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Characterization of side-directed mutations in conserved domains of MalK, a bacterial member of the ATP-binding cassette (ABC) family

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Site-directed mutagenesis was used to change four amino acid residues (Q82, P152, L179, H192) in the MalK subunit of *S. typhimurium* maltose transport system which are highly conserved among members of the ATP-binding cassette (ABC) family. Replacement of H192 caused complete failure to complement the transport defect of a *malK* strain whereas changes of the other residues resulted in reduced or wild-type activity. The purified mutant proteins exhibited ATPase activity comparable to wild-type MalK.

Maltose transport; MalK; ATP-binding cassette family; Site-directed mutagenesis; *Salmonella typhimurium*

1. INTRODUCTION

Enteric bacteria, like *E. coli* and *S. typhimurium* can utilize maltose and maltodextrins as their sole sources of carbon and energy. The uptake of these substrates is accomplished by a binding protein-dependent transport system at the expence of ATP (for review, see [1,2]). In addition to a specific porin in the outer membrane (LamB) and the substrate-specific binding protein in the periplasm (MalE), three proteins localized to the cytoplasmic membrane are required for the accumulation of the sugars. MalF and MalG are very hydrophobic and presumably span the membrane several times [3,4]. In contrast, MalK is hydrophilic and thought to be anchord to the membrane at the cytoplasmic side by interaction with MalG [5]. A $MalFGK_2$ complex has been purified and reconstituted into liposomes. Accumulation of radiolabelled substrate was observed in the presence of MalE and ATP [6]. The MalK subunit, which contains nucleotide-binding motifs in its primary structure [7], was recently purified and demonstrated to catalyze the hydrolysis of ATP [8]. Thus it belongs to a growing family of procaryotic and eucaryotic proteins (referred to as ATP-binding cassette proteins [9] or traffic ATPases [1]), all believed to be involved in ATP-dependent transport processes. Recent additions include the medically important mammalian P-glycoprotein (Mdr), which is responsible for multidrug resistance [10], the CFTR protein, which is mutated in patients affected by cystic fibrosis [11], and a peptide transport protein from the major histocompatibility locus [12]. In addition to the nucleotide binding motifs (Walker sites A and B [13]) there are several other regions shared by these proteins (previously designated sites C–F, [14]) which contain highly conserved amino acid residues of unknown function. In order to elucidate their putative role in the transport process, we have chosen Q82, P152, L179 and H192 of MalK of *S. typhimurium* as representatives of these sites and subjected them to mutational analysis.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains*

E. coli strain JM109 [15] was used for general cloning purposes. *S. typhimurium* strain ES25 (*dhuA1 ΔhisF645 malK786 galE503 recA56*) lacking any immunologically detectable MalK protein [16] was used for complementation and transport studies.

2.2. *Plasmids*

pCW14 carries the *malK* wild-type allele on pJLA502 downstream of the λ promoters [16]; pCW11, pCW25, pCW27, pCW28, and pCW29 carry various mutant *malK* alleles on pJLA502 (see Table II); pSW7 carries the *malK* wild-type allele on pSE380 [17] down-stream of the trc-promoter; pSW3, pSW5, pSW6, and pSW10 carry various mutant *malK* alleles on pSE380 (See Table II). Plasmid pCW11 was constructed by replacing the first four codons of *malK* by a synthetic oligonucleotide linker lacking basepairs encoding amino acids S3 and V4.

2.3. *DNA techniques*

Site-directed mutagenesis was performed according to [18] using a derivative of phage M13mp18 which carries the *malK* wild-type gene as a template. Oligonucleotides were synthezised by Dr. Lill (Osnabrück). Introduced base changes were confirmed by nucleotide sequence analysis [19] prior to and after subcloning into pJLA502 or pSE380, respectively, using the T7 sequencing kit from Pharmacia (Freiburg).

2.4. *Biochemical methods*

Wild-type and mutant MalK proteins were purified on a small scale

as described previously [8]. Briefly, MalK aggregates (10 mg) from an overproducing strain were dissolved in urea and bound to a red agarose column (Sigma, Deisenhofen) (1 × 5 cm). Upon removal of urea, renatured MalK protein was eluted with ATP (10 mM), dialyzed to remove ATP and stored in liquid nitrogen. ATPase activity was measured in microtiter plates according to [20]. Maltose transport assays were performed as in [16]. Sodium dodecylsulfate gel electrophoresis according to [21] was carried out using 10% acrylamide. Protein concentrations were determined by the bicinchoninic acid method [22], following the manufacturer's instructions (Pierce, Rockford).

