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Evidence for a close association between helix IV and helix XI in the melibiose carrier of *Escherichia coli*

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Abstract

The melibiose carrier of *Escherichia coli* is a cation–sugar cotransport protein. Asp124 in membrane-spanning helix IV of the carrier protein was replaced with Ser, Ile or Phe by site-directed mutagenesis of a plasmid containing the *melB* gene. Each of these mutants failed to show membrane transport of melibiose and melibiose-positive revertants could be isolated on melibiose MacConkey indicator plates. D124F showed only one type of revertant (D124C) and D124I showed only revertants to the normal (D124). Second site revertants were not found with either of these mutants. S124, however, showed two types of second site revertants: D124S/V375A and D124S/V375G. The revertant D124S/V375A had lost melibiose/proton cotransport but showed 25% of normal melibiose (20 mM) uptake in the presence of 10 mM sodium ion at 37°C. The value for the parental strain D124S was 2%. The second revertant D124S/V375G showed greater activity than S124 in the presence of 100 mM NaCl at both 20°C and 37°C. It was concluded that in the normal carrier protein Asp124 in helix IV is probably close to Val375 in helix XI. Since Lys377 is close to Val375, it is possible that Asp124 may interact with Lys377 to form a salt bridge. 0005-2736/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The melibiose carrier of *Escherichia coli*, coded for by the *melB* gene, is a cation–substrate cotransport system (for a review see Ref. [1]). This carrier protein can transport the α-galactoside melibiose in association with either Na^+, H^+ or Li^+ [2]. The *melB* gene has been sequenced [3] and codes for an extremely hydrophobic protein. A topological model for the carrier, consisting of 12 transmembrane α-helices, has been proposed based on hydropathy plots [3], phoA fusion experiments [4,5] and proteolytic digestion experiments [6].

One approach to the question of the arrangement of helices in the membrane is to study second site revertants from site-directed mutants with low activity. In the lactose carrier of *E. coli*, for example, evidence has been obtained by this method for salt bridges between certain positively charged residues and negatively charged residues in different helices [7–9]. Such experiments suggest the close proximity of one helix with another.

In the melibiose carrier, the proximity of helix II to helix IV has been indicated by a second site revertant of Asp55 → Ser [10]. The change of Asp55 to Ser resulted in loss of transport activity. A melibiose positive revertant was isolated which showed a change of Gly117 to aspartic acid. It was concluded that the new carboxyl group at position 117 compen-

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sated for the loss of the carboxyl group at position 55. Presumably in the 3-dimensional structure of the protein position 117 in helix IV is close to position 55 in helix II.

There is evidence that negatively charged amino acid residues located in membrane-spanning helices (Asp19, Asp55, Asp59 and Asp124) are important for the function of the carrier [1]. When neutral amino acid residues were substituted for each of these aspartic acid residues, the transport of melibiose and TMG was abolished [11–14]. Sugar was bound to the carrier in the absence of Na^+ but Na^+ stimulation of binding was lost [11,13]. When Glu was substituted individually for the Asp residues, Na^+ stimulation of melibiose accumulation was retained although with a much reduced affinity for Na^+ [15,16]. It was suggested that these four Asp residues form part of a cation binding pocket [1].

In this paper, second site revertants were isolated from site directed mutants of Asp124 (helix IV). Three mutants, Ser124, Phe124 and Ile124, were constructed and were found to show extremely low activity. Cells containing a plasmid with the melA gene (coding for α-galactosidase) plus a plasmid containing the mutated Asp124 were grown on melibiose MacConkey indicator plates. Since melibiose was not taken up by these cells, the clones were white. After several days, red revertants appeared on the plates. The DNA of these revertants was then sequenced. Many of the revertants contained the original mutation at position 124 plus a second site mutation in which Val375 (helix XI) was changed to either alanine or glycine. It is postulated that there may be an interaction between Asp124 in helix IV and Val375 in helix XI.

2. Materials and methods

2.1. Reagents

Melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) was purchased from Sigma. [^{3}H]Melibiose was a generous gift from Dr. Gerard Leblanc. [α-^{33}P]dATP was purchased from Andotek and [^{35}S]Protein A was purchased from Amersham. Bacteriological media were from Difco. Plasmid DNA was isolated with the Qiagen kit.

2.2. Bacterial strains and plasmids

E. coli DW1 (*lacI*$^+$ *ΔlacZY ΔmelAB*) [17] was used as the host strain. pSUmelA is a derivative of pACYC184 (New England Biolabs) into which was cloned the melA gene subcloned from pSTY91 [3]. The plasmid, pKKmelB [18], contains the gene for the temperature-resistant form of the melibiose carrier inserted into the vector pKK223-3 (Pharmacia Biotech).

