Mutational analysis eliminates Glu^{64} and Glu^{94} as candidates for 'catalytic carboxylate' in the bacterial ATP-binding-cassette protein MalK

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Abstract MalK is the ATP-hydrolyzing subunit of the binding protein-dependent ATP-binding-cassette (ABC) transport system for maltose from *Salmonella typhimurium*. In a recent hypothesis, Glu^{64} and Glu^{94} of MalK were proposed as candidates for 'catalytic carboxylate', common to ATP- and GTP-hydrolyzing proteins [Yoshida and Amano (1995) FEBS Lett. 359, 1–5]. Substitution of both residues and, additionally, Glu^{74} by either glutamine or glycine and valine, respectively, had no deleterious effect on maltose transport. Thus, our data disprove the above notion.

Key words: ABC transporters; ATP-binding domain; Catalytic carboxylate; Binding-protein-dependent transport systems; MalK; *Salmonella typhimurium*

1. Introduction

ATP-binding-cassette (ABC) transport systems comprise an extremely diverse class of proteins that couple the energy of ATP hydrolysis to the translocation of solutes across biological membranes. Members of the family not only accomplish the uptake of nutrients but play crucial roles in a large variety of processes, such as signal transduction, drug and antibiotic resistance, antigen presentation, bacterial pathogenesis and sporulation [1]. Typically, an ABC transporter is composed of four parts: two membrane-integral domains each of which spans the membrane six times, and two ATP-hydrolyzing domains. In eukaryotic systems, these domains are mostly fused to yield a single polypeptide chain, while bacterial ABC transporters are build up from individual subunits [1]. When compared between different systems and organisms, the membrane-spanning modules usually differ in their primary structure, while the ATP-hydrolyzing proteins/domains display a high degree of sequence identity. This is mainly due to the presence of a set of Walker A and B motifs that are supposed to constitute a nucleotide binding fold [2]. Moreover, a short sequence motif immediately preceding the B-site ('linker peptide') is also highly conserved and unique to the ABC transport family. Intensive mutational analyses has led to suggesting a role in transmitting the energy of ATP hydrolysis to the membrane-integral components [1,3]. In the absence of structural data, two slightly differing secondary structural predictions of the ATP-hydrolyzing domains have been proposed on the basis of ATP- and GTP-binding proteins whose structures are known [4,5]. According to these models, both Walker motifs are linked by an extended peptide fragment of approx. 100 residues that largely folds into an α-helical conformation ('helical domain') (Fig. 1). Being less conserved and moderately hydrophobic, the latter is thought to function in the interaction with the membrane-spanning domains. Evidence in line with this view has recently been presented [6]. On the molecular level, neither the precise role of ATP in the transport process nor the individual steps in hydrolysis of the nucleotide are understood. However, the presence of the typical Walker A and B motifs give rise to the speculation that ABC transport proteins might just represent a variation on a common theme. Thus, the availability of crystal structures of other ATP-hydrolyzing proteins, such as the RecA protein of *E. coli* [7] and the F_1-ATPase from beef heart mitochondria [8] has led to the proposal that the attack of the γ-phosphate of ATP by a water molecule requires activation by a ('catalytic') carboxylate that is rather equidistantly positioned between the Walker A and B motifs. In a recent hypothesis based on sequence alignments, Yoshida and Amano [9] identified two glutamic acid residues as putative candidates for such a function in ABC transporters. Here, we report on the mutational analysis of the corresponding residues Glu^{64} and Glu^{94}, respectively, of MalK, the ATP-hydrolyzing subunit of the binding-protein-dependent ABC transport system for maltose ($MalFGK_2$) from *Salmonella typhimurium*. In addition, Glu^{74} that is moderately conserved in a subfamily of bacterial ABC proteins was also investigated. Our data clearly indicate that none of the residues is essential for a functional transport complex.

