Disulfide Cross-linking Reveals a Site of Stable Interaction between C-terminal Regulatory Domains of the Two MalK Subunits in the Maltose Transport Complex*

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Understanding the structure and function of the ATP-binding cassette (ABC) transporters is very important because defects in ABC transporters lie at the root of several serious diseases including cystic fibrosis. MalK, the ATP-binding cassette of the maltose transporter of Escherichia coli, is distinct from most other ABC-binding transporters in that it contains an additional C-terminal regulatory domain. The published structure of a MalK dimer is elongated with C-terminal domains at opposite poles (Diederichs, K., Diez, J., Greller, G., Muller, C., Breed, J., Schnell, C., Vonrhein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 5951–5961). Some uncertainty exists as to whether the orientation of MalK in the dimer structure is correct. Superpositioning of the N-terminal domains of MalK onto the ATP-binding domains of an alternate ABC dimer, in which ATP is bound along the dimer interface between Walker A and LSGGQ motifs, places both N- and C-terminal domains of MalK along the dimer interface. Consistent with this model, a cysteine substitution at position 313 in the C-terminal domain of an otherwise cysteine-free MalK triggered disulfide bond formation between two MalK subunits in an intact maltose transporter. Disulfide bond formation did not inhibit the function of the transporter, suggesting that the C-terminal domains of MalK remain in close proximity throughout the transport cycle. Enzyme IIA\textsuperscript{C} still inhibited the ATPase activity of the disulfide-linked transporter indicating that the mechanism of inducer exclusion was unaffected. These data support a model for ATP hydrolysis in which the C-terminal domains of MalK remain in contact whereas the N-terminal domains of MalK open and close to allow nucleotide binding and dissociation.

ATP-binding cassette (ABC) transport systems constitute the largest family of transporters known. These transporters utilize ATP to either import or export an extremely diverse array of substrates across cell membranes. They share a common structural organization consisting of two hydrophobic transmembrane-spanning domains or subunits and two nucleotide-binding domains or subunits. Forty-eight ABC transporters have been identified in the human genome, and defects in these transporters cause a variety of diseases including cystic fibrosis, macular dystrophy, and hyperinsulinemia (1).

The maltose transporter of Escherichia coli is well characterized and serves as a model system for study of the ABC transport mechanism. The transporter complex (MalFGK\textsubscript{2}) comprises four subunits; one copy each of MalF and MalG, the transmembrane subunits; and two copies of MalK, the ATP-binding subunit (2, 9). A periplasmic maltose-binding protein (MalE or MBP) is also required for transport, functioning as a high affinity receptor for maltose that also stimulates the ATPase activity of the transporter by binding tightly to and stabilizing the catalytic transition state conformation of the transporter (4). The crystal structures of several of the ATP-binding subunits have been determined (5–11) as well as those of two intact transporters (12, 13). The structures of the ATP-binding subunits are highly conserved and usually consist of two domains. One domain is predominantly \(\beta\)-sheet and forms a classic nucleotide-binding fold as observed in the F\textsubscript{1}-ATPase (14), and the second domain is \(\alpha\)-helical and is specific to ABC transporters. The MalK protein differs in that it contains a third domain at its C terminus that is involved in regulatory functions, as deduced from mutational analysis (15, 16). MalK binds MalT, a positive transcription factor that is required for expression of the genes involved in maltose transport and metabolism (17). Overexpression of MalK apparently sequesters MalT, preventing expression at the mal operons, whereas deletion of MalK leads to constitutive expression (18, 19). Evidence of a direct protein-protein interaction between isolated MalK and MalT has been presented (17). IIA\textsuperscript{C}, an enzyme participating in the transport and phosphorylation of glucose, also appears to bind directly to MalK, inhibiting maltose transport when glucose is present by a mechanism known as inducer exclusion (15, 20, 21).

