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Transport properties of Asp-51 → Glu and Asp-120 → Glu mutants of the melibiose carrier of *Escherichia coli*

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Abstract

Asp-51 → Glu and Asp-120 → Glu mutants of the melibiose carrier of *Escherichia coli* were investigated for their cation/sugar cotransport properties. The carrier containing Glu-51 showed proton/melibiose cotransport but was extremely defective in Na^+ or Li^+ stimulation of sugar accumulation. On the other hand, the carrier containing Glu-120 had lost the ability to couple protons with melibiose uptake while retaining considerable Na^+ or Li^+ cotransport with melibiose (40-fold accumulation versus 90-fold for the wild type in the presence of Na^+). It is concluded that both Asp-51 and Asp-120 are important for cation recognition.

Key words: Melibiose carrier; Cation cotransport

1. Introduction

The melibiose carrier of *Escherichia coli* is a sugar/cation cotransport system which can utilize H^+, Na^+ or Li^+ for the translocation of a range of α-galactosides, β-galactosides and monosaccharides across the cytoplasmic membrane (see Refs. 1 and 2 for reviews). The gene coding for this carrier (*melB*) has been sequenced [3] and the secondary structure deduced from hydropathy plots [3] and from pho A fusion experiments[4]. During study of the effect of cations on this carrier it was observed that cells growing on melibiose were inhibited by the presence of 10 mM LiCl [5]. As a result, lithium-resistant mutants could be isolated. The first mutant of this type to be sequenced showed a change of Pro-122 to Ser [6]. This cell was dependent on Li^+ or Na^+ for melibiose transport as it no longer coupled melibiose transport to protons. Additional Li^+-resistant mutants have been described which use Na^+ but not Li^+ or H^+ for cotransport with melibiose [7]. Using a different method of mutant selection 23 sugar recognition mutants were isolated as cells that grew on melibiose in the presence of a competitive inhibitor, thiomethylgalactoside [8]. An interesting finding was that all but one of the mutants showed increased resistance to Li^+ as well as altered sugar affinity. Thus, the mutants showed alterations in both sugar and cation recognition.

Recently site specific mutagenesis has been used to substitute Cys or Asn for Asp-31, Asp-51, Asp-55 or Asp-120 in putative transmembrane helices 1, 2, and 4 [9–11]. These replacements result in loss of TMG transport activity in all cases except that of Asp-31 → Asn. Sugar binding still occurs but it is no longer enhanced by Na^+ suggesting that these amino acids may be important for Na^+ binding. The role of two of these aspartic acid residues, Asp-51 and Asp-120, was studied in this laboratory [12] by changing these residues individually to amber codons. Plasmids containing Amb-51 or Amb-120 were placed in eight different amber suppressor strains. Of the eight substitutions at each position only Glu-51 and Glu-120 showed activity.The purpose of the present study was to investigate in greater detail the changes in the physiology of sugar and cation transport of Glu-51 and Glu-120.

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Abbreviations: TMG, methyl β-D-thiogalactopyranoside; LB, Luria-Bertani broth; Mops, 4-morpholinepropanesulfonic acid.

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2. Materials and methods

2.1. Materials

Bacteriological media were from Difco. [^{3}H]Melibiose was a generous gift from Dr. Gerard Leblanc. [*Me*-^{14}C]Thiomethylgalactoside (TMG) was from Du Pont New England Nuclear. [*Me*-^{14}C]Lactose was from Amersham. The three radioactive sugars were purified by descending paper chromatography (Whatman No. 1 paper) using propanol/water (3 : 1, v/v). Other chemicals were of the highest quality commercially available.

2.2. Strains and plasmids

E. coli strain DW2R (*lacI*$^+$ *Δ(ZY) melA*$^+$ *ΔB Tn10 :: recA*) [13] was used in this work. The plasmid pKKMB is pKK223-3 with the *melB* insert [14]. By site-directed mutagenesis, ASP 51 and ASP 120 of the *melB* gene on plasmid pKKMB had been separately replaced by an amber codon and transformed into DW2R [12]. The resultant strains gave white colonies on melibiose MacConkey plates (plus ampicillin and tetracycline) at 37°C. After 2–3 days red revertants appeared. When the plasmid DNA was sequenced some of the revertants were found to have glutamic acid as the replacement for aspartic acid. The plasmids containing these mutations were designated pKKGlu-51 and pKKGlu-120, respectively.

