A Cys-less variant of the bacterial ATP binding cassette protein MalK is functional in maltose transport and regulation

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Abstract The cysteine residues of the ABC protein MalK from *Salmonella typhimurium* maltose transport system (C40, C350, C360) were consecutively replaced by serines. Cys-less MalK was fully functional in maltose transport in vivo. Moreover, the activity of MalK as a repressor of other maltose-regulated genes was also retained. The absence of cysteine residues in the purified protein was verified by its failure to react with fluorescein-5-maleimide. In contrast to purified wild-type MalK, the ATPase activity of the C40S variant was insensitive to inhibition by *N*-ethylmaleimide.

Key words: ABC transporter; ATP binding cassette; Binding protein-dependent transport system; MalK; Maltose regulon; *N*-ethylmaleimide

1. Introduction

In enterobacteria, such as *Escherichia coli* and *Salmonella typhimurium*, the uptake of maltose and maltodextrins is accomplished by a binding protein-dependent transport system that is a member of the ATP binding cassette (ABC) superfamily of proteins [1]. The membrane-associated complex is composed of one copy each of the hydrophobic subunits MalF and MalG, that presumably form a translocation pore [2], and of two copies of the ATP-hydrolyzing subunit (also referred to as ABC protein) MalK, that is thought to provide the energy for the transport process [3]. MalK is characterized by a typical set of Walker A and B motifs that are suggested to constitute a nucleotide binding fold [4]. In addition, and unique to this subclass of ABC transporters, an extracellular (periplasmic) substrate binding protein (MalE) is also required for activity. It is proposed that the presence of substrate is signalled by maltose-loaded binding protein via interaction with externally exposed peptide loops of MalF/MalG. As a consequence, conformational changes of the latter are transmitted to the MalK subunits which, in turn, become activated. Hydrolysis of ATP would then trigger subsequent conformational changes that eventually lead to the translocation of the substrate molecule [5]. Besides being indispensable for the transport process, MalK exhibits additional regulatory functions. The protein is the target for unphosphorylated enzyme IIA^{Glc} of the phosphoenolpyruvate phosphotransferase system in the process of inducer exclusion [6] and also acts as a repressor of genes belonging to the maltose regulon [7,8]. The latter activity has been largely attributed to the carboxy-terminal domain of MalK [8–11] (Fig. 1). Although the molecular basis of the repressing function is currently not understood, evidence in support of a direct interference with the positive regulator of the maltose system, MalT, has been presented [7]. Thus, the various functions of MalK in transport and regulation require specific protein-protein interactions the chemical nature of which are only beginning to emerge. Analysis of suppressor mutations suggested that in the assembled $MalFGK_2$ complex MalK might interact with MalF/MalG via a peptide fragment connecting the Walker A and B motifs ('helical domain') [12,13]. Moreover, the analysis by Mourez et al. [13] also revealed that conserved cytoplasmic peptide loops of MalF and MalG (referred to as 'EAA loop') might be in contact with the helical domain. Most recently, reconstitution experiments provided first biochemical evidence supporting the involvement of the helical domain in subunit-subunit interactions in the complex [14]. In order to identify individual amino acid residues from both the helical domain and the EAA loops of MalF/MalG that might be in close physical contact to each other, we intend to use site-directed chemical crosslinking. In particular, introduction of cysteine residues and subsequent analysis of disulfide bond formation has proven to be a powerful tool in elucidating neighboring residues in proteins in the absence of a crystal structure [15–17]. The construction of a Cys-less variant of MalK that has retained its functions in transport and regulation is a prerequisite for such a study. MalK contains three native cysteine residues at positions 40, 350, and 360, respectively (Fig. 1). C40 is located within the Walker A motif and, as revealed by database searches, is almost invariant in the subfamily of MalK-related ABC proteins (present in 23 out of 24 sequences). In other ABC proteins a cysteine residue is also frequently found at that position. C350 and C360 are both located in the carboxy-terminal extension of MalK that is unique to members of the MalK subfamily (see also [12]). Although the degree of sequence identity between the proteins is significantly less pronounced in this region, C350 is found within a conserved short sequence motif (consensus: R<u>C</u>HLFD) of yet unknown function. In contrast, no cysteine residue corresponding to C360 is present in other MalK-like proteins listed in the database to date. Here, we report on the functional consequences of consecutively replacing the native cysteine residues of MalK by serines. Our results clearly demonstrate that the Cys-less variant is fully active in transport and also has retained the repressing activity. Moreover, we show that the previously observed inhibition of the MalK ATPase activity by *N*-ethylmaleimide [18] is due to modification of C40.