Biochemical evidence of cooperativity in ATP hydrolysis in many systems suggests that the two ATP-binding subunits of ABC transporters interact (22–27). The first three structures of ABC proteins, those of transport proteins HisP from Salmonella typhimurium and MalK from Thermococcus litoralis, and of recombinase protein Rad50 from Pyrococcus furiosus (5–7) maltose-binding protein; DTT, dithiothreitol; CuPhen, Cu(1,10-phenanthroline)\textsubscript{2}SO\textsubscript{4}.

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The abbreviations used are: ABC, ATP-binding cassette; MBP, maltose-binding protein; DTT, dithiothreitol; CuPhen, Cu(1,10-phenanthroline)\textsubscript{2}SO\textsubscript{4}.
each reported a dimer of ATP-binding subunits. However, sites of interaction between the subunits differed in each crystal igniting a controversy over the true nature of the interaction between nucleotide-binding subunits. A Rad50-like dimer interface was again seen in the crystal of the ABC subunit LolD (MJ0796) but only when a mutation was introduced into the ATP-binding site that stabilized the dimeric conformation of this protein (11, 28). In both Rad50 and MJ0796 dimers, the family signature or LSGGQ motif of one subunit makes contact with ATP bound to the nucleotide-binding or Walker A motif of the opposing subunit generating a configuration with two ATPs buried along the dimer interface (6, 11). Independent biochemical evidence of a Rad50-like dimer interface in the intact MalFGK2 transporter was obtained in our laboratory from the pattern of photooxidative cleavage induced by vanadate trapped in the position of the γ-phosphate of ATP (29). MalK is cleaved by vanadate at both Walker A and LSGGGQQ, indicating that both motifs lie in close proximity to ATP. Because the LSGGGQQ motif is too far from the ATP-binding site in the monomer to be cleaved by vanadate, it must contact ATP across the dimer interface. A similar architecture is observed in the structure of the intact vitamin B12 transporter BtuCD (13) but not in the ABC subunit of the ABC transporter of the intact vitamin B12 transporter BtuCD (13) but not in the ABC subunit of the ABC transporter that expressed both MalF and MalK as judged by Western blotting were frozen at −70 °C in 8% glycerol and used to inoculate overnight cultures for larger scale preparations. Large scale preparations were grown in terrific broth (Invitrogen) containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 50 μg/ml spectinomycin until the A600 reached an OD of 6–10 μl isopyrrol-1-thio-β-d-galactopyranoside and grown an additional 24 h. Expression of some of the mutants was improved if the temperature was maintained at 23 °C both before and after induction and if less isopyrrol-1-thio-β-d-galactopyranoside (5 μl) was used in the induction. Cells were harvested and total membrane was isolated as described previously (30).

Expression of the MalFGK2 Complex—A plasmid carrying the desired malK gene was cotransformed with pMR81 into strain HN741 (32) containing plasmid pMS421 that carries the lacI gene. Transformants that expressed both MalF and MalK as judged by Western blotting were frozen at −70 °C in 8% glycerol and used to inoculate overnight cultures for larger scale preparations. Large scale preparations were grown in terrific broth (Invitrogen) containing 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 50 μg/ml spectinomycin until the A600 reached an OD of 6–10 μl isopyrrol-1-thio-β-d-galactopyranoside and grown an additional 24 h. Expression of some of the mutants was improved if the temperature was maintained at 23 °C both before and after induction and if less isopyrrol-1-thio-β-d-galactopyranoside (5 μl) was used in the induction. Cells were harvested and total membrane was isolated as described previously (30).

Protein Purification—Histidine-tagged transport complexes were purified by affinity chromatography as previously described (27) with some modification. Briefly, membranes were washed with 50 mM Tris-HCl and 1 mM EDTA (pH 8), resuspended at a protein concentration of 3 mg/ml, and solubilized in a solution containing 20 mg Tris-HCl (pH 8), 5 mM MgCl2, 10% glycerol, and 1% β-octyl-d-maltoside. Solubilized membranes were bound to 5 ml affinity resin (Mal) equilibrated with Buffer A (20 mM Tris-HCl (pH 8), 100 mM NaCl, 10% glycerol, and 0.01% dodecyl maltoside). After washing the column with Buffer A to remove unbound protein, bound protein was eluted with Buffer A containing 100 mM imidazole. Proteins were dialyzed against 20 mM Tris-HCl (pH 8), 10% glycerol, 5 mM MgCl2, and 0.01% dodecyl maltoside. Purified preparations were either stored frozen at −70 °C or reconstituted into E. coli phospholipid vesicles by detergent dilution (3) and then stored at −70 °C.

