the transmembrane subunits MalF and/or MalG are characterized by a decrease in affinity for maltose, a failure to accept maltodextrins as substrates (30) and by a spontaneous ATPase activity (25). Fluorescence spectroscopy revealed that the conformation of the nucleotide-binding sites in BPI more closely resemble the transition state as represented by the vanadate-trapped complex than the ground state of the wild type complex (22). The data presented here are fully consistent with this notion.

Our results also demonstrate that mutations affecting conserved motifs in the ABC subunit MalK lock the transporter in a similar state. The glutamate immediately following the aspartate in the Walker B site is highly conserved in ABC domains and was suggested to represent the catalytic carboxylate polarizing the water molecule that attacks the bond between the \(\beta\)- and \(\gamma\)-phosphate of ATP (43). Mutation to glutamine abolished or severely impaired ATPase activity of all ABC domains studied (44,45,58). In case of MJ0796 it was first demonstrated that the mutation stabilized ATP-induced dimer formation (44). Our result that the proteolytic profiles of MalFG of the complex containing MalKE159Q in plain buffer resembles that of the wild type complex in the vanadate-trapped state suggests that the mutation locks the transporter in a transition-like state. A similar conclusion was drawn by Urbatsch \textit{et al} (45) who studied the equivalent residues E552 and E1197 of mouse P-glycoprotein. Mutations to glutamine abolished drug-stimulated ATPase activity. However, drugs did stimulate vanadate trapping of 8-azido-ATP. Thus, the authors interpreted their results to mean that a step following hydrolysis, possibly the release of MgADP, were impaired in the mutant proteins.

The results obtained with transport complexes carrying different mutations in the conserved glutamine residue Q140 provide further evidence for a crucial role of the ABC signature motif in ATP-induced signaling during transport (32,56,57). Interestingly enough, the observed tryptic digestion profiles depended on the chemical nature of the substituting amino acid. A lysine residue at position 140 appears to lock the complex in the ground state, thus being unable to transmit ATP binding to the transmembrane subunits.
In contrast, replacement by leucine mimicks
the ATP-bound form of the transporter that
however, is unable to proceed to the next step
in the transport cycle.

ATP-driven dimer formation is thought
to represent the power stroke of ABC
transporters (6,45). The monomer-dimer
transition, that is the transition from the open,
nucleotide free to the closed ATP-bound form
would be coupled to conformational changes in
the transmembrane domains (8). Our results
present first evidence for such alterations in an
ABC import system. Signaling might involve
residues from the ABC signature (e.g. Q140)
(32, this study), the Q loop (8, 24,31) and the
EAA loops of the membrane-integral subunits
(11,24).

According to a model of maltose
transport proposed by Chen et al (8) substrate-
loaded maltose binding protein binds to the
nucleotide-free MalFGK2 complex at the
extracellular (periplasmic) side. ATP-binding
to the MalK subunits causes closing of the
MalK dimer, simultaneous opening of the
binding protein (29) and of a gate at the
periplasmic side, thereby giving the substrate
molecule access to binding sites in the
translocation pore. It is tempting to speculate
that the ATP-induced exposure of trypsin
cleavage sites in periplasmic loops P2 and P1
in MalF and MalG, respectively, is indicative
for such changes.

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FOOTNOTES

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Abbreviations used are: ABC, ATP-binding cassette; ACN, acetonitrile; DTT, dithiothreitol; NBD, nucleotide binding domain; TMD, transmembrane domain; DDM, β-D-dodecylmaltoside; MIANS, 2-(4′-maleimidoanilino)naphthalene-6-sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

FIGURE LEGENDS

Fig. 1. SDS-PAGE analysis of purified maltose transport complex variants used in this study. The transport complexes were purified as described in 'Materials and methods'. Lanes were loaded with 2 µg (1-7) and 10 µg (8) of protein, respectively. Lanes: 1, wild type; 2, MalFGKQ140L; 3, MalFGKQ140K; 4, BPI; 5, MalFGKE159Q; 6, MalFG(P78C)K(C40S); 7, MalF(S252C)GK(C40S); 8, wild type trapped with vanadate in the presence of MalE/ maltose and re-chromatographed on Ni-NTA (please note that under these conditions a stable complex with MalE is partially formed (26)).