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Abbreviations: NEM, *N*-ethylmaleimide; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Bacterial strains

E. coli strain JM109 (Stratagene) was used for general cloning purposes. Complementation and transport studies were performed using *S. typhimurium* strain ES25 (*dhuA1 ΔhisF645 malK786 galE503 recA56*) that lacks a functional *malK* gene as a host [9]. The regulatory activity of MalK variants was analyzed in *E. coli* strain SK1280 (MC4100 Θ*[malK::lacZ] hyb1113 recA*) [8] as described in [11]. BL21DE3(pLysS) (Stratagene) was used as a host for overexpression of *malK* alleles under the control of the T7 promoter.

2.2. Plasmids

Plasmid pSW7 carries the *malK* wild-type allele on pSE380 downstream of the trc promoter [19]. Derivatives of pSE380 carrying the mutant alleles *malK796* (C40S), *malK797* (C40S/C350S), *malK798* (C40S/C350S/C360S), *malK812* (C360S), and *malK826* (C350S) were designated pSH25, pSH27, pSH39, pSH33, and pCB1, respectively. Plasmids pES67 and pSH31 carry the *malK* wild-type allele and the *malK796* allele, respectively, on expression vector pRSET5d downstream of the T7 promoter [20]. Plasmids pGS91-1 (Schmees and Schneider, unpublished) and pSH53 carry the *malK* wild-type allele and the *malK798* allele, respectively, on the his-tag fusion vector pQE9 (Qiagen).

2.3. DNA techniques

Site-directed mutagenesis using Stratagene's QuikChange protocol or a PCR-based method (for pCB1 only) [21] was done with pSW7 as template vector. Oligonucleotide primers were purchased from MWG-Biotech (Freiburg). Introduced base changes were confirmed by nucleotide sequence analysis.

2.4. Biochemical methods

The uptake of [^{14}C]maltose (3.7 MBq ml^{-1}, ICN) in cells of strain ES25 harboring the described plasmids, and SDS-PAGE were performed as described in [9]. The MalKC40S variant was purified from the overproducing strain BL21DE3(pSH31, pLysS) according to [20]. The Cys-less variant of MalK was prepared as urea-soluble fraction from the low speed pellet of strain JM109(pSH53) after cell disrupture, as described in [22]. Wild-type protein was prepared by both procedures using the overproducing strains BL21DE3(pES67, pLysS) and JM109(pGS91-1), respectively. ATP hydrolysis was assayed by monitoring the release of inorganic phosphate from [γ-^{32}P]ATP (424 MBq ml^{-1}, Amersham) as described previously [22].

3. Results and discussion

The native cysteine residues of MalK (C40, C350, C360) were consecutively replaced by serine residues using site-directed mutagenesis. The resulting plasmids carrying the mutant *malK* alleles *malK796* (C40S), *malK826* (C350S), *malK812* (C360S), *malK797* (C40S/C350S), or *malK798* (C40S/C350S/C360S) were subsequently analyzed for their capability to restore maltose transport activity in *S. typhimurium* strain ES25 that lacks a functional *malK* gene [9]. As shown in Fig. 2, all transformants displayed initial rates of [^{14}C]maltose uptake comparable to wild-type. Thus, none of the native cysteine residues, including the highly conserved C40, is essential for transport. This result confirms the recent finding that a C40G mutation did not affect transport activity of the *E. coli* MalK protein either [23].

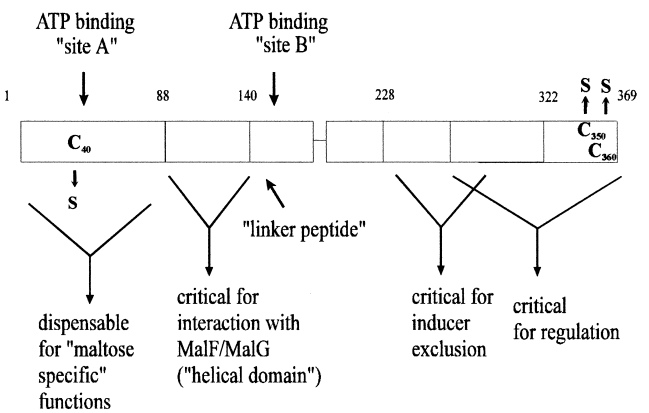

Fig. 1. Linear representation of MalK, with the assignment of putative functional domains (modified from [12]). The relative positions of the nucleotide binding motifs A and B, and of the native cysteine residues that were replaced by serines in this study are indicated. For further details, see text and references [11,12].