2. Materials and methods

2.1. Bacterial strains

E. coli strain JM109 [10] was used for general cloning purposes. Complementation studies using *S. typhimurium* strain ES25 (*dhuA1 ΔhisF645 malK786 galE503 recA56*) that lacks a functional *malK* gene were performed in minimal salt medium, containing 0.5% maltose as carbon source and supplemented with ampicillin (50 μg ml^{-1}), as described in [11].

2.2. Plasmids

Plasmid pSW7 carries the *malK* wild-type allele on pSE380 downstream of the trc-promoter [12]. Plasmids carrying the mutant alleles *malK804* through *malK808*, were designated pAS1 (E64Q), pAS2 (E64G), pAS3 (E74G), pAS4 (E94Q), and pAS5 (E94V), respectively.

2.3. DNA techniques

Site-directed mutagenesis, using pSW7 as a template was performed as in [12]. Oligonucleotide primers were purchased from MWG-Biotech (Freiburg). Introduced base changes were confirmed by nucleotide sequence analysis as in [12], but using a non-radioactive system (GATC, Konstanz) with biotin-labelled dideoxynucleotides.

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2.4. Biochemical methods

The uptake of [^{14}C]maltose (final concentration: 3 μM, 0.6 μCi ml^{-1}) in cells of strain ES25 harbouring the described plasmids, SDS-PAGE, and immunoblotting using a polyclonal anti-MalK antiserum were performed as in [11].

3. Results

Site-directed mutagenesis was used to study the role of the proposed candidates for catalytic carboxylate in maltose transport. To this end, Glu^{64} was changed to either glutamine or glycine, and Glu^{94} was substituted for by glutamine or valine, respectively. Subsequently, *S. typhimurium* strain ES25, deficient in utilizing maltose as sole source of carbon and energy due to the lack of a functional *malK* gene was transformed with the resulting plasmids pAS1 (E64Q), pAS2 (E64G), pAS4 (E94Q), and pAS5 (E94V), respectively. Analysis of the transformants for growth on maltose-containing indicator plates (MacConkey) and on minimal salt medium, supplemented with 0.5% maltose, revealed that all plasmid-borne mutant alleles conferred growth indistinguishable from wild-type (pSW7). Furthermore, in liquid medium the mutants grew with doubling times that compared favourably with wild-type (Table 1). These results were further confirmed by measuring the uptake of [^{14}C]maltose in cells of ES25 harbouring the described plasmids. The initial rates of transport were within the same range as monitored with control cells (Table 1). All experiments were carried out in the absence of the inducer IPTG in order to eliminate the repressing activity of MalK [13]. Under these conditions, relatively low amounts of protein are synthesized, as compared to fully induced cells (Fig. 2). The data also demonstrate that the levels of expression remained unaffected by the base substitutions.

The above results clearly indicate that Glu^{64} and Glu^{94} are dispensable for a functional transport system and consequently, can be eliminated as candidates for catalytic carboxylate. Due to variations in the length of the peptide segment linking the Walker A and B sites in the ATP-hydrolyzing domains of ABC transporters, other residues, specific to individual proteins or subgroups might have to be considered. Thus, we have also analyzed the function of a carboxylate that is moderately conserved in the 'MalK'-subfamily of bacterial ATP-hydrolyzing proteins, involved in the uptake of certain sugars [6,14]. This residue corresponds to Glu^{74} in MalK. Again, replacement by glutamine did not affect transport activity (Table 1).

