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GLY113→ASP CAN RESTORE ACTIVITY TO THE ASP51→SER MUTANT IN THE MELIBIOSE CARRIER OF ESCHERICHIA COLI

Dorothy M. Wilson, Hiroko Hama and T. Hastings Wilson*

Department of Cell Biology, Harvard Medical School, Boston,MA 02115

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SUMMARY: ASP51 in the putative membrane-spanning helix 2 of the melibiose carrier of Escherichia coli was replaced by SER. This mutation caused failure of the cell to transport melibiose and failure to ferment melibiose on indicator plates. A melibiose-positive revertant was isolated from these plates and was found to have two additional mutations, GLY113→ASP (in helix 4) and PHE16 →LEU (in helix 1). The double mutant ASP51→SER/GLY113→ASP was constructed and showed accumulation of melibiose. On the other hand ASP51→SER/PHE16→LEU showed no activity. It is concluded that the new carboxyl group at position 113 compensates for the loss of the carboxyl group at position 51.

The melibiose carrier of E.coli transports α-galactosides and some β-galactosides across the cell membrane coupled with a cation which may be H^+, Na^+ or Li^+ [for a recent review see Leblanc et. al. (1)]. The sequence of the melB gene has been determined (2). Hydropathy plots of the deduced amino acids (2) and phoA fusion experiments (3,4) have been employed to predict the secondary structure of the protein (4) (Fig.1).

Four aspartic acid residues believed to lie within the membrane-spanning region of the protein have been implicated in the cation coupling of the carrier. When neutral amino acid residues were substituted for ASP31, ASP51, ASP55 or ASP120, the accumulation of melibiose or thiomethylgalactoside was greatly reduced or abolished (5-8). Sugar was bound in the absence of Na^+ but Na^+ stimulation of the binding was lost (7). It was suggested that these ASP residues are in or near the cation binding site. When GLU was substituted individually for the ASP residues, Na^+

*To whom correspondence should be addressed. FAX: 617-432-1144.

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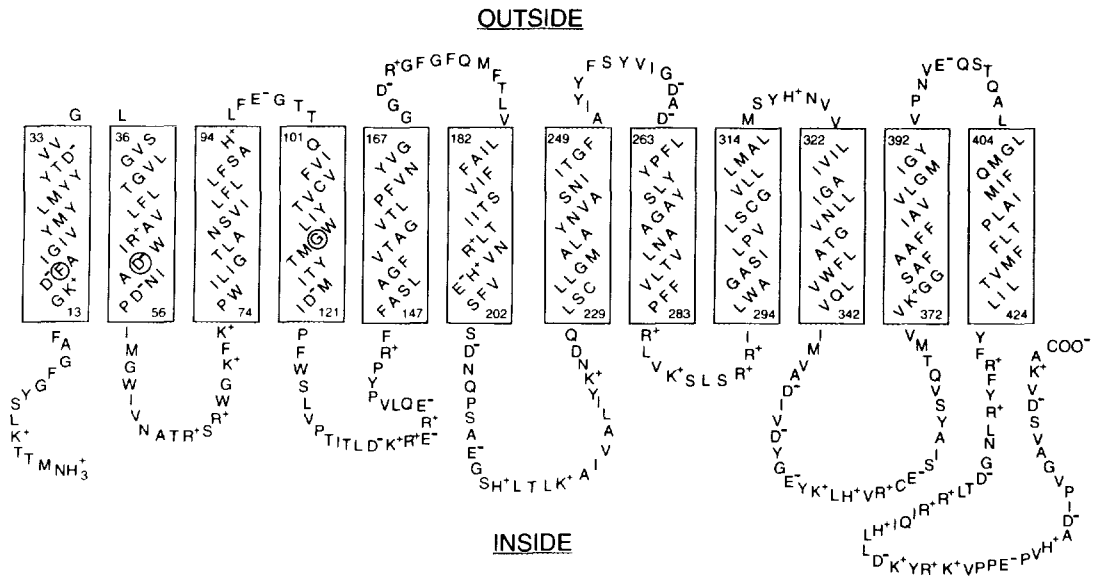

Fig. 1. Model for the melibiose carrier . The boxes represent membrane-spanning regions. The positions of the mutations are circled. The model is based on Zani et. al. (4).

stimulation of melibiose accumulation was retained although with much reduced affinity in the case of GLU51 (1,4,9).