2.3. Site-directed mutagenesis

The Chameleon double-stranded site-directed mutagenesis kit of Stratagene was used to produce D124F and D124I. D124S was prepared with the Bio-Rad Muta-Gene Phagemid kit. The mutagenic primers are given in Table 1.

2.4. DNA sequencing

DNA sequencing was performed with the AmpliCycle kit of Perkin Elmer.

2.5. Sugar transport assays

Overnight grown cells were diluted 1:100 in LB medium plus the appropriate antibiotic (100 μg/ml ampicillin for pKK 223-3 plasmid and 30 μg/ml chloramphenicol for pSUmelA plasmid). Cells were grown to late log phase. Cells were centrifuged and washed twice with 0.1 M MOPS/0.5 mM $MgSO_4$/Tris (pH 7.0). Transport assays were carried out in the same buffer with different concentrations of [^{3}H]melibiose and NaCl. At appropriate time points, 200-μl aliquots were removed and filtered rapidly through 0.65-μm pore size cellulose nitrate filters (Sartorius). Filters were then washed with 5 ml of the same buffer. The filters with adherent cells were then dissolved in 4 ml of Liquiscint (National Diagnostics) and counted in a liquid scintillation counter (Tracor).

Melibiose entry was measured in cells containing α-galactosidase (DW1/pSUmelA/pKKmelB). In these cells sugar that enters is metabolized so that the internal concentration of melibiose is low. Thus the entry of melibiose is thermodynamically 'downhill'. As a control, the uptake by DW1/pSUmelA/

Table 1
Oligonucleotide primers used for site-directed mutagenesis

Oligonucleotide	Sequence	Amino acid change
D124F	5′-CCAGAAGGGAAT(G*A*A*)CATAATGGTGTAAGTCATGCCCC-3′	Asp124 to Phe
D124I	5′-CCAGAAGGGAAT(G*A*T*)CATAATGGTGTAAGTCATGCCCC-3′	Asp124 to Ile
D124S	5′-CACCATTATG(T*C*C*)ATTCCCTTCTGG-3′	Asp124 to Ser

Mismatches between the *melB* gene and the primer are followed by an asterisk and the resulting codon is given in parentheses.

pKK223-3 (vector without insert) was subtracted from all experiments.

In the experiment to assay accumulation of melibiose against a concentration gradient, DW1/pKKmelB was used. This cell contains no α-galactosidase.

2.6. Immunoblots

Immunoblots were performed by the method of Lolkema et al. [19]. The amount of melibiose carrier protein in each strain was determined using a polyclonal antibody directed against the ten carboxyl terminal amino acids (diluted 1:1000). [^{35}S]Protein A was used to label the bound antibody and the product was washed thoroughly. The radioactivity was determined in each sample in a Tracor liquid scintillation counter. To correct for non-specific adsorption, the counts for DW1/melA/pKK223-3 (which does not express the melibiose carrier) were subtracted from each sample. Values were expressed as the percent of the wild-type *melB*.

3. Results

3.1. Site directed mutagenesis

Site directed mutagenesis was employed to make the following three substitutions: Asp124 → Ser, Asp124 → Phe, and Asp124 → Ile.

3.2. Phenotype on MacConkey plates

Fermentation of melibiose was indicated by the appearance of red colonies on MacConkey indicator plates containing 30 mM melibiose. Cells of DW1/pmelA/pmelB appeared as bright red clones after overnight incubation at 37°. Sugar was transported into the cell by the melibiose carrier and the sugar was metabolized by α-galactosidase. Cells containing any of the three mutants of Asp124 (S124, F124 and I124) gave rise to white clones on these plates.

3.3. Isolation of revertants

Each of the three Asp124 mutants was streaked on melibiose MacConkey indicator plates and incubated for several days. After 2 or 3 days, small red clones appeared on the original white areas of the culture. These red regions were restreaked on MacConkey plates to purify the clones. Plasmid DNA was then isolated from these melibiose positive clones and used to transform cells of DW1/pmelA. In most cases the transformed cell gave red clones on the plate, indicating that the phenotype was present in the *melB* gene on the plasmid. Plasmid DNA was isolated from a transformed cell and sequenced. Three different revertants were isolated from Asp124 → Ser (Table 2) and their appearance on MacConkey plates is shown in Table 3. The revertant Asp124 → Cys showed only weak activity (pink clone). The other two revertants were red and bore the original mutation (Asp124 → Ser) plus a second mutation (Val375 → Ala or Val375 → Gly). The most frequent revertant was Asp124 → Ser/Val375 → Ala (41 out of a total of 97 revertants).