Assay of ATPase Activity—The ATPase activity of reconstituted transport complexes was measured at 57 °C in 20 mM HEPES (pH 8) with 2 mM [γ-32P]ATP, 10 mM MgCl2, 5 mM MnCl2, and 0.1 mM ATPase and 0.1 mU of ATPase as described previously (33). The activity is measured using standard procedures. MBP stimulates the ATPase activity of the reconstituted transporter. Disulfide Cross-linking by Cu(I,10-phenanthroline)SO3− (CuPhen)—Prior to treatment with CuPhen, purified proteins (5 μg) or membranes (1 mg/ml) were treated with 10 mM DTT. The reaction mixtures (100 μl) were spun through Sephadex G-50 spin columns to remove DTT, and MalFGK2 reconstituted with the intact pyridoxal phosphate domain for 20 min as described. Essentially the same results were obtained at 0 or 37 °C. The reaction was terminated by adding 5% SDS-PAGE sample loading buffer containing 5 mM N-ethylmaleimide and no reducing agent (34). The samples were then subjected to SDS-PAGE on 7.5% gels, transferred onto nitrocellulose paper, and probed with antibody against MalK. Oligonucleotides used in mutagenesis

| Oligonucleotide   | Sequence                  |
|------------------|---------------------------|
| MalF C85S       | 5'-CGGTCGCTGCAAAAGG-3'    |
| MalP C304S      | 5'-CCACGCTGCAAGACCC-3'    |
| MalF C388S      | 5'-CGAACCATCAGTAGGATC-3'  |
| MalK C106S      | 5'-GTCAGCAGTTCG-3'        |
| MalK C40G       | 5'-CTGCGGTCTCAT-3'        |
| MalK C352S      | 5'-CCAGTACCATCCATTT-3'    |
| MalK C362S      | 5'-GGCCATGACTGCTTCCAC-3'  |
| MalK S162C      | 5'-GTGACGAGTCTGACG-3'     |
| MalK L295C      | 5'-CTACACCGGGCTCATCATCT-3'|
| MalK G311C      | 5'-GTCGTTGCTGACG-3'       |
| MalK E313C      | 5'-GACCGAGTCTGACG-3'      |
| MalK D333C      | 5'-GCTGCTGCTGACG-3'       |
amplify the gene encoding IIA<sup>tec</sup> from a colony of <i>E. coli</i> K-12, and a sequence encoding an additional six histidines was added to the 3′-end of the gene. The DNA was subcloned into the pET21 vector (Novagen) and transformed into BL21(DE3) for expression. Approximately 20 mg of protein, affinity-purified on Talon resin, was obtained from 1 liter of cells.

Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (36) with the indicated percentage of acrylamide. Samples in SDS-loading buffer were not heated prior to loading.

Protein Determination—Protein concentrations were determined as described previously (3) by the method of Schaffner and Weissmann (37).