In the present study the carrier containing ASP51→SER was constructed. The cell containing this carrier showed no fermentation on melibiose MacConkey indicator plates. A melibiose positive revertant was isolated and found to have the mutations GLY113→ASP and PHE16→LEU in addition to the starting mutation ASP51→SER. The roles of the two extra mutations were analyzed by studying the transport of single and double mutants.

MATERIALS AND METHODS

Reagents-Melibiose and thiomethyl-β-D-galactoside (TMG) were obtained from Sigma. [^{14}C]TMG was purchased from Du Pont-New England Nuclear. [^{3}H]-Melibiose was a generous gift from Dr Gerard Leblanc. ^{35}S-dATP was purchased from Amersham Corp. Restriction enzymes and T4 DNA ligase were from New England Biolabs. The Sequenase kit was purchased from United States Biochemical.

Strains-E.coli strains DW1 [lacI^{+} Δ(ZY) Δmel(AB)] (10) and DW2R [lacI^{+} Δ(ZY) melA^{+}ΔB Tn10::RecA] (11) were used as host strains for the plasmids. ES953 (mutD5) (12) was used to mutagenize the plasmid containing ASP51→SER.

Plasmids- pKKMBPst is pKKMB (13) with a Pst1 site in the melB gene (obtained by making a silent one base change at position 627). In addition this construct differs from pKKMB in that the EcoR1 site of pKKMBPst is immediately preceeding the start codon and the Hind 111 site is immediately following the stop codon. pSUMelA is pSU2718 (related to pACYC184) containing the melA gene subcloned

from pSTY91 (2). The melA gene is enclosed by two Pst1 sites and is under the regulation of a lac promoter.

Selection for revertants-Plasmid pKKMBPst-S51 was mutagenized by transforming it into ES953 mutD5. The plasmid DNA was reisolated and transformed into DW1/pSUmelA which was then spread on 1% melibiose MacConkey indicator plates containing ampicillin (100μg/ml) and chloramphenicol (30μg/ml). The colonies were white but after 5 days at 37°C a red clone appeared. Plasmid DNA was isolated from this purified red clone. The red phenotype was transferred to DW1/pSUmelA by transformation with this DNA.The melB gene was completely sequenced from the double-stranded plasmid DNA (14)and showed ASP51→SER/GLY113→ASP/PHE16→LEU.

Mutagenesis-Site-directed mutagenesis of uracil-labeled DNA was carried out as described in the BIORAD kit.The sense strand primers used to prepare the 3 mutations were as follows: i)ASP51→SER,5'-GCG AGG ATC TGG tcT GCT ATT AAC G-3' ii)PHE16→LEU,5'-CGG GAA GGA TcT TGC GAT CGG C-3'; iii)GLY113→ASP,5'-CAT CCT CTG GGa CAT GAC TTA CAC C-3'(mutation indicated in lower case). The EcoR1-Pst1 fragments from these mutants were ligated into the wild type plasmid from which the EcoR1-Pst1 fragment had been removed.

Transport of sugars-DW2R/plasmid was grown overnight in LB medium plus ampicillin (100μg/ml) and tetracycline (10μg/ml)and then diluted 50-fold and grown to mid log phase at 30°C.Cells were centrifuged and washed twice with 100mM MOPS pH7 plus 0.5mM MgSO4. Cells were exposed to radioactive melibiose or TMG for various times, then rapidly filtered, washed and the filter disk counted.

RESULTS AND DISCUSSION

The mutation Asp51→Ser (S51) in the E. coli melibiose carrier results in loss of transport (6) and cells containing this mutation (DW1/pSUmelA/pKKMBPst-S51) fail to ferment (white clones) on 1% melibiose indicator plates (Table 1). After 5 days at 37°C a red revertant was found on such a plate. When sequenced it showed S51 and two additional mutations,PHE16→LEU (L16) and GLY113→ASP (D113). To evaluate the role of these mutations, the double mutants S51/D113 and S51/L16 and the single mutants D113 and L16 were

Table 1. Fermentation of melibiose on MacConkey indicator plates

Plasmid	Colony appearance
pKK223-3 (no insert)	WHITE
pKKMBpst (wild type)	RED
pKKS51	WHITE
pKKS51/D113/L16	RED
pKKS51/D113	RED
pKKS51/L16	WHITE
pKKD113	WHITE
pKKL16	RED

Plasmids were in strain DW1/pSUmelA. Plates were grown for 2 days at 30°C.

constructed by site-directed mutagenesis. The results given in Table 1 show that while the single mutants S51 and D113 fail to ferment melibiose, the double mutant S51/D113 has good activity. On the other hand L16 shows good activity as a single mutant but fails to restore activity to S51 in the double mutant S51/L16.