4. Discussion

The crystal structures of the ATP-hydrolyzing proteins RecA of *E. coli* [7] and F1-ATPase [8] from beef heart mitochondria imply that an ATP molecule bound in the nucleotide binding fold mainly by residues of the Walker A and B motifs is attacked at its γ-phosphate by a catalytic water molecule which needs to be polarized for function. This is achieved by a carboxylate that is positioned at a distance of 24 ± 2 residues from the invariant lysine in site A, immediately following the β2-strand. By sequence alignments, Yoshida and Amano [9] noticed that such a residue is conserved in a large variety of proteins that contain in their primary structure a set of Walker boxes. Consequently, they proposed that these residues might play identical roles in catalysis. Moreover, the authors also suggested candidates for catalytic carboxylate in the ATP-hydrolyzing domains/subunits of the ABC transport family. In the case of the MalK protein, E64 was identified as a likely candidate on the basis of its position at a distance of 22 residues from the invariant lysine of the Walker A motif. However, although frequently found in this region of ATP-hydrolyzing domains a carboxylic acid residue is not at all highly conserved. Thus, a better conserved carboxylate at a position 59 ± 8 residues after the lysine (E94 in MalK) was alternatively proposed. While their hypothesis was recently supported (but not proven) in the case of the SecA-ATPase of the preprotein translocase from *E. coli* by demonstrating that substitution of the (proposed) residue D133 by asparagine severely affected its function [15], the results presented in this report clearly disprove the notion on ABC proteins. Our data eliminate both putative candidates for catalytic carboxylate, E64 and E94, in the MalK protein by showing that neither of the residues is essential for function in the binding protein-dependent uptake of maltose by the $MalFGK_2$ complex in vivo. The functional consequences of mutations in the corresponding residues of other members of the ABC family have not been reported yet. Interestingly however, substitutions of residues D1270 (→N; corresponding to MalKE64) and of E504 (→Q; corresponding to MalKE94) in the C- and N-terminal halves, respectively, of the CFTR protein, which is mutated in patients affected by cystic fibrosis are listed in the database (http://www.genet.sickkids.on.ca/cftr/) as naturally occurring but rare mutations. Since the phenotype of cystic fibrosis does not necessarily require a non-functional protein but can be caused by a defect in processing [16], the effects on CFTR protein functions are unknown at this stage. In the

Table 1
Summary of activities of strain ES25 harbouring the described plasmids

Plasmid	Protein	Doubling time[a] (h)	Transport rate[b] (nmoles min^{-1} per 10^9 cells)	(%)
pSW7	MalK	2.6	3.4	100
pAS1	MalKE64Q	2.4	3.2	94
pAS2	MalKE64G	2.7	2.4	71
pAS3	MalKE74G	3.3	3.5	103
pAS4	MalKE94Q	2.5	3.4	100
pAS5	MalKE94V	2.8	2.7	79

[a]Cells of strain ES25 (*malK*), harbouring the described plasmids, were grown in minimal salt medium with maltose (0.5%) as sole source of carbon and energy [11]. Growth was monitored by measuring the optical density at 650 nm.

[b]Cells were grown as in [a], harvested at $OD_{650} = 1$, and washed twice in salt medium, lacking maltose. The initial rate of uptake of [^{14}C]maltose was determined as in [11]. Rates are corrected for background values which were obtained by measuring uptake in cells harbouring the vector plasmid pSE380 that were grown with glycerol (0.5%) as carbon source.