RESULTS

Structural Alignment of MalK and the MJ0796 Dimer—Based on recent data indicating that the MalK subunits in the MalFGK<sub>2</sub> transporter complex interact such that the LSGGGQ motif of one subunit interacts with nucleotide bound to the Walker A of the opposing subunit (29), we have superposed the two MalK monomers A and B from the structure of the <i>T. litoralis</i> dimer (7) onto the structure of the MJ0796 dimer (Fig. 1) (11). The MalK monomers are in a different conformation than the MJ0796 monomer, which appears to result from rotation of the helical domain containing the LSGGGQ relative to the nucleotide-binding domain containing the Walker A (7, 10). As a consequence, alignment of the nucleotide-binding domains of MalK and MJ0796 resulted in the displacement of the helical domain and the LSGGGQ motif away from the dimer interface in the MalK model (Fig. 1, compare A with E). In both MJ0796 and Rad50 dimers, serines from both the Walker A and the LSGGGQ motif form hydrogen bonds with the γ-phosphate of ATP (6, 11). In our MalK dimer model (Fig. 1), the Walker A motif is similarly placed within hydrogen-bonding distance of ATP, but the oxygen of the serine of LSGGGQ was either 8 (subunit B) or 12 Å (subunit A) from the corresponding oxygen in the γ-phosphate. It has been suggested that rotation of the helical domain and movement of the LSGGGQ motif into the nucleotide-binding site upon binding of ATP may be part of the mechanism of activation of ATP hydrolysis and transport (8, 13, 38). Hence, this open dimer model (Fig. 1A) may approximate a resting state of the MalK dimer in the MalFGK<sub>2</sub> transporter complex, and the structure of MJ0796 (Fig. 1E) may approximate an active state. In contrast to the crystallographic dimer of MalK from <i>T. litoralis</i> where the two C-terminal regulatory domains are at opposite poles of the dimer (Fig. 1F), our model, based on alignment with MJ0796, suggests that these domains may be in contact (Fig. 1B). Alignment of the MalK subunits onto the Rad50 (6) or BtuCD (13)
impairment of transport function, but these have been removed with minimal purification. Cross-linking experiments were performed on the intact, functional transporter—The MalF, MalG, and MalK proteins that constitute the maltose transporter complex from E. coli are 95% identical including the locations of the three cysteines; and finally, the cysteine at position 40 of our MalK is replaced by glycine rather than serine. The cysteine-free MalF and MalG proteins are from E. coli in both systems and contain cysteine to serine substitutions at all sites. We found that the cysteine-free versions of MalF and MalG co-purified with cysteine-free HisTagMalK on metal-affinity resin (Fig. 2), as seen for the wild-type proteins although the yield of purified protein (0.6 mg/liter of cells) was substantially reduced from that generally obtained with the wild type (6 mg/liter of cells). The yield of the various mutants was generally improved using lower temperatures for growth and lower isopropyl-1-thio-β-D-galactopyranoside concentrations (see “Experimental Procedures”) that improved the detergent solubility of the overexpressed proteins. The purified cysteine-free transporter complex when reconstituted into proteoliposomes exhibits MBP-stimulated ATPase activity, a good in vitro measure of function (32). The rates of ATP hydrolysis (0.7 – 2 μmol/min/mg in the presence of MBP and maltose, 0.03 in the absence) generally range from 50–100% of that seen in parallel experiments with the wild-type transporter. Addition of Single Cysteine Substitutions into MalK—Single cysteines were individually introduced into E. coli MalK at positions 162, 295, 311, 313, and 333, residues that are predicted to be close to each other across the dimer interface in our model (Fig. 1, C and D). We also studied a transporter that contained one native cysteine at position 40 within the Walker A motif of MalK because disulfide bond formation between these residues has been reported in the P-glycoprotein ABC transporter (39). The mutant MalK proteins were coexpressed with Cys-free versions of MalF and MalG. Purification of the fully Cys-free complex and complexes containing a single Cys at either position 40 or position 313 is shown in Fig. 2A. HisTagMalK typically migrates at 45 kDa on an 11% acrylamide gel just above the MalF protein, and MalG migrates at 27 kDa (27). When 2-mercaptoethanol is omitted from the SDS-PAGE running buffer and dodecyl maltoside is used as detergent, MalF migrates at 45 kDa, but MalG migrates at 27 kDa (27). When 2-mercaptoethanol is included and dodecyl maltoside is used, MalF and MalG both migrate at 34 kDa (27). When purified transporter is incubated with 50 μM CuPhen for 30 min at 37 °C and then passed over a desalting column to remove DTT and CuPhen, MalK migrates at 53 kDa (Fig. 3A) and MalG migrates at 27 kDa (Fig. 3B). The cross-linking reactions were stopped by treatment with gel-loading buffer containing 5 mM N-ethylmaleimide and no DTT. Samples were run on 7.5% SDS-PAGE gels and analyzed by Western blot analysis to visualize MalK. The position of prestained molecular mass markers (Bio-Rad) is indicated for the gel on the right. B, purified MalFGK2 containing the E313C substitution was treated with 10 mM DTT, desalted, and then incubated with 50 μM CuPhen for 30 min at 37 °C at decreasing protein concentrations in 0.01% dodecyl maltoside. Positions of MalK monomer and dimer are indicated.
Cross-linking between C-terminal Domains of MalK