Transport studies were carried out with the plasmids in DW2R. Melibiose (0.2 mM) accumulation was measured in the wild type and mutants in the absence of added cation and in the presence of either 10 mM NaCl or 10 mM LiCl. A representative experiment is shown in Figure 2. The wild type gave a low level of accumulation without added cation. When 10 mM Li^+ was added there was strong stimulation of melibiose uptake, while NaCl (10 mM) caused even greater stimulation. The strain containing the original mutation (S51) showed no accumulation of melibose under any of the three conditions. In contrast, the revertant (S51/D113/L16) had regained ability to accumulate melibiose although the level reached was low. Stimulation of melibiose uptake by Na^+ was 2.5-fold in the revertant compared to 20-fold for the wild type, while stimulation by Li^+ was 4.5-fold compared to the wild type figure of 13-fold. The double mutant,S51/D113 showed similar properties to those of

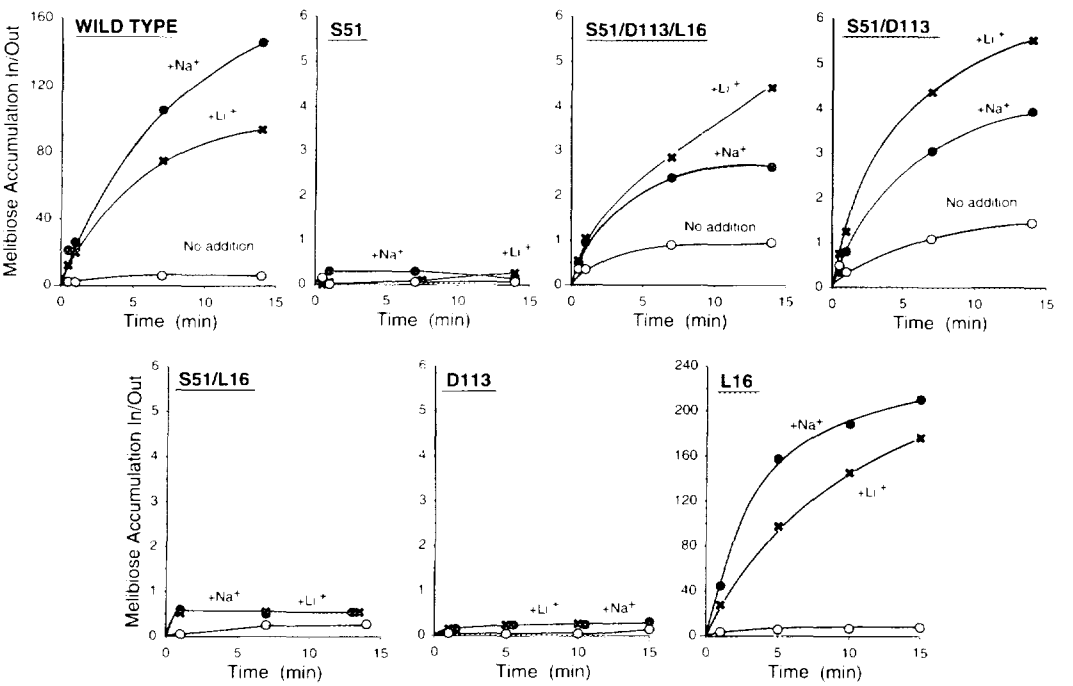

Fig. 2. Melibiose(0.2mM) accumulation by cells of DW2R containing mutant plasmids. Na^+ or Li^+ concentration was 10mM.

S51/D113/L16. Accumulation of melibiose by S51/D113 was slightly higher than that for S51/D113/L16 in spite of the smaller amount of carrier protein found in the membrane (Fig. 3). The single mutant L16 shows considerably greater activity than normal especially in the presence of Li^+. However the double mutant S51/L16 showed no accumulation of melibiose under these conditions.