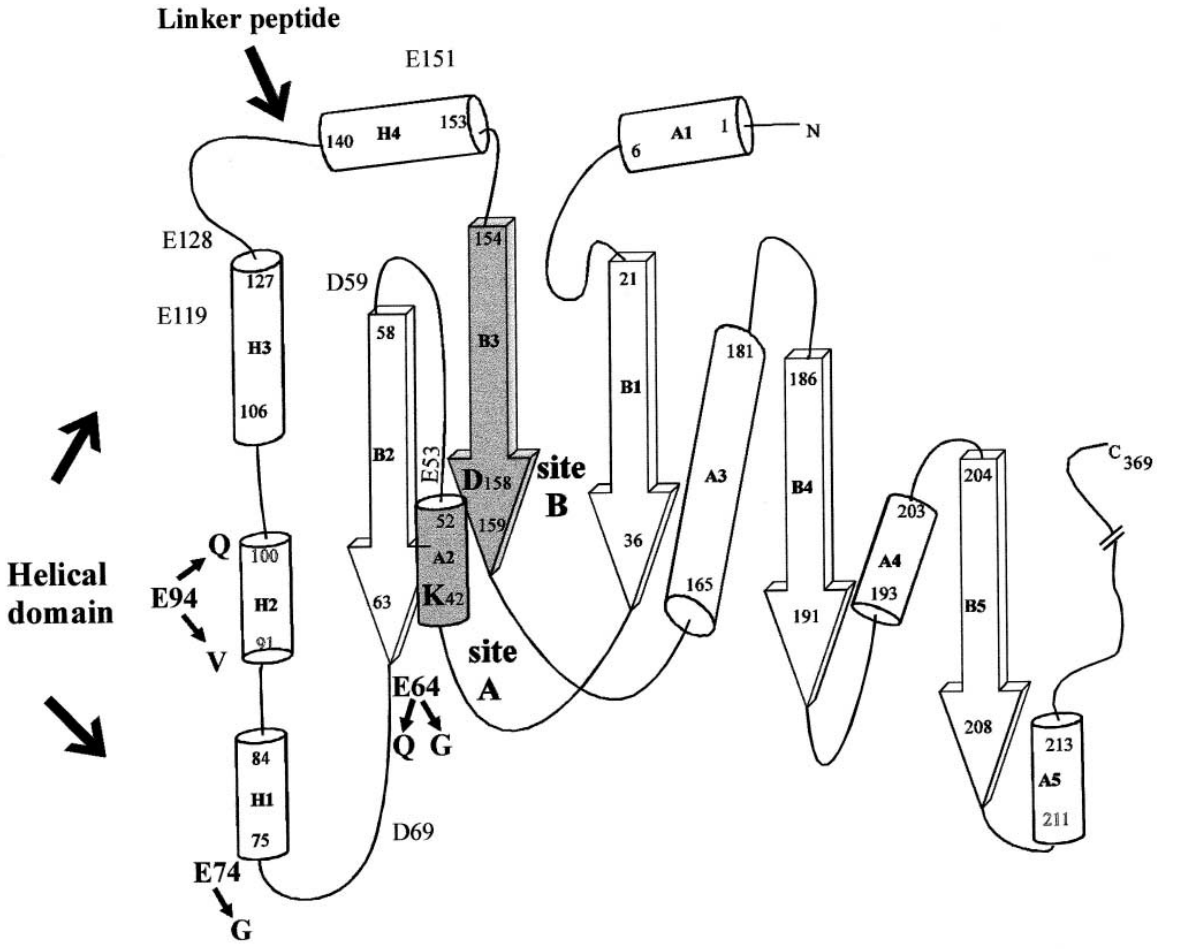

Fig. 1. Secondary structural model of MalK (based on [4] and modified from [12]). The relative positions of the Walker boxes A and B, and of the carboxylate residues in the connecting peptide segment are indicated. Cylinders represent proposed α-helices (A1–A5) and arrows represent proposed β-sheets (B1–B5). Helices H1–H4 encompass the putative 'helical domain' and the 'linker peptide'. Structural elements comprising the A and B sites are shown in grey.

absence of structural informations on any ABC protein, we can only speculate on the implications of our results on the hydrolysis mechanism. When assuming that proteins comprising both Walker motifs operate by the same basic mechanism then structural variations might have to be considered in order to explain the above results. A major feature by which the ATP-hydrolyzing domains of ABC transport systems differ from other enzymes that catalyze the cleavage of ATP, is the variation in length of the peptide fragment that links the Walker A and B motifs. Thus, it seemed reasonable to assume that in these proteins, individual rather than positionally conserved carboxylic residues might function as catalytic carboxylate in each case. Besides E64 and E94, seven carboxylates are located between the A and B motifs in the MalK protein (Fig. 1). If residues E53, D59, and E151 are excluded on the basis of the topology of the ATP-binding domain given in [9], as to close to the A and B sites, residues D69, E74, E119, and E128 might have to be taken into account. Among those, E119 can be eliminated due to the fact that substitution by lysine in the *E. coli* MalK protein was shown to cause a defect in a regulatory function but did not affect transport [17]. Thus, for additional analysis we have chosen residue E74 due to its position in a region where carboxylic residues are conserved in proteins that belong to the 'MalK'-subfamily of