**Fig. 4.** CuPhen-induced disulfide bond formation between MalK proteins containing the S162C substitution. Purified MalFGK₂ contains the S162C substitution. A, in lane 1, the untreated transporter complex was blotted with antibody to MalF to demonstrate copurification of this protein with HisTagMalK. Transporter complex was treated with either 10 mM DTT at 0 °C for 30 min (lane 2) or 50 μM CuPhen for 30 min at 37 °C (lane 3) prior to electrophoresis. Samples were run on a 7.5% SDS-PAGE gel and analyzed by Western blot analysis to visualize MalK. B, crude membrane fraction was treated with 3 mM CuPhen to promote cross-linking of MalK. MalK was visualized by Western blot analysis.

loading buffer as in Fig. 2A, a higher molecular mass band migrating near 100 kDa is often seen in the purified sample. This band appears to be a dimer of MalK stabilized in SDS through disulfide bond formation, because it reacts with antibody to MalK but not MalF or MalG and is less apparent following treatment with fresh 2-mercaptoethanol or DTT (Fig. 2B). This dimer is absent in the Cys-free transporter complex. Treatment with CuPhen to promote disulfide bond formation triggers reformation of the dimer (and higher order) bands in the sample containing Cys-40 but not in the Cys-free construct. The smearing of MalK at a higher Mr on the gel, most commonly seen following treatment with CuPhen, disappears if the sample is treated with DTT prior to SDS-PAGE and may result from disulfide bond formation with contaminants in the preparation or from the formation of aggregates that are not dissociated by SDS in the absence of DTT. Cys-free MalK containing the E313C substitution is notable in that MalK is depleted in the position of the monomer relative to the amount of MalF, and MalK dimer is the prominent species on the gel (Fig. 2A). In contrast, disulfide bond formation between MalK proteins containing Cys-40 is inefficient. Quantitation of gels similar to those shown in Fig. 2, A and B, indicated that only ~10% of Cys-40 MalK migrates as a dimer, even following CuPhen treatment.

**Disulfide Cross-linking of the C-terminal Domains of MalK**—Purified transporter complexes containing single cysteines at positions 295, 311, 313, and 333 in the C-terminal domain were treated with DTT to reduce any disulfide bonds that may have formed by air oxidation during isolation and purification, desalted, and incubated with CuPhen to promote disulfide bond formation (Fig. 3A). The position of migration of MalK was shifted either partially or completely from monomer to dimer with a cysteine at position 295, 313, or 333, consistent with formation of a disulfide bond between two MalK proteins upon CuPhen treatment. Addition of DTT following CuPhen treatment brings MalK back to the position of a monomer, as anticipated for a disulfide bond (data not shown). Transporter containing the E313C substitution was selected for further experimentation because it was well expressed and cross-linking appeared to go to completion.