3. RESULTS AND DISCUSSION

The four amino acid residues (Q82, P152, L179, H192), which we have chosen for a site-directed mutagenesis study as presented in this communication, are highly conserved among both procaryotic and eucaryotic members of the ABC family (Table I). All but one (H192) are likely to be located within helical domains of the proteins (Fig. 1) [9,23], but do not participate in the nucleotide binding site which is constituted by the Walker motifs A and B [13]. We have studied the consequences of replacing these amino acid residues in the MalK protein of *S. typhimurium* by transforming a *malK* strain, ES25, with plasmid-borne alleles of *malK* and observing maltose uptake. Furthermore, the mutant proteins were purified by a procedure developed recently that allows the preparation of large quantities of functional MalK. The results are summarized in Table II.

Replacement of H192 by either leucine or arginine resulted in the complete loss of transport activity, thereby confirming results obtained by Shyamala et al. [24] for the respective histidine residue (H211) in the HisP protein. In their study, natural mutations to aspartate, tyrosine and arginine, respectively, were found to abolish histidine transport. Due to the finding that binding of 8-azido-ATP to the membrane-bound mutant proteins was unchanged as compared to wild-type, the authors suggested that H211 might be critical for

Table I

Conservation of amino acid residues, which have been changed in this study, among bacterial and eucaryotic ATP-binding proteins

MalK	Binding protein systems[a]	C F T R[b]	M D R[b]
Q82	17/21	432 (N)	475 (N)
		1280 (C)	1119 (C)
P152	17/21	–	550 (N)
		–	1195 (C)
L179	20/21	–	–
		–	–
H192	18/21	–	596 (N)
		1402 (C)	1240 (C)

[a] The number of sequences which contain the respective residue per total number of 21 sequences. Data were compiled from [23], [29] and [30].

[b] The numbers represent the position of the respective residue in the N- and/or C-terminal half of the proteins. Data were taken from [9].

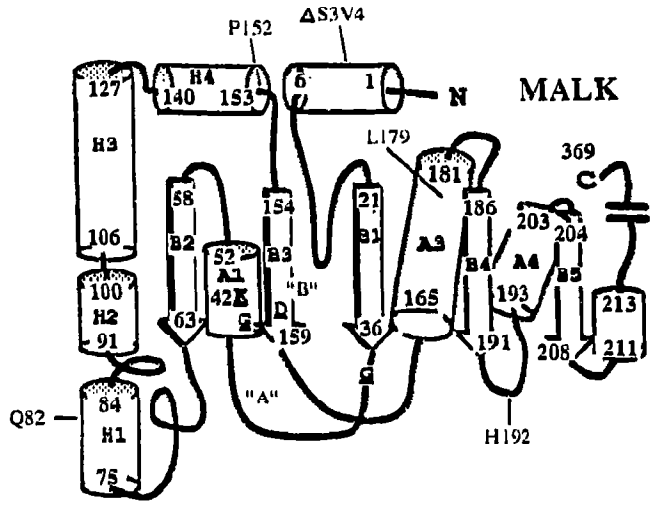

Fig. 1. Secondary structural model of MalK (adapted from [23] with permission). Sequence information was taken from [25]. The positions of residues changed in this study and of the Walker sites A and B are indicated.

conformational changes following ATP binding [24]. We provide strong support in favor of this view by demonstrating that even the purified mutant MalK proteins encoded by pSW6 and pSW10 exhibit normal ATPase activity (Table II).

In the case of L179, a neutral change to glutamine had no effect on transport, whereas a bulkier-charged residue like arginine, in its place caused a 50% inhibition

Table II

Functional consequences of mutations in *MalK*

Plasmid	Residue change	Transport[a] (%)	ATPase activity[b] (%)
pCW14	none	100	100
pCW11	ΔS3V4	0	85
pCW25	P152L	90	40
pCW27	P152Q	84	39
pCW28	Q82K	29	61
pCW29	Q82E	24	84
pSW7	none	100	100
pSW3	L177R	52	130
pSW5	L177Q	103	372
pSW6	H192L	0	78
pSW10	H192R	0	89

[a] Plasmids carrying genes encoding wild-type MalK and MalK proteins bearing site-directed mutations were used to transform ES25, a *malK* strain of *S. typhimurium*. Initial-rate transport assays were performed as in [16]. Wild-type cells exhibited uptake rates of 800 pmol/min/10^9 cells (pCW14) or 400 pmol/min/10^9 cells (pSW7), respectively.

[b] ATPase activity of purified and dialyzed wild-type and mutant MalK proteins was measured as described in [20]. Control values correspond to 27.1 nmol P_i/min/mg (pCW14) and 12.8 nmol P_i/min/mg (pSW7), respectively. Average values of duplicates are presented for both assays.