Asp124 → Phe showed only one revertant type (Asp124 → Cys) in addition to mutations back to the

Table 2
Revertants from the position 124 mutants

Initial strain	Revertant	Number of revertants
D124S	D124S/V375A	41
D124S	D124S/V375G	8
D124S	D124C	4
D124S	D124 (normal *melB*)	17
D124F	D124C	11
D124F	D124 (normal *melB*)	7
D124I	D124 (normal *melB*)	9

Table 3
Fermentation of melibiose on melibiose MacConkey indicator plates

Cell	Color after 18 h at 37°C
DW1/pSUmelA/pKKmelB	Red
DW1/pSUmelA/pKK223-3	White
DW1/pSUmelA/pKKS124	White
DW1/pSUmelA/pKKF124	White
DW1/pSUmelA/pKKI124	White
DW1/pSUmelA/pKKS124/A375	Red center
DW1/pSUmelA/pKKS124/G375	Red
DW1/pSUmelA/pKKC124	Pink

normal Asp124 genotype. The Asp124→Ile mutant showed few revertants, all of which had returned to the original wild-type, Asp124.

3.4. Measurement of the melibiose carrier protein

The quantity of melibiose carrier protein produced by the site-directed mutants and the revertants was determined by the antibody assay (Table 4). S124 and F124 gave about 75% of the normal amount of protein. I124 gave almost zero. The revertants gave between 58% and 120% of normal.

3.5. Accumulation of melibiose in DW1

The ability of the mutants to accumulate melibiose against a concentration gradient was tested with DW1 containing the plasmid pKKmelB or pKKmutant. DW1 contains a deletion through the mel operon and lacks both of the genes *melA* and *melB*. When DW1/pKKmelB was exposed to 0.2 mM melibiose for 10 min, the cell accumulated sugar so that the internal cellular concentration of sugar was 200 times the concentration outside the cell (Table 5). On the other hand, all mutants showed an internal concentration less than the external concentration.

Table 4
Measurement of melibiose carrier protein levels in wild-type and mutant strains

Strain	Melibiose carrier protein (% of wild-type)
MelB	100
S124	72
C124	120
S124/A375	106
S124/G375	58
F124	75
I124	1

The amount of melibiose carrier protein was determined by using an antibody directed against the ten carboxyl-terminal amino acids. Data given represent the mean of three experiments.

Table 5
Accumulation of melibiose by cells of DW1-containing mutants

Cell	Melibiose accumulation IN/OUT	
	5 min	10 min
DW1/pKKmelB	150	200
DW1/pKKF124	0.1	0.2
DW1/pKKI124	0.2	0.2
DW1/pKKS124	0.2	0.1
DW1/pKKC124	0.3	0.3
DW1/pKKS124-A375	0.1	0.2
DW1/pKKS124-G375	0.1	0.2

Cells were exposed to 0.2 mM [^{3}H]melibiose in a medium containing 10 mM NaCl. Data represent the mean values of three experiments.

3.6. Melibiose transport

Melibiose transport was measured in the cells containing α-galactosidase (DW1/pSUmelA/pKKmelB). Under these conditions entry can occur 'downhill' without accumulation as the sugar is metabolized within the cell. When transport was measured at room temperature with 20 mM radioactive melibiose and 100 mM NaCl, the cell containing the wild-type *melB* gene gave uptake of 64 nmol/mg protein. The two revertants C124 and S124/A375 gave the same low value as the parent S124. The revertant S124/G375, however, showed distinctly greater transport than S124 (Table 6).

The revertant S124/G375 in DW1/pSUmelA was assayed for transport at different sugar concentrations with 100 mM NaCl at room temperature (data not shown). A K_m value of 17 mM was obtained. Sugar uptake by the S124 and the two revertants (C124 and S124/A375) at room temperature was so low that it was not possible to determine K_m values.

The revertants had been isolated at 37°C on plates containing 30 mM melibiose and NaCl of the order of 100 mM. In the case of revertants C124 and S124/A375, transport experiments at room temperature (20°C) with 20 mM melibiose and 100 mM NaCl

Table 6
Melibiose uptake by revertants of S124

Cell	nmol/mg protein	% Normal
MelB	64	100
S124	2	3
C124	0.5	1
S124/A375	1.5	2
S124/G375	9.1	14

DW1/pSUmelA/pKKmelB plus four mutants in the same construction were exposed to 20 mM [^{3}H]melibiose and 100 mM NaCl for 5 min at room temperature. Data represent mean values of three experiments.

failed to show any sugar transport greater than the low value for the parent S124 (Table 6). In the present experiments the transport of 20 mM melibiose in the presence of 0 mM, 10 mM or 100 mM NaCl was measured at 37°C. Fig. 1 shows that the cell containing the normal carrier (pKKmelB) gave considerable uptake in the absence of NaCl but less uptake at 100 mM NaCl. On the other hand, the three revertants all showed considerably greater uptake of sugar in the presence of 100 mM NaCl than in the absence of NaCl. It should be noted that the three revertants showed much greater melibiose uptake in the presence of 100 mM NaCl than the cell from which they arose (S124). None of the revertants showed any significant melibiose transport in the absence of Na^+.