Our model for a MalK dimer (Fig. 1B) suggests that the 313 linkage forms between the two MalK subunits in the same transporter complex (intratransporter cross-linking); however, the formal possibility exists that cross-linking occurs between two different transporters (intertransporter cross-linking). Based on the assumption that the efficiency of inter-transporter cross-linking would be an inverse function of protein concentration, CuPhen-induced disulfide bond formation was assessed as a function of protein concentration. No decrease in the efficiency of disulfide bond formation was seen when the concentration of MalFGK₂ in detergent solution was reduced from 1.2 μM down to 0.036 μM suggesting that the cross-linking is occurring within the MalFGK₂ complex rather than between transporter complexes (Fig. 3B). In fact, a 2–4-fold reduction in protein concentration actually increased the efficiency of disulfide bond formation in this experiment suggesting that 50 μM CuPhen concentration might be limiting at high protein concentration. Migration of fully cross-linked, or uncross-linked MalFGK₂ transporter complexes through a G3000SWXL high pressure liquid chromatography gel filtration column (Tosoh Biosciences) in 0.01% dodecyl maltoside was also compared and no difference in the time of elution was detected, as might be expected if disulfide bond formation had generated a stable transporter dimer (MalFGK₂₂). Whereas this column is able to separate soluble proteins in the range of 10,000–500,000 Da, it should be noted that the ability of gel filtration to resolve membrane proteins of similar size in detergent micelles has not been carefully studied.

**Dissulfide Cross-linking of Residues in the N-terminal Domain of MalK**—In our model of a MalK dimer only 6.5 Å separates the α-carbons of the serines at positions 162 in the N-terminal nucleotide-binding domain; hence cysteines at this position may form a disulfide bond. In the structure of the Rad50 dimer the corresponding position is the point of closest contact between identical residues in opposing subunits (6). Placement of a cysteine at this position resulted in efficient formation of a MalK dimer as judged by Western blot analysis (Fig. 4A, lanes 2 and 3); however, purified transporter complex was obtained in very low yield despite the production of large amounts of MalK protein in the membrane pellet (Fig. 4B). A positive reaction of anti-MalF antibody with the purified sample (Fig. 4A, lane 1) was used to verify that MalF copurified with the mutant HisTagMalK, as expected for an intact transporter. This MalK dimer formed spontaneously during purification and, like the E313C mutant, could be reduced with DTT and reoxidized, essentially to completion, with CuPhen treatment (data not shown). No further characterization of this preparation was performed because of the poor yield of assembled complex.

**Influence of Disulfide Bond Formation on ATPase and Inducer Exclusion Activities**—Because disulfide bond formation went essentially to completion with the E313C substitution, the effect of the presence of a disulfide bond on transporter function could be assessed. As in the wild-type MalFGK₂ transporter, little or no ATPase activity was detected in the oxidized transporter following reconstitution when MBP was absent (data not shown). MBP plus maltose efficiently stimulated the ATP activity of the oxidized transporter, and DTT enhanced this activity by only 30% (Table II). Similar rates of ATP

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**Table II**

| Mutant MalFGK₂ | MBP-stimulated ATPase activity* |
|----------------|---------------------------------|
|                | Oxidized (+ CuPhen) | Reduced (+ DTT) |
| E313C (100% oxidized) | 1540 ± 570 | 2050 ± 600 |
| Cys-40 (~10% oxidized) | 1920 ± 400 | 3100 ± 500 |
| Cys-free         | 1830              | 1950              |

* Measurements in duplicate or triplicate (where mean ± S.D. is given).
hydrolysis were seen in the cysteine-free transporter and the transporter containing the Cys-40 substitution where only about 10% of total MalK migrated as a dimer on SDS-PAGE.

In inducer exclusion, IIAglc of the glucose phosphotransferase system inhibits maltose transport when glucose is present in the medium. Because several mutations that prevent inducer exclusion are located in the C-terminal domain of MalK (15, 20) it was of interest to determine whether tethering the C-terminal domains together would interfere with this regulatory activity. In Fig. 5, the pattern of inhibition of MBP-stimulated ATPase activity by IIAglc was very similar in the E313C and cysteine-free constructs whether the disulfide bond between MalK subunits was present or absent (Fig. 5, inset). The concentration of IIAglc yielding 50% maximal inhibition of wild-type MalFGK2 was ~1.5 μM, comparable with the data obtained in the cysteine-free background (Fig. 5).