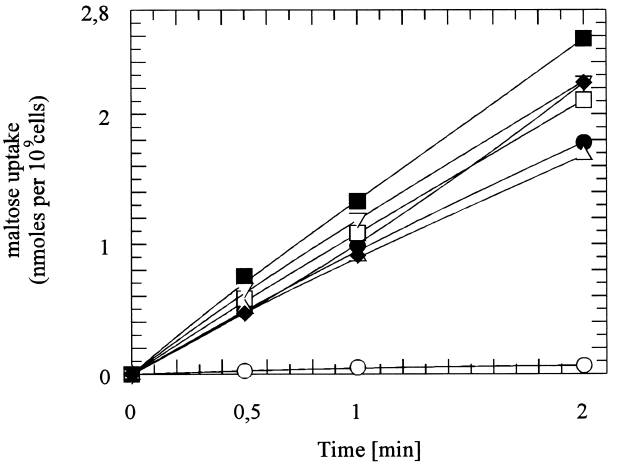

Fig. 2. Effect of cysteine to serine mutations on maltose transport. Cells of *S. typhimurium* strain ES25 (*malK*) harboring the described plasmids were grown in nutrient broth, supplemented with 0.5% maltose, to $A_{650} = 1$, washed twice in minimal salts, and immediately analyzed for maltose uptake as described in [9]. ●, MalK wild-type (pSW7); □, C40S (pSH25); ■, C350S (pCB1); ▽, C360S (pSH33); △, C40S/C350S (pSH27); ◆, C40S/C350S/C360S (pSH30); ○, (plasmid vector pSE380). Means of three experiments are shown.

Linker mutagenesis of the *E. coli malK* gene has recently revealed that insertion of a hydrophilic peptide after positions 346 or 364 abolished the repressing activity of MalK while an insertion after position 361 did not [10]. However, as the authors pointed out, the effect of the insertion after residue 364 might be caused by a structural alteration. Consequently, we have analyzed the implications of Cys to Ser mutations at positions 350 and 360 on this activity. To this end, plasmids encoding the MalK variants were introduced into *E. coli* strain SK1280. This strain carries a chromosomal *malK-lacZ* fusion that results in a maltose-negative phenotype but places the *lacZ* gene under the control of the p_{malK} promoter [8]. Thus, the repressing activities of plasmid-encoded MalK variants are conveniently monitored by assaying the β-galactosidase activity of the cells. In the presence of the vector plasmid (pSE380), full enzymatic activity was obtained, while a plasmid carrying the *malK* wild-type allele (pSW7) caused repression of the fusion gene (Fig. 3). As demonstrated in Fig. 3, the β-galactosidase activities measured with any of the variants compared favorably to that observed with wild-type. These results clearly indicate that both C-terminally located cysteine residues are dispensable for the regulatory function of MalK. Whether other residues of the short sequence motif encompassing C350 (RCHLFD, see Section 1) are essential for this activity remains to be elucidated.

To determine that the MalK variant encoded by the

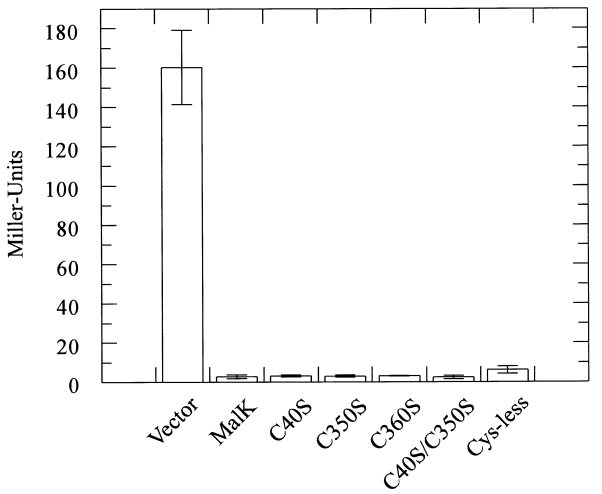

Fig. 3. Effect of serine for cysteine substitutions on the regulatory activity of MalK. Cells of *E. coli* strain SK1280, harboring the described plasmids were grown in Luria broth to $A_{600} = 0.4$, and subsequently analyzed for β-galactosidase activity as described in [11]. The data represent the means of two independent experiments. Standard deviations are indicated by error bars.