Fig. 2. Trypsin digestion profiles of wild type MalFGK2 complex. Purified wild type complex was incubated in buffer D alone (lanes 1-4), and in buffer D supplemented with 15 mM ATP (lanes 5-8), 15 mM MgATP + 6 µM MalE + 0.1 mM maltose (lanes 9-12), or 15 mM MgATP + 6 µM MalE + 0.1 mM maltose + 1 mM vanadate (lanes 13-16) for 20 min at 37 °C, cooled to 25 °C, and subsequently treated with trypsin (31:1, w/w) as described in 'Materials and methods'. Samples were taken after 1, 4 and 8 min. The reactions were stopped by the addition of soybean trypsin inhibitor (5-fold excess over trypsin, w/w) followed by SDS-PAGE loading buffer. Controls (at time zero) were incubated with the indicated co-factors as above, a mixture of trypsin and soybean trypsin inhibitor, followed by loading buffer was added, and the samples were immediately shock-frozen in liquid nitrogen. The peptide fragments from each sample (1.5 µg/lane) were separated on three SDS gels (15 % acrylamide) to account for the MalF, MalG and MalK subunits, and subsequently transferred on nitrocellulose membranes. The full-length proteins and their fragments were detected by polyclonal antisera (see 'Materials and methods' for further details).

Fig. 3. Topological models of MalF (A) and MalG (B) of S. typhimurium indicating the localization of the proposed tryptic cleavage sites (arrow heads) and of the residues that were mutated to cysteines for fluorescence spectroscopy (encircled). The assignment of membrane-spanning helices is based on experimental topological analyses of E. coli MalF (59) and MalG (60).

Fig. 4. MIANS labeling of complex variants. Transport complexes were labeled with MIANS in detergent solution and analyzed by SDS-PAGE as described under 'Materials and methods'. Lanes: 1, MalFG(P78C)K(C40S)2; 2, MalF(S252C)GK(C40S)2; 3, wild type; 4, cys-less transport complex.

Fig. 5. Fluorescence spectra of MIANS-labeled complexes. Transport complexes were labeled with MIANS in detergent solution after incubation for 20 min at 37 °C in buffer D alone or in buffer D supplemented with MgATP, MalE, maltose and vanadate as described under 'Materials and methods'. Fluorescence spectra were recorded using an excitation wavelength of 340 nm and an emission range of 360-600 nm. Maxima were as follows: A, MalFG(P78C)K(C40S)-MIANS in buffer D (solid line): 427 nm; MalFG(P78C)K(C40S)-MIANS with vanadate (broken line): 423 nm; B, MalF(S252C)GK(C40S)-MIANS in buffer D alone (solid line): 430 nm; MalF(S252C)GK(C40S)-MIANS with vanadate (broken line): 424 nm.

Fig. 6. Trypsin digestion profiles of a binding protein-independent transport complex (MalF500GK2). Experimental conditions of trypsin treatment and analysis of fragments were as described in legend to Fig. 2.

Fig. 7. Trypsin digestion profiles of a complex containing MalKQ140K. Purified transport complex was incubated in buffer D alone or under vanadate-trapping conditions prior to treatment with trypsin. Samples were taken after 0 (controls), 1, 4 and 8 min. Experimental details were otherwise as described in legend to Fig. 2.
Fig. 8. Trypsin digestion profiles of a complex containing MalKQ140L. Purified transport complex was incubated in buffer D alone or under vanadate-trapping conditions prior to treatment with trypsin. Samples were taken after 0 (controls), 1, 4 and 8 min. Experimental details were otherwise as described in legend to Fig. 2.

Fig. 9. Trypsin digestion profiles of a complex containing MalKE159Q. Purified transport complex was incubated in buffer D alone or under vanadate-trapping conditions prior to treatment with trypsin. Samples were taken after 0 (controls), 1, 4 and 8 min. Experimental details were otherwise as described in legend to Fig. 2.

TABLE 1. Plasmids used in this study.

| Plasmid  | Relevant genotype/description                          | Source or reference |
|----------|--------------------------------------------------------|---------------------|
| pBB1     | pT5 malKht, pT5 malFmalG, amp′/(MalFG His6-MalK)       | (36)                |
| pVE1     | pT5 malK801ht, pT5 malFmalG, amp′/(MalFG His6-MalKQ140L) | This study          |
| pDE08    | pT5 malK848ht, pT5 malFmalG, amp′/(MalFG His6-MalKE159Q) | This study          |
| pAW3     | pT5 malF(Cys-/S403C/G338R/N505I)malG(Cys-) lacF′, amp′ | (24), This study    |
| pDE17    | pT5 malF(Cys-)malG(Cys-/P78C) lacF′, amp′              | This study          |
| pMM38    | pT5 malF(Cys-/S252C)malG(Cys-) lacF′, amp′             | This study          |
| pES62    | pT5 malFG cam′                                       | (32)                |
| pGS91-1  | pT5 malKht, amp′/(His6-MalK)                          | (32)                |
| pJS08    | pT5 malKht cam′/(His6-MalK)                           | This study          |
| pMM37    | pT5 malK796ht cam′/(His6-MalK40S)                     | This study          |
| pGS98-1  | pT5 malK809ht amp′/(His6-MalKQ140K)                   | (32)                |

TABLE 2. Functional characterisation of MalFGK2 complexes used in this study.