DISCUSSION

Repositioning of the N-terminal domains of the two MalK subunits from the T. litoralis dimer to conform to the dimeric structure of MJ0796 and Rad50, where ATP is bound along the dimer interface between the Walker A and LSGGQ motifs (6, 11), creates a model for MalK in which the C-terminal regulatory domains lie in close proximity. Justification for this realignment came initially from the observation that vanadate trapped in the nucleotide-binding site of MalK mediated oxidative photocleavage of both the Walker A and the LSGGQ motifs (29). Our demonstration of efficient disulfide bond formation between cysteines placed along the proposed dimer interface in the C-terminal domain of MalK provides additional support for this arrangement of subunits, as opposed to that seen in the crystal of MalK from T. litoralis. In the structure of the T. litoralis dimer (7) the C-terminal domains are well separated (Fig. 1F). The MBP-stimulated ATPase activity of the transporter was only modestly affected, if at all, by the presence of the disulfide bond between cysteines at position 313, effectively ruling out the possibility that the dimer conformation seen in the structure of the T. litoralis protein represents some type of conformational intermediate in the translocation cycle. This possibility also seems unlikely based on the location of residues predicted to interact with the transmembrane region from the structure of BtuCD (13), which are partially obscured by the dimer interface in the published MalK structure (7).

In previous studies, disulfide bond formation was reported between MalK proteins with single cysteines placed at either position 85 or position 106 in the N-terminal domain of MalK from Salmonella (34). The cross-link at position 85, cited in support of the T. litoralis dimer (7), is also consistent with the model for dimerization depicted in Fig. 1A. Although the α-carbon atoms of these residues are 32 Å apart in our MalK dimer

FIG. 5. Inhibition of ATPase activity by IIAglc. MalFGK2 containing the E313C substitution (circles) or no cysteines (squares) was treated with 200 μM CuPhen for 30 min at 37 °C and then reconstituted into proteoliposomes. ATPase assays were performed in the presence (open symbols) or absence (closed symbols) of 10 mM DTT to reduce the disulfide bond. The indicated concentration of IIAglc was present in the assay. Values are means of duplicate determinations. Inset, Western blot of samples assayed with positions of MalK monomer and dimer indicated. Lane 1, E313C + DTT; lane 2, E313C − DTT; lane 3, Cys-free + DTT; lane 4, Cys-free − DTT.

FIG. 6. Location of mutations that inhibit the regulatory functions of MalK. Model of MalK dimer from Fig. 1 with location of mutations highlighted in magenta (16, 21). A, mutations that prevent inducer exclusion (119, 124, 228, 241, 278, 284, 302, and 322 in E. coli MalK) (viewed from side). Domains belonging to MalK subunit A are colored in darker hues, as in Fig. 1. B, mutations that prevent MalK from inhibiting the function of MalT, the transcriptional activator of the mal operon (residues 72, 248, 250, 262, 267, 268, 289, 291, 297, 346, and 350 in E. coli MalK) (viewed from bottom).
Cross-linking between C-terminal Domains of MalK

Mutations affecting the regulatory interaction of MalK with the transcriptional activator MalT appear to define a binding site for MalT toward the bottom of the C-terminal regulatory domain (Fig. 6B). The ability of MalK to modulate MalT function appears to be affected by the conformation of MalK within the transporter complex because an inactive form of MalK down-modulates MalT and decreases transcription, whereas an active form of MalK does not (17). Once an in vitro assay for this regulatory function has been developed, it will be possible to determine whether MalT function is influenced in any way by cross-linking of C-terminal regulatory domains.