malK798 allele was indeed devoid of cysteine residues, labelling experiments were carried out with the cysteine specific reagent fluorescein-5-maleimide to directly monitor modification of the protein. Wild-type and 'Cys-less' MalK prepared from the low speed pellet fractions of the overproducing strains JM109(pGS91-1) and JM109(pSH53), respectively, by solubilization with 8 M urea were incubated with fluorescein-5-maleimide and subjected to SDS-PAGE. As shown in Fig. 4, visualization of the gel under UV light revealed modification of the wild-type protein that could be prevented by preincubation with *N*-ethylmaleimide (NEM). In contrast, the reagent failed to modify the Cys-less variant, thereby strongly indicating that the protein was lacking any cysteine residue.

We have recently shown that the purified MalK protein exhibits a spontaneous ATPase activity that is inhibited by NEM [18]. Since inhibition was blocked by preincubation with ATP, Cys-40, that is located within the nucleotide binding motif A (GPSGCGKST; see also Fig. 1) was proposed to be the likely candidate for modification. This view was supported by the finding that modification of the corresponding cysteine residues in either of the two nucleotide binding domains inhibited the ATPase activity of P-glycoprotein, a mammalian member of the ABC transport family involved in multi-drug resistance [24]. The ATPase activity of HisP, another bacterial homolog of MalK, was also reported to be sensitive to NEM [25]. Interestingly however, the protein is lacking a cysteine residue within the Walker A motif. The authors speculated on a carboxy-terminally located cysteine residue (C51) as the possible target, although this residue is not involved in the interaction with ATP as revealed from the recently determined crystal structure of HisP [26]. Thus, in order to clarify the role of Cys-40 in the inhibition of MalK ATPase activity by NEM, the MalKC40S variant was purified from strain BL21DE3(pLysS, pSH31) by a published procedure [20], and analyzed for its capability to hydrolyze ATP in the presence and absence of NEM. As demonstrated in Fig. 5 and confirming recent results, 20 μM NEM caused substantial inhibition (>80%) of the catalytic activity of the wild-type protein. In contrast, under the same conditions the ATPase activity of the MalKC40S variant was basically insensitive to the inhibitor. These results are fully consistent with the notion that the observed loss of enzymatic activity in the presence of NEM is caused by covalent modification of C40. Moreover, while the cysteine residue as such is dispensable for function, the introduction of a bulky group probably prevents ATP from getting proper access to the nucleotide binding fold and thus, is not tolerated. This view is supported by the crystal structure of HisP which demonstrated the formation of a hydrogen bond between the main-chain nitrogen of S43 (corresponding to C40 in MalK) and the β-phosphate of ATP [26].

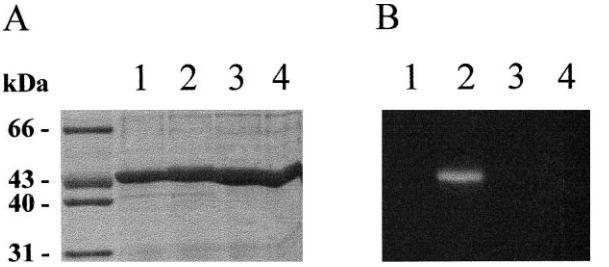

Fig. 4. Modification of MalK variants with fluorescein-5-maleimide. Urea-solubilized native and Cys-less MalK (6 μg each) were incubated with fluorescein-5-maleimide (Pierce) (0.5 mM) for 10 min on ice and subsequently subjected to SDS-PAGE. Labelling was visualized under UV light (A) prior to protein staining with Coomassie brilliant blue (B). Preincubation with NEM (0.5 mM) was performed for 1 h on ice. Lanes: 1, wild-type (+NEM); 2, wild-type (−NEM); 3, Cys-less (+NEM); 4, Cys-less (−NEM).

Taken together, we have shown that the native cysteine residues of MalK can be replaced by serines without affecting functions. The Cys-less variant can thus be subjected to site-directed chemical modification in order to elucidate subunit-subunit interactions in the transport complex. Such studies are in progress in this laboratory.