The *melB* gene in each of these revertants was completely sequenced and no other mutations were found.

2.3. Growth of cells

Cells of DW2R were routinely grown to mid log phase in Luria-Bertani broth (LB) to which was added ampicillin (100 μg/ml) and tetracycline (10 μg/ml). Under these conditions the chromosomal α-galactosidase is not induced. However, for measurement of the K_m for melibiose transport by the pKKGlu-51 carrier the cells were induced with α-methylgalactoside or melibiose.

2.4. Transport of sugars

Cells were washed three times in 120 mM Mops (adjusted to pH 7 with tetramethyl ammonium hydroxide) and were resuspended in the same buffer to a density corresponding to 1.1 mg dry wt./ml. NaCl or LiCl was added where indicated. The reaction was started by the addition of radioactive sugar to the cell suspension at 22°C. At given time intervals samples were filtered rapidly through 0.65 μm pore size nitrocellulose filters (Sartorius Filters) washed and counted in a liquid scintillation counter using Liquiscint (National Diagnostics).

2.5. Proton movement

Proton uptake was measured by the method of West [15]. The cells were washed twice in 120 mM choline chloride and then resuspended in the same medium with the addition of 10 mM KSCN. The cell density in the assay was equivalent to 8 mg dry wt./ml. Potassium thiocyanate was added to give 10 mM. The cell suspension (2.5 ml) was placed in a 3 ml plastic vial and was stirred by a magnetic stirring bar. The combined pH electrode was from Radiometer, Copenhagen (GK 2321-C). This electrode was inserted through a hole in the vial lid. A small vent in the lid was used for passage of nitrogen over the fluid in the vial; a second vent served for introduction of the sugar. The assay was performed at approx. pH 6 which was the pH of the cell suspensions without adjustments. After passage of nitrogen through the vial for at least 30 min, anaerobic sugar solution (pH 6) was added. Proton movement was recorded on a Linear Instruments recorder.

2.6. Sodium uptake

Sodium uptake was measured with a sodium electrode (Radiometer Copenhagen G502Na). The cells were washed twice and resuspended in 100 mM Tricine buffer pH 8 at a cell density of 8 mg dry wt./ml. The final mixture contained 10 mM KSCN and 50 μM NaCl. A 5 ml plastic vial (2 cm in diameter) had two holes in the lid for the sodium electrode and the calomel electrode (Radoiometer Copenhagen K401) plus two additional small vents for introduction of nitrogen or substrates.

3. Results

The uptake of melibiose by the two mutant carriers was compared with that of the wild-type carrier (pKKMB) in DW2R. As this strain contains an inducible α-galactosidase the cells were grown in the absence of inducer. Production of the melibiose carrier expressed from the plasmid does not require inducer and cells grown under these conditions accumulate melibiose when exposed to the sugar. Since it was of interest to determine any change in cation cotransport shown by the altered carriers, the transport of melibiose was measured in the absence of added cations (H^+ cotransport) or in the presence of 20 mM Na^+ or Li^+. It can be seen in Fig. 1 that there is a 7-fold accumulation of melibiose by the wild type (DW2R/pKKMB) in the absence of added cations. The addition of Na^+ or Li^+ (20 mM) caused marked stimulation of uptake of the

sugar in this strain. In the case of the Glu-120 carrier there was 40-fold accumulation of melibiose in the presence of 20 mM Na^+ and 30-fold accumulation in the presence of Li^+. With either ion the rate of transport by Glu-120 was approx. 20% of the wild type and the level of accumulation reached 50% of normal. In contrast to the wild type, however, Glu-120 showed no uptake of sugar in the absence of added cations, indicating that proton/melibiose cotransport was not functioning under these conditions. A different pattern of uptake was shown by the mutant Glu-51. In this case approx. 50% of the melibiose uptake activity shown by the wild type in the absence of Na^+ or Li^+ was retained by the Glu-51 mutant. The enhancement of melibiose accumulation in the presence of Na^+ or Li^+ however, was drastically reduced. On the addition of 20 mM Na^+ Glu-51 accumulated melibiose 7-fold compared with 90-fold for the parent.