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REFERENCES

1. Dean, M., Hamon, Y., and Chimini, G. (2001) J. Lipid Res. 42, 1007–1017
2. Boos, W., and Lucht, J. M. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., Curtiss, R. III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) Vol. 1, 2nd ed., pp. 1175–1209, ASM Press, Washington, D.C.
3. Davidson, A. L., and Nikaido, H. (1991) J. Biol. Chem. 266, 8946–8951
4. Chen, J., Sharma, S., Quiocho, F. A., and Davidson, A. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15290–15293
5. Kung, L. H., Wang, I. X., Nikaido, K., Liu, P. Q., Ames, G. F., and Kim, S. H. (1998) Nature 396, 703–707
6. Hamburger, P., Karcher, A., Kuzin, D. S.,Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Cell 101, 789–800
7. Diederichs, K., Diez, J., Grellier, G., Muller, C., Breed, J., Schnell, C., Vonrhein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 3081–3091
8. Yuan, Y. R., Bleeker, S., Martinekevich, O., Millen, L., Thomas, P. L., and Hunt, J. F. (2001) J. Biol. Chem. 276, 32313–32321
9. Karpowich, N., Martinekevich, O., Millen, L., Yuan, Y., Dai, P. L., MacVey, K., Thomas, P. L., and Hunt, J. F. (2001) Structure 9, 571–586
10. Gaultier, R., and Diederichs, K. (2001) EMBO J. 20, 4964–4972
11. Smith, F. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. L., and Hunt, J. F. (2002) Mol. Cell. 10, 139–149
12. Chang, G., and Roth, C. B. (2001) Science 293, 1783–1800
13. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) Science 296, 1091–1098
14. Abrams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
15. Kuhnau, S., Reyes, M., Sievertsen, A., Shuman, H. A., and Boos, W. (1991) J. Bacteriol. 173, 2180–2186
16. Bohn, A., Diez, J., Diederichs, K., Welte, W., and Boos, W. (2002) J. Biol. Chem. 277, 3708–3717
17. Panagioudis, C. H., Boos, W., and Shuman, H. A. (1998) Mol. Microbiol. 30, 525–546
18. Reyes, M., and Shuman, H. A. (1988) J. Bacteriol. 170, 4598–4602
19. Buka, B., Ehrmann, M., and Boos, W. (1986) J. Bacteriol. 166, 884–891
20. Davidson, A. L., Reizer, J., Nikaido, H., and Saier, M. H. J. (1990) J. Biol. Chem. 265, 21005–21010
21. Stein, A., Seifert, M., Volkmann-Engert, R., Siepelmyer, J., Jahreis, K., and Schneider, C. E. (2002) Eur. J. Biochem. 269, 4074–4085
22. Azzaria, M., Schurt, E., and Gros, P. (1989) Mol. Cell. Biol. 9, 5289–5297
23. Berkower, C., and Michaelis, S. (1991) EMBO J. 10, 3777–3785
24. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 22957–22961
25. Senior, A. E., Al-Shawi, M. K., and Urbatsch, I. L. (1995) FEBS Lett. 377, 285–289
26. Davidson, A. L., Lagaieria, S. S., and Mannering, D. E. (1996) J. Biol. Chem. 271, 4555–4563
27. Davidson, A. L., and Sharma, S. (1997) J. Biol. Chem. 179, 5458–5464
28. Moody, J. E., Millen, L., Binns, D., Hunt, J. F., and Thomas, P. J. (2002) J. Biol. Chem. 277, 2111–2114
29. Fetsch, P. E., and Davidson, A. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9685–9690
30. Diederichs, K., Diez, J., Shuman, S. S., and Davidson, A. L. (2001) J. Biol. Chem. 376, 12362–12368
31. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1257–1261
32. Hanzlik, S., Mourez, M., Jehanno, M., Dassa, E., and Schneider, E. (2000) J. Biol. Chem. 275, 15526–15534
33. Davidson, A. L., and Nikaido, H. (1990) J. Biol. Chem. 265, 4254–4260
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. Schaffner, W., and Weissmann, C. (1973) Eur. J. Biochem. 44, 502–514
36. Davidson, A. L. (2002) J. Bacteriol. 184, 1225–1233
37. Urbanek, I. L., Gros, P., Wilke-Mounts, S., Lerner-Marmarosh, N., Rousseau, M. E., Gros, P., and Senior, A. E. (2001) J. Biol. Chem. 276, 26880–26887
38. Kerr, I. D. (2002) Biochim. Biophys. Acta 1561, 47–64