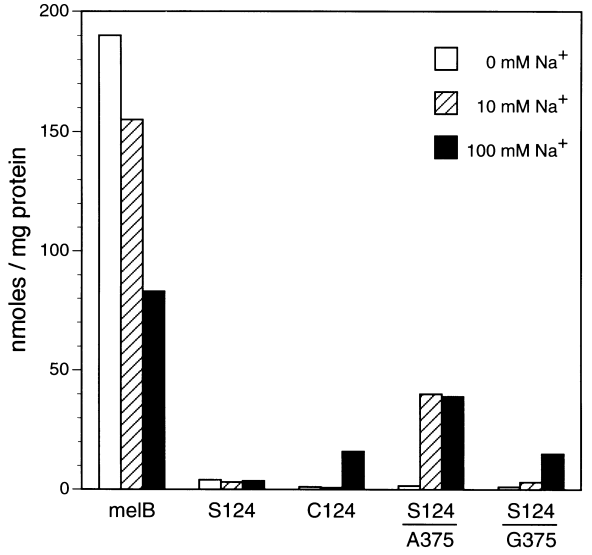

Fig. 1. The effect of Na^+ concentration on melibiose transport at 37°C. Cells (DW1/pSUmelA/plasmid) were exposed to 20 mM [^{3}H]melibiose for 2 min at 37°C. Data represent the mean values of three experiments.

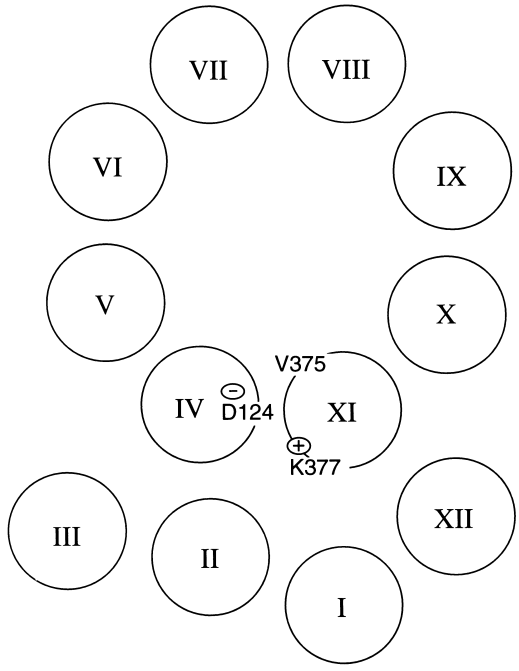

Fig. 2. Model showing the possible interaction between D124 and V375.

4. Discussion

Previous studies have shown that Asp124 is important for the activity of the melibiose carrier. When Asp124 was changed to Ser, Gln, Tyr, Lys, Leu, Ala, His, Cys or Asn transport of melibiose was lost [11–14]. When Asp124 was changed to Glu, the carrier retained Na^+ stimulated melibiose transport although the affinity for the sugar and for the Na^+ was greatly reduced [15,16]. In the present study the mutant S124 was streaked onto melibiose MacConkey plates. The white clones which appeared on the plate were incubated for an additional several days until small red revertants appeared. On sequencing the plasmid *melB* DNA, it was found that many of these revertants retained the original S124 mutation and showed a second site mutation at Val375 (either V375A or V375G). This observation suggests that there is some type of interaction between Asp124 and Val375. The simplest explanation is that these two residues are anatomically close together which

would indicate that helix IV in the N-terminal region is close to the C-terminal helix XI, as shown in Fig. 2. Another piece of evidence suggesting that there is proximity between some N-terminal helices and the C-terminal domain of the melibiose carrier comes from a tryptophan fluorescence analysis [20]. In this case the C-terminal helices involved were IX and X.

Although the present experiments suggest an interaction between Asp124 and Val375, it is possible that the physiological interaction of importance is a salt bridge between Asp124 and Lys377. If there is normally such a salt bridge, the loss of the negative charge at position 124 would leave the positive charge at 377 uncompensated which might alter the structure sufficiently to lose transport function. It is possible that the effect of a mutation in Val375 is to alter the position of Lys377 in relation to Asp124.

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