The effect of sodium ion concentration on 0.2 mM melibiose transport was tested in the normal and the two mutants. Fig. 2 shows that a very high sodium concentration is necessary to achieve maximal stimulation of melibiose uptake in Glu-51. The K_m for sodium in Glu-51 was approx. 75 mM while that for Glu-120 was 7 mM (Table 1). Thus, the affinity for Na^+ in the two mutants was much less than the normal ($K_m = 0.35$ mM). The effect of sugar concentration on the transport rate was measured in the presence of saturating concentrations of sodium ion (Table 2). In the case of Glu-51 the K_m for melibiose transport was slightly higher than normal while the V_{max} was 23% of normal. With Glu-120 the K_m was more than 20-fold higher than that of the wild type while the V_{max} was only slightly less than normal.

The effect of pH on melibiose uptake by the normal cell and mutants was measured in the presence of 20 mM Na^+ (Fig. 3). The uptake by the normal cell at pH

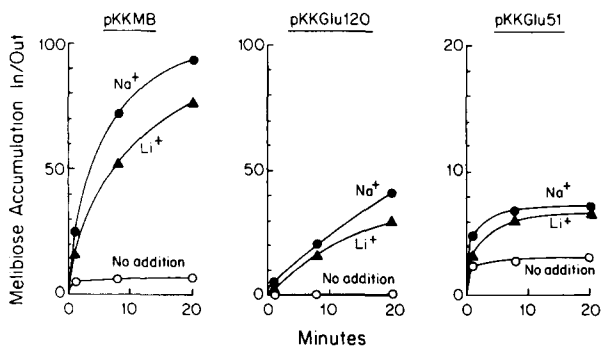

Fig. 1. Melibiose accumulation by cells of DW2R containing pKKMB, pKKGlu120 or pKKGlu51. Cells were grown in LB and washed three times in 120 mM Mops buffer (pH 7). Cells were exposed to 0.2 mM [^{3}H]melibiose in media without added cation or with 20 mM Na^+ or Li^+. Control values obtained for melibiose uptake by DW2R/pKK223 (no *melB* insert) have been subtracted. Note that a 5-fold larger scale is used for the pKKGlu51 than for the other two.

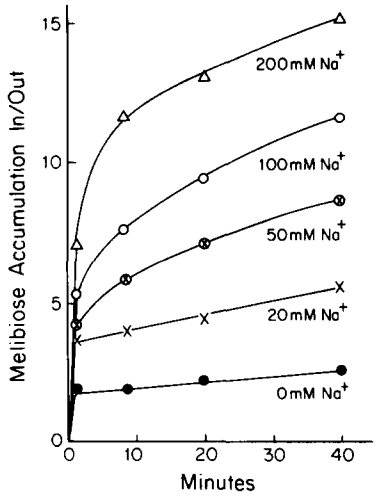

Fig. 2. The effect of Na^+ concentration on 0.2 mM melibiose accumulation by DW2R/pKKGlu51. Cells were incubated in medium of 50 mM Mops (pH 7) plus a mixture of NaCl and choline Cl to give a final total concentration of 200 mM.

8 was maximal while uptake at pH 7 and pH 6 was progressively less. In the case of Glu-120 melibiose uptake at pH 6 was comparatively poor (10-fold at 20 min compared with 100-fold for the normal). On the other hand, at pH 8 uptake by Glu-120 reached 100-fold at 20 min. Since the Glu-120 mutant no longer shows proton cotransport this pH effect is presumably due to an effect on the Na^+ cotransport mechanism alone. Melibiose uptake by Glu-51 was only slightly affected by pH.