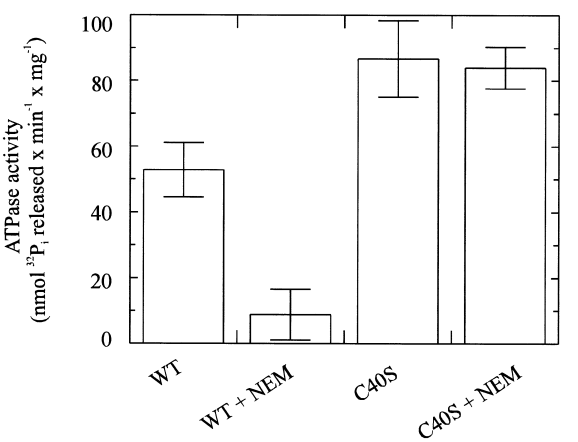

Fig. 5. Effect of *N*-ethylmaleimide on ATPase activity of purified MalK variants. Native MalK (WT) and MalKC40S (10 μg each) were incubated with 20 μM NEM in assay buffer for 15 min at room temperature and subsequently analyzed for ATPase activity by monitoring the release of phosphate from [γ-^{32}P]ATP as described in [22]. The data represent the means of two independent experiments. Standard deviations are indicated by error bars. Note that the somewhat lower ATPase activity of the wild-type was a property of the particular preparation used. The C40S mutation did not cause an increase in enzymatic activity.

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References

[1] Boos, W. and Lucht, J.M. (1996) in: F.C. Neidhardt et al. (Eds.), Escherichia coli and Salmonella. Cellular and Molecular Biology, American Society for Microbiology, Washington, D.C., pp. 1175–1209.
[2] Ehrmann, M., Ehrle, R., Hofmann, E., Boos, W. and Schlösser, A. (1998) Mol. Microbiol. 29, 685–694.
[3] Schneider, E. and Hunke, S. (1998) FEMS Microbiol. Rev. 20, 1–20.
[4] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–961.
[5] Davidson, A.L., Shuman, H.A. and Nikaido, H. (1992) Proc. Natl. Acad. Sci. USA 89, 2360–2364.
[6] Dean, D.A., Reizer, J., Nikaido, H. and Saier Jr., M.H. (1990) J. Biol. Chem. 265, 21005–21010.
[7] Panagiotidis, C.H., Boos, W. and Shuman, H.A. (1998) Mol. Microbiol. 30, 535–546.
[8] Kühnau, S., Reyes, M., Sievertsen, A., Shuman, H.A. and Boos, W. (1991) J. Bacteriol. 173, 2180–2186.
[9] Schneider, E. and Walter, C. (1991) Mol. Microbiol. 5, 1375–1383.
[10] Lippincott, J. and Traxler, B. (1997) J. Bacteriol. 179, 1337–1343.
[11] Schmees, G. and Schneider, E. (1998) J. Bacteriol. 180, 5299–5305.
[12] Wilken, S., Schmees, G. and Schneider, E. (1997) Mol. Microbiol. 22, 555–666.
[13] Mourez, M., Hofnung, M. and Dassa, E. (1996) EMBO J. 16, 3066–3077.
[14] Mourez, M., Jéhano, M., Schneider, E. and Dassa, E. (1998) Mol. Microbiol. 30, 353–363.
[15] Wu, J. and Kaback, H.R. (1997) J. Mol. Biol. 270, 285–293.
[16] Chen, X. and Koshland Jr., D.E. (1997) Biochemistry 36, 11858–11864.
[17] Jiang, W. and Fillingame, R.H. (1998) Proc. Natl. Acad. Sci. USA 95, 6607–6612.
[18] Morbach, S., Tebbe, S. and Schneider, E. (1993) J. Biol. Chem. 268, 18617–18621.
[19] Walter, C., Wilken, S. and Schneider, E. (1992) FEBS Lett. 303, 42–44.
[20] Schneider, E., Linde, M. and Tebbe, S. (1995) Protein Expr. Purif. 6, 10–14.
[21] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 51–59.
[22] Walter, C., Höner zu Bentrup, K. and Schneider, E. (1992) J. Biol. Chem. 267, 8863–8869.
[23] Panagiotidis, C.H., Reyes, M., Sievertsen, A., Boos, W. and Shuman, H.A. (1993) J. Biol. Chem. 268, 23685–23696.
[24] Loo, T.W. and Clarke, D.M. (1995) J. Biol. Chem. 270, 22957–22961.
[25] Nikaido, K., Liu, P.-Q. and Ames, G.F.-L. (1997) J. Biol. Chem. 272, 27745–27752.
[26] Hung, L.-W., Wang, I.X., Nikaido, K., Liu, P.-Q., Ames, G.F.-L. and Kim, S.-H. (1998) Nature 396, 703–707.