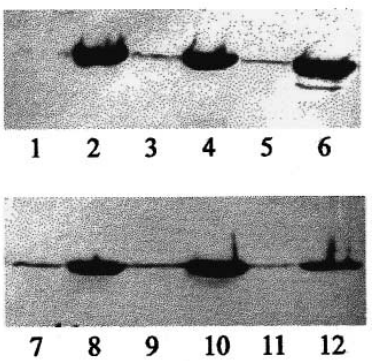

Fig. 2. Immunochemical analysis of wild-type and mutant MalK proteins. Cells of host strain ES25, harbouring the described plasmids were grown in the absence (odd-numbered lanes) or presence (even-numbered lanes) of IPTG. At early stationary phase, the cells were harvested and subjected to SDS-PAGE. Subsequently, the proteins were electrotransferred to nitrocellulose and probed with a MalK-specific polyclonal antiserum. The blots were developed with the enhanced chemoluminescence kit from Amersham. Lanes: 1, 2, MalK (pSW7); 3, 4, MalKE64Q (pAS1); 5, 6 MalKE64G (pAS2); 7, 8, MalKE74G (pAS3); 9, 10, MalKE94Q (pAS4); 11, 12, MalKE94V (pAS5).

bacterial ABC proteins [6,14]. Members of this subfamily share a higher degree of sequence identity as compared to the consensus sequence and, more importantly, the Walker motifs are connected by peptide segments of equal lengths. Again, the residue proved not to be essential.

Taken together, our results do not support the hypothesis by Yoshida and Amano [9] with respect to a member of the ABC transport family. Thus, we conclude that the identification of a residue that functions as catalytic carboxylate in these proteins might have to await structural informations.

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References

[1] Higgins, C.F. (1992) Annu. Rev. Cell Biol. 8, 67–113.
[2] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[3] Ames, G.F.-L. and Lecar, H. (1992) FASEB J. 6, 2660–2666.
[4] Mimura, C.S., Holbrook, S.R. and Ames, G.F.-L. (1991) Proc. Natl. Acad. Sci. USA 88, 84–88.
[5] Hyde, S.C., Emsley, P., Hatshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1990) Nature 346, 362–365.
[6] Wilken, S., Schmees, G. and Schneider, E. (1996) Mol. Microbiol. 22, 555–666.
[7] Story, R.M. and Steitz, T.A. (1992) Nature 355, 374–376.
[8] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[9] Yoshida, M. and Amano, T. (1995) FEBS Lett. 359, 1–5.
[10] Yanish-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[11] Schneider, E. and Walter, C. (1991) Mol. Microbiol. 5, 1375–1383.
[12] Walter, C., Wilken, S. and Schneider, E. (1992) FEBS Lett. 303, 41–44.
[13] Reyes, M. and Shuman, H.A. (1988) J. Bacteriol. 170, 4598–4602.
[14] Heekstra, D. and Tommassen, J. (1993) J. Bacteriol. 175, 6546–6552.
[15] Sato, K., Mori, H., Yoshida, M. and Mizushima, S. (1996) J. Biol. Chem. 271, 17439–17444.
[16] Welsh, M.J. and Smith, A.E. (1993) Cell 73, 1251–1254.
[17] Kühnau, S., Reyes, M., Sievertsen, A., Shuman, H.A. and Boos, W. (1991) J. Bacteriol. 173, 2180–2186.