The carrier protein present in the membrane was assayed immunologically (Fig. 3). The two double mutants showed about 50% of the wild type value; S51 and D113 showed 12% and 8% respectively. It is interesting that the presence of the L16 mutation increases the amount of carrier protein in the membrane in all cases. For example, addition of L16 to S51 caused a 5-fold increase although melibiose transport was still negative.

The possibility that the low melibiose transport activity of the revertant might be due to poor affinity for the co-transported cation was tested in the case of Na^+. In Fig. 4 is shown the effect of increasing Na^+ concentrations on the accumulation of 0.2mM melibiose by the wild type and the revertant. The wild type showed inhibition of uptake by concentrations of Na^+ greater than 10 mM whereas the transport activity of S51/D113 and S51/D113/L16 increased with concentrations of Na^+ up to 100mM.

The melibiose concentration was then varied at a fixed concentration of NaCl (50mM). S51/D113/L16 and S51/D113 showed an uptake of melibiose at 2mM 7 or 8-fold greater than that observed at 0.2mM (Table 2) indicating a poor affinity of the carrier for sugar. The wild type cell showed only 1.2-fold increase in uptake.

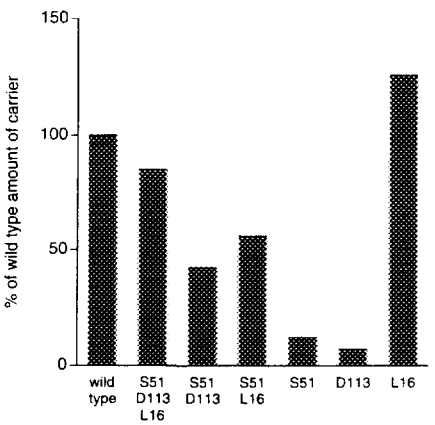

Fig. 3. Carrier protein in the membrane assayed immunologically. An antibody to the C-terminal 10 amino acids (15) was used in the immunoblot assay (16).

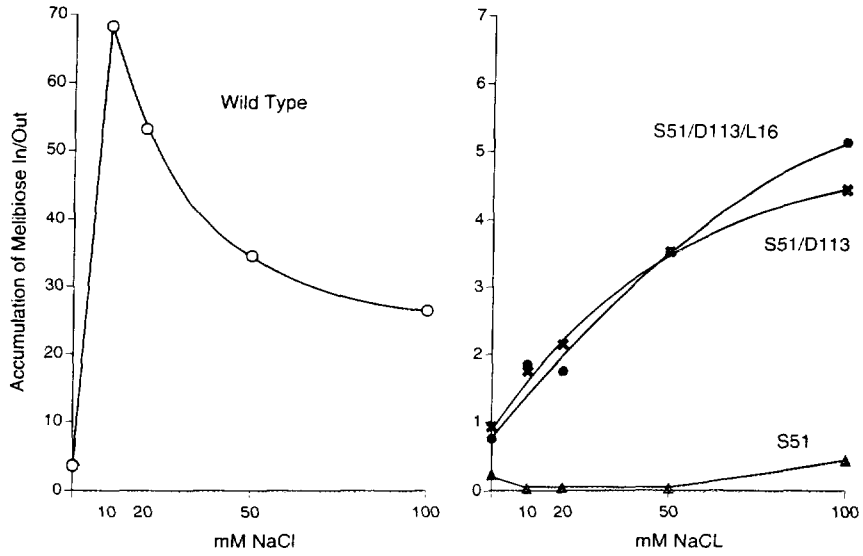

Fig. 4. Effect of Na^+ concentration on melibiose (0.2mM) accumulation by wild type and mutants. Plasmids expressed in DW2R.

S51, D113 and S51/L16 showed no significant uptake when tested with 1mM melibiose in the presence of 50 mM NaCl (data not shown).

Table 3 shows the accumulation of TMG by various strains. There was no uptake of TMG by the mutant cells except in the case of L16 where transport was identical to the wild type.

The major interest of this study is that the loss of an aspartic acid residue at position 51 can be partially compensated for by the introduction of an aspartic acid at position 113. Presumably in the 3-dimensional structure of the protein position 113 in helix 4 is close to position 51 in helix 2. Apparently a carboxyl group in this general region is important for function.