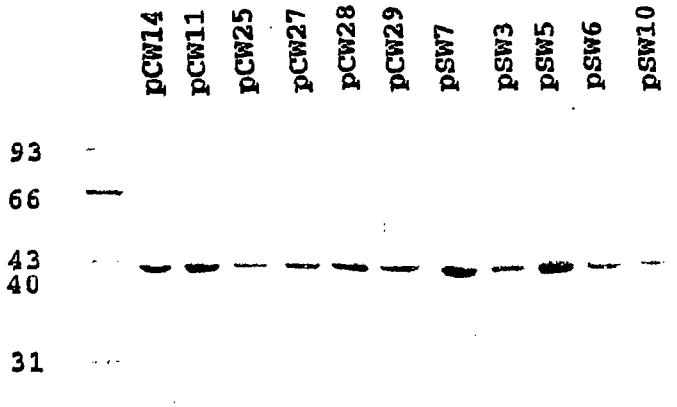

Fig. 2. Sodium dodecylsulfate-gel electrophoresis of purified wild-type and mutant proteins. Cells of *E. coli* strain JM109, harboring the respective plasmids were grown in LB medium under inducing conditions, harvested and fractionated by passage through a Ribi press. MalK proteins were purified from aggregates by red agarose chromatography as described [8]. Equal volumes (20 μl) of dialyzed protein fractions eluted with ATP were subjected to gel electrophoresis [21]. Molecular mass markers are indicated.

of the maltose uptake rate. Interestingly in the MalK sequence, L179 separates R178 from three consecutive positive charges [25]. Given that these positive charges are compensated in the tertiary structure by aspartate or glutamate residues, an additional positive charge at position 179 might disturb this arrangement. On the other hand, replacement by arginine had no effect on the ATPase activity of the mutant protein, whereas glutamine at this position caused a significant increase in the specific ATPase activity. It will be worthwhile studying the biochemical properties of wild-type and mutant protein to elucidate its role in the enzymatic process.

Q82 is located at the N-terminal site of the large helical domain between B2 and B3 (see Fig. 1). This domain is moderately hydrophobic, less conserved in the primary structure, and believed to have no counterpart in adenylate kinase [8]. Together, this has been taken as evidence for the view that it might interact with the membrane-bound components of the transport systems [9,23]. Both changes to either a positively or negatively charged residue severely affected but did not abolish transport. Again, the mutant proteins exhibited ATPase activity (Table II). In line with this is the observation that even an uncharged residue (L) in place of the corresponding Q100 in HisP caused almost complete loss of function [24]. In contrast, in the STE6 protein of *Saccharomyces cerevisiae*, which mediates the export of mating factor a and is composed of two homologous halves, no effect on activity was observed when either one of the two analogous glutamine residues (440/1,135) were replaced by arginine [26]. It seems that in this case a conserved residue is critical in bacterial *uptake* systems whereas in a eucaryotic *export* protein any residue in this position is apparently tolerated.

According to the secondary structural model the conserved P152 connects the large helical domain with site B of the nucleotide binding pocket (Fig. 1), suggested to reside in a β-structural conformation. In HisP, changes of the corresponding P172 to either T or L caused a reduction in transport activity [24], although no effects on growth characteristics were observed [27]. In contrast, no effects on either growth (not shown) or transport were found when P152 in MalK was replaced by Q or L, respectively (Table II). This result is not explained by a high copy number effect because the number of maltose transport complexes in the membrane is limited by the levels of transmembrane components (MalF, MalG) expressed from chromosomal alleles. Interestingly, however, histidine permease mutants with replaced P172 in HisP were selected by suspension of a mutant binding protein (HisJ) [27]. Since in the maltose system such a suppressor phenotype was observed only with certain *malG* or *malF* mutants [28] this might be an indication for different functions of P152/172 in both proteins.

Basically no conserved residues are positioned at the amino-terminal domain of ATP-binding proteins. However, a deletion mutation (S3V4) in MalK failed to complement the transport defect of strain ES25, although ATPase activity was observed with the purified protein (Table II). Our recent finding that the N-terminal portion of MalK can be replaced by a corresponding portion of HisP in a chimeric protein and still allows function in maltose transport argues against a maltose-specific role of the N-terminus [16]. Thus it seems more likely that the relative length of the polypeptide in this early helical domain is important rather than the precise amino acid sequence.

All mutant proteins could be purified by a procedure which, as a prerequisite, requires the formation of a correctly folded nucleotide-binding site (Fig. 2) [8]. Therefore, none of the introduced amino acid changes prevented this process in general, although in some cases the protein yield differed from that obtained with wild-type MalK. Moreover, an intrinsic ATPase activity, recently demonstrated for purified wild-type MalK was also found with all mutant proteins. In summary, we have provided evidence that highly conserved residues in MalK which do not take part of site A and B are neither essential for ATP binding nor subsequent hydrolysis. Differences in their specific activities as listed in Table II might, however, reflect secondary effects on the tertiary structure in the proximity of the ATP binding fold.

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