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The interaction between aspartic acid 237 and lysine 358 in the lactose carrier of *Escherichia coli*

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The *lacY* from *Escherichia coli* strains 020 and AE43 have been cloned on plasmids which were designated p020-K358T and pAE43-D237N. These *lacY* mutants contain amino acid substitutions changing Lys-358 to Thr or Asp-237 to Asn, respectively. The charge neutralizing effect of each mutation is associated with a functional defect in melibiose transport which we exploited in order to isolate second site revertants to the melibiose-positive phenotype. Eleven melibiose-positive revertants of p020-K358T were isolated. All contained a second-site mutation converting Asp-237 to a neutral amino acid (8 to Asn, 1 to Gly, and 2 to Tyr). Twelve melibiose-positive revertants of pAE43-D237N were isolated. Two were second-site revertants converting Lys-358 to a neutrally Gln residue, while the remainder directly reverted Asn-237 to the wild-type Asp-237. We conclude that the functional intimate relationship between Asp-237 and Lys-358 suggests that these residues may be closely juxtaposed in three-dimensional space, possibly forming a 'charge-neutralizing' salt bridge.

Introduction

The lactose carrier of *Escherichia coli* is a well studied example of a cation substrate cotransport system in which the coupling ion is H^+ (for reviews, see Refs. 1, 2). As a result of the sequencing studies of Büchel et al. [3] it is known that the molecule contains 70% hydrophobic amino acids. On the basis of hydropathy plots and α-helical content Foster et al. [4] proposed a model of 12 transmembrane segments (mainly α-helical) with the N-terminus and C-terminus on the cytoplasmic side. This topological model of secondary structure has been supported by studies of Calamia and Manoil [5] who used alkaline phosphatase gene fusions to identify segments of the lactose carrier having exposure to either the cytoplasmic or periplasmic spaces.

Recent studies of Bibi and Kaback [6] suggest that secondary structural elements in the N-terminal and C-terminal halves of the carrier molecule can be independently organized into functional domains. They have constructed two separate plasmids, one expressing the N-terminal half of the lactose carrier and the other expressing the C-terminal half of the protein. Coexpression of the N-terminal and C-terminal polypeptides within a single strain resulted in 30% normal transport function, whereas expression of either terminal polypeptide without the other failed to produce functional carrier activity [6]. Such observations imply: (1) that there must be interactions between the two domains which allow them to functionally associate within the memorane, and (2) that within either domain there must be a set of interactions which contribute to the intrinsic stability of the domain. The nature of these interactions is at present unknown.

Our laboratory has begun to approach this problem in a general way by using mutagenesis to look for evidence that certain amino acid side chains interact with one another, even though they may be separated

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Abbreviations: TMG, methyl β-D-thiogalactopyranoside; XG, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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by a considerable linear distance in the polypeptide backbone of the lactose carrier. There is a particularly interesting interaction between two functionally important charged residues (Asp-237 and Lys-358) located within the second half of the carrier molecule.

Because of the large energy requirement for placing a single charged amino acid residue in the hydrophobic environment of a membrane such residues are a relatively infrequent occurrance in many membrane-spanning α-helices. On the other hand when present they frequently have specific physiological functions. The importance of His-322, Glu-325 and