Table 2. Effect of increasing concentrations of melibiose on sugar transport

Mutant	Melibiose uptake (nmol/mg protein/5 min)			
	0.2mM	0.5mM	1mM	2mM
Wild type	57.7	52.3	56.9	71.6
S51	0.2	0.4	0	0.7
S51/D113/L16	4.4	12.2	25.6	35.2
S51/D113	3.1	6.3	16.0	20.7

Plasmids were in strain DW2R. Cells were grown at 30°C, with α-methylgalactoside (10mM) added to induce α-galactosidase.

Table 3. Accumulation of thiomethylgalactoside (TMG) at 10 min

Plasmid	Concentration IN/Concentration OUT		
	No addition	10mM Na^+	10mM Li^+
pKKMBpst	7.3	196	235
pKKS51	0	0	0
pKKS51/D113/L16	0.1	0.3	0.1
pKKS51/D113	0	0.3	0.2
pKKS51/L16	0	0.1	0.1
pKKL16	3.9	195	212

The value for pKK223-3 (no insert) was subtracted. External concentration of TMG was 0.1mM.

This phenomenon of substitution of carboxyl groups has been observed in the c subunit of the F_o portion of the F_1F_o ATP-ase in E.coli (17). The loss of ASP61 can be compensated for by the mutation ALA24→ASP in the adjacent α-helix. Similar cases of negatively charged substitutions have been described for the tetracycline carrier (18) and the photosynthetic reaction center of Rhodobacter sphaeroides (19). The latter three are examples of proton translocation systems. The present study is the first report of a comparable type of second site revertant in a cotransport system involving Na^+.

ACKNOWLEDGMENT

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REFERENCES

1. Leblanc,G.,Pourcher,T. and Zani,M-L. (1993) in Molecular Biology and Function of Carrier Proteins (Reuss,L.J.M., Russel,J.Jr. and Jennings, M.C.,eds) pp213-227,Rockefeller Univ Press
2. Yazyu,H.,Shiota-Niiya,T.,Shimamoto,T.,Kanazawa,H.,Futai,M.and Tsuchiya,T. (1984) J.Biol.Chem. 259: 4320-4326
3. Botfield,M.C.,Naguchi,K.,Tsuchiya,T.and Wilson,T.H.(1992) J. Biol. Chem. 267: 1818-1822
4. Zani,M.L.,Pourcher,T.and Leblanc,G. (1994) J.Biol.Chem. 269: 24883-24889
5. Pourcher,T.,Deckert,M.,Bassilana,M.and Leblanc,G. (1991) Biochem. Biophys. Res. Comm. 178: 1176-1181
6. Wilson,D.M. and Wilson,T.H. (1992) J.Bacteriol. 174:3083-3086
7. Pourcher,T.,Zani,M-L.,and Leblanc,G. (1993) J. Biol. Chem. 268: 3209-3215
8. Zani,M-L., Pourcher,T. and Leblanc,G. (1993) J. Biol. Chem. 268: 3216-3221
9. Wilson,D.M. and Wilson,T.H.(1994) Biochim. Biophys.Acta 1190: 225-230

10. Wilson,D.M. and Wilson,T.H. (1987) Biochim. Biophys. Acta 904: 191-200
11. King,S.C. and Wilson,T.H. (1989) J.Biol. Chem. 264: 7390-7394
12. Vaccaro,K.K. and Siegel,E.C. (1977) Mutant Res. 42: 443-446
13. Botfield,M.C. and Wilson,T.H. (1989)J.Biol.Chem.264:11643-11648
14. Sanger,F.,Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 5463-5467
15. Botfield,M.C. and Wilson,T.H. (1989)J.Biol.Chem.264:11649-11652
16. Lolkema,J.S., Puttner,I.B. and Kaback,H.R. (1985) Biochemistry 27: 8307-8310
17. Miller,M.J.,Oldenberg,M. and Fillingame,R.H. (1990) Proc. Natl. Acad. Sci U.S.A. 87: 4900-4904
18. Yamaguchi,A.,Oyauchi,R.,Someya,Y.,Akasaka,T.and Sawai,T. (1993) J. Biol. Chem. 268: 26990-26995
19. Rongey,S.H.,Paddock,M.L.,Feher,G. and Okamura,M.Y. Proc.Natl. Acad. Sci. U.S.A. 90: 1325-1329