Sugar-induced sodium ion uptake at pH 8 was measured. In these experiments cells were made anaerobic

Table 1
Effect of sodium on melibiose transport

Plasmid	K_m for Na^+
Normal	0.35 mM
Glu-51	75 mM
Glu-120	7 mM

Melibiose concentration was 0.2 mM.

Table 2
Effect of melibiose concentration on transport

Plasmid	K_m	V_{max} (nM/min per /mg dry wt.)
Normal	0.14 mM	37.6
Glu-51	0.23 mM *	8.8 *
Glu-120	3.3 mM	29

The Na^+ concentration was that which gave maximal melibiose transport for each cell (20 mM Na^+ for pKKMB and pKKGlu-120 and 200 mM Na^+ for pKKGlu-51).

* In these experiments sugar uptake was measured in cells induced for α-galactosidase. Under these conditions entry of melibiose is thermodynamically 'downhill' since it is metabolized by the cell.

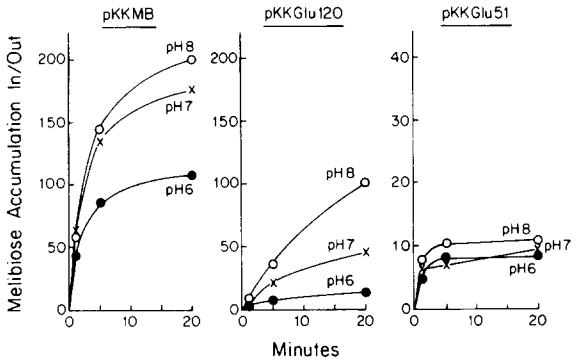

Fig. 3. The effect of pH on 0.2 mM melibiose accumulation by cells of DW2R containing pKKMB, pKKGlu120 and pKKGlu51. Cells were incubated in a medium containing 120 mM Mops with the pH adjusted by the addition of tetramethylammonium hydroxide. Note that a 5-fold larger scale is used for the pKKGlu51 than for the other two.

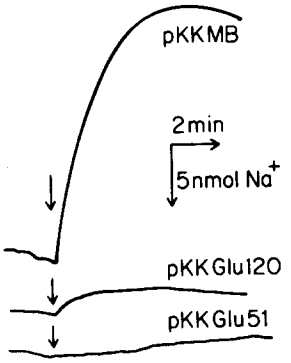

Fig. 4. Melibiose-induced Na^+ uptake. Anaerobic cells were incubated in 100 mM Tricine buffer (pH 8) plus 10 mM KSCN and 50 μM NaCl in the presence of a Na^+-specific electrode. Melibiose was added at the arrow to give a final concentration of 10 mM.

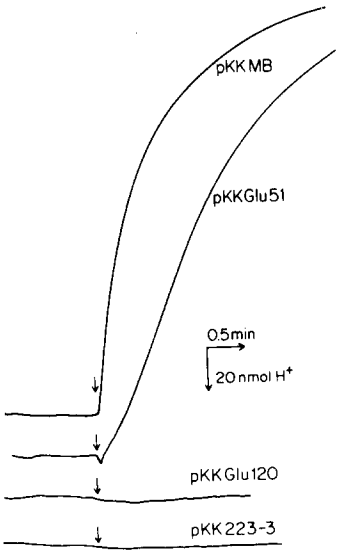

Fig. 5. Melibiose-induced proton uptake. Anaerobic cells were incubated in 120 mM choline Cl and 10 mM KSCN. Initial pH was approx. pH 6. Melibiose was added at the arrow to give a final concentration of 10 mM.

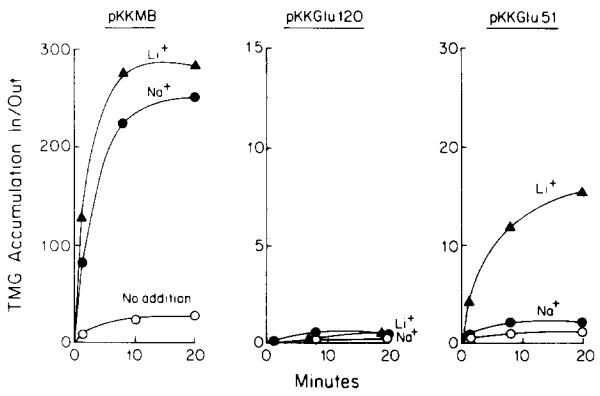

Fig. 6. TMG accumulation by cells of DW2R containing pKKMB, pKKGlu120 or pKKGlu51. Cells were incubated in the presence of 0.1 mM [^{14}C]TMG with or without 20 mM Na^+ or Li^+. Note that the scale differs in the three graphs. Control values for TMG uptake by cells without the *melB* insert have been subtracted.

to block the normal respiration-driven proton pumping and secondary Na^+ movement. The addition of 10 mM melibiose to wild-type cells caused uptake of sodium ion as shown by the fall in external sodium ion concentration which was measured with a sodium electrode (Fig. 4). A small but distinct sodium uptake was observed with Glu-120 but extremely slow uptake occurred with Glu-51. Because of the poor affinity of the mutants for sodium (Table 1), Na^+ uptake was measured with a higher external Na^+ concentration (100 μM). However, the elevated 'background' caused decreased sensitivity and the recordings showed even less deflection than in Fig. 4.

Proton uptake induced by melibiose addition to anaerobic cells was measured (Fig. 5). The cell suspension containing the wild-type melibiose carrier (pKKMB) showed a prompt alkalinization following the addition of 10 mM melibiose. The initial rate of proton uptake by Glu-51 was approx. 60% of normal and the

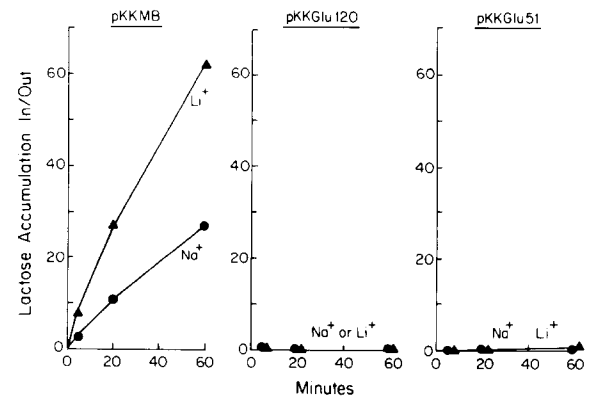

Fig. 7. Lactose accumulation by cells of DW2R containing pKKMB, pKKGlu120 and pKKGlu51. Cells were incubated in the presence of 0.1 mM [^{14}C]lactose with or without 20 mM Na^+ or Li^+. Control values obtained for lactose uptake by DW2R/pKK223-3 have been subtracted.

equilibrium value was similar to the normal. Glu-120 showed no proton uptake. The latter observation is consistent with the failure of Glu-120 to accumulate melibiose in the absence of added cations (Fig. 1).

The normal cell accumulated TMG 25-fold in the absence of added Na^+ or Li^+, 250-fold in the presence of Na^+ and 270-fold in the presence of Li^+ (Fig. 6). Glu-51 accumulated TMG only about 3-fold with Na^+ and 15-fold in the presence of Li^+. Glu-120 failed to accumulate TMG under any of the conditions tested.

Lactose was accumulated 27-fold by the normal cell in the presence of sodium (Fig. 7). In the presence of lithium accumulation was 63-fold. No lactose accumulation was observed by either of the two mutants.

4. Discussion

There are striking alterations in the effect of cations on sugar transport in both of the mutants, Glu-51 and Glu-120. In the case of Glu-51 the affinity of Na^+ for stimulation of melibiose transport is extremely poor. Fig. 2 shows that a very high concentration of Na^+ is required to give maximal uptake. The calculated K_m based on initial rates of transport was 75 mM compared with the value of 0.35 mM for the wild type. However, the K_m for melibiose in the presence of 200 mM Na^+ did not differ to any great extent from the wild type. In addition the Glu-51 carrier demonstrates considerable proton/sugar cotransport activity. The melibiose-induced proton uptake (Fig. 6) is approx. 50% of normal and proton coupled accumulation of melibiose (Fig. 4) is 3-fold compared with a normal of 7-fold. Since the carrier protein of Glu-51 is present in the membrane to the extent of 70% of normal by an immunological assay [12] the proton/sugar transport activity per carrier molecule in the membrane is rather similar to that of the wild type. Thus, the recognition of melibiose does not seem much changed in the Glu-51 carrier whereas the recognition of sodium is drastically changed.

The physiological properties of Glu-120 differ from those of Glu-51. Proton/sugar cotransport could not be demonstrated in the case of Glu-120. Similar observations have been made by Leblanc et al. [2] who found that cells containing the Glu-120 mutation exposed to 0.8 mM melibiose showed no significant sugar uptake in the absence of Na^+ and Li^+. On the other hand Na^+/melibiose cotransport was active. In the experiment shown in Fig. 1 the rate of Na^+-stimulated melibiose accumulation was approx. one quarter that of the normal in the presence of 20 mM NaCl. The affinity for Na^+ however, was very poor (K_m for Glu-120 was 7 mM compared with 0.35 mM for the normal). Similarly the affinity for melibiose was very low (a K_m more than 20 times that of the parent) although the V_{max} was approx. normal. Thus, it appears that proton/melibiose cotransport is completely lost in the Glu-120 mutant while Na^+/melibiose cotransport is retained with a reduced affinity for both sugar and cation.

Because of the substantial melibiose uptake at pH 8 (100-fold accumulation) by Glu-120 in the presence of 20 mM Na^+ it is probable that sodium uptake under these conditions is very significant. Distinct sugar stimulation of Na^+ uptake at pH 8 was in fact observed with Glu-120 in the Na^+ electrode experiments but it was found to be relatively weak at the extremely low concentration of Na^+ (50 μM) required for this particular assay. With a K_m of 7 mM the rate of uptake of Na^+ at 50 μM would be expected to be extremely low. We suspect that the total Na^+ uptake is large when the Na^+ concentration is high (20 mM) and the melibiose concentration is high (10 mM) but this has not been measured experimentally.

Previously several amino acid substitutions for Asp-51 and Asp-120 had been prepared by changing the residues individually to amber codons and placing a plasmid containing these mutations into amber suppressor strains. The following substitutions were made by this method: Ser, Gln, Tyr, Lys, Leu, Ala, His, and Glu. Only the glutamic acid substitutions showed melibiose transport. Cysteine and asparagine substitutions of Asp-51 have been studied by Zani et al. [11]. They showed that Cys-51 retains some measure of proton/melibiose cotransport but has lost the Na^+-stimulated melibiose uptake and the Na^+-stimulated α-pNPG binding. On the other hand Asn-51 had lost all transport activity although it retained α-pNPG and melibiose binding activity. The substitution of Cys or Asn for Asp-120 resulted in the failure of Na^+ to stimulate transport or sugar binding [10]. Thus, all of the neutral amino acid substitutions for Asp-51 and Asp-120 lead to a carrier inactive for Na^+. However, the presence of a negative charge (Glu substitution) permitted Na^+ stimulation of sugar transport although the affinity for Na^+ was extremely poor. Thus, both Asp-51 and Asp-120 are important for Na^+ cotransport.

There are several possible explanations for the effect of the mutations on the recognition of Na^+. The most attractive of these is that Asp-51 and Asp-120 might be part of the cation binding site, as has been proposed by Leblanc and co-workers [2,9–11]. However, one cannot yet exclude the possibility that the two residues have an allosteric effect on a binding site in another location.

An interesting observation is that the substitution of Glu for Asp-51 and Asp-120 results in an apparent change in sugar recognition by the carrier. Glu-51 shows a reduced rate of transport of TMG and lactose. The K_m for melibiose in Glu-120 is increased more than 20-fold and TMG and lactose were not trans-

ported. Thus, both sugar recognition and cation recognition are altered in these mutants, consistent with similar observations on lithium-resistant mutants [6,7] and TMG-resistant mutants [8]. One possibility is that the cation binding site and the sugar binding site communicate through relatively long-range allosteric interactions. An alternative explanation is that the sugar and cation binding sites overlap [8].

5. Acknowledgements

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