| Complex variant | ATPase activity a (µmol Pi/min/mg) | Inhibition by vanadate b (%) | Maltose uptake c (nmol maltose/min/mg) |
|-----------------|------------------------------------|-----------------------------|----------------------------------------|
|                 | - + MalE/maltose                   |                            |                                        |
| MalFGK (WT)     | 0.2 0.4                            | 86.7                        | 120                                    |
| MalF500GK (BPI) | 1.1 1.1                            | 95.0                        | nd                                     |
| MalFGK(Q140L)   | 0.01 0.02                          | nd                          | nd                                     |
| MalFGK(Q140K)   | 0.03 0.03                          | nd                          | nd                                     |
| MalFGK(E159Q)   | 0.01 0.015                         | nd                          | nd                                     |
| MalF(S252C)GK(C40S) | 0.33 0.57  | 53.5                        | 106                                    |
| MalFGK(P78C)K(C40S) | 0.32 0.52 | 60.0                        | 43                                     |

a Specific ATPase activities of purified transport complex variants in detergent solution (0.08 mg/ml) measured in the absence and presence of purified MalE (0.2 mg/ml) and maltose (10 µM); b measured as in (a) but preincubated with ortho-vanadate (1 mM) for 20 min at 37 °C; BPI was analyzed in the absence of MalE; c Initial rates of maltose uptake in proteoliposomes containing the indicated complex
variants. Values are average from 2-5 independent experiments. See Reference 39 and Materials and methods for details. *nd*, not determined.

**TABLE 3. Peptides determined by nanoLC-MS/MS**

| Peptide sequence | Residues in mature protein |
|------------------|-----------------------------|
| MDVIK            | 1-5                         |
| MDVIKK           | 1-6                         |
| HWQSDLQK        | 8-16                        |
| KTYAWR          | 61-66                       |
| TYAWR           | 62-66                       |
| AQQVLMDR        | 105-112                     |
| HYLSDAFSFGEQK   | 143-156                     |
| LQLK            | 157-160                     |
| LQLKETDALPGGER  | 157-170                     |
| ETDALPGGER      | 161-170                     |
| ETDALPGGERANLR  | 161-174                     |
| LITQNR          | 175-180                     |
| IITQNRALNQITAVLPDESK | 175-195   |
| LALNQITAVLPDESK | 181-195                     |
| LQLK            | 196-202                     |
| LQLKETDALPGGER  | 196-202                     |
| ETDALPGGER      | 228-250                     |
| YRPNNDSGYQSNADGSWGDEK | 228-250 |
| LSPGYTVTIGAK    | 251-262                     |
| AMVQPK          | 2-7                         |
| LALGFSVEADGR    | 61-73                       |
| MNDIPPAER       | 67-75                       |
| MNDIPPAER       | 67-75                       |
| TLVAEPR         | 147-153                     |
| TMIYVTHDQVEAMTLADK | 186-203   |
| IVVLADAGR       | 204-211                     |
| VAQVGKPLELYHYPADR | 212-228                 |
| VAQVGKPLELYHYPADR | 212-228                 |
| VAQVGKPLELYHYPADR | 212-228                 |
| FVAGFIGSPK      | 229-238                     |
| FVAGFIGSPK      | 229-238                     |
| MNFLPVK         | 239-245                     |
| VTATAIEQVQVELPNR | 246-261                |
| VTATAIEQVQVELPNR | 246-261                |
| QQIWLPVESR      | 262-271                     |
| QNLVYR          | 323-328                     |

see Materials and methods for details.
**FIGURE 7**

| MW (kDa) | 0 | 1 | 4 | 8 | 0 | 1 | 4 | 8 |
|----------|---|---|---|---|---|---|---|---|
| 45       |   |   |   |   | MalF |   |   |   |
| 31       |   |   |   |   | F1 | F2 |   |   |
| 21.5     |   |   |   |   | MalG |   |   |   |
| 14.4     |   |   |   |   | G1 |   |   |   |
| 6.5      |   |   |   |   |   |   |   |   |

**FIGURE 8**

| MW (kDa) | 0 | 1 | 4 | 8 | 0 | 1 | 4 | 8 |
|----------|---|---|---|---|---|---|---|---|
| 45       |   |   |   |   | MalF |   |   |   |
| 31       |   |   |   |   | F1 |   |   |   |
| 31       |   |   |   |   | MalG |   |   |   |
| 21.5     |   |   |   |   | G1 |   |   |   |
| 14.4     |   |   |   |   |   |   |   |   |
| 6.5      |   |   |   |   |   |   |   |   |
FIGURE 9

[Diagram of protein bands with molecular weight (kDa) and time points (min) for MalF, F1, MalG, G1, MalK, K1, K2, K3, K4 under conditions: MgATP, MalE, maltose, vanadate]
ATP induces conformational changes of periplasmic loop regions of the maltose ATP-binding cassette (ABC) transporter

Martin L. Daus, Heidi Landmesser, Andreas Schlosser, Peter Mueller, Andreas Herrmann and Erwin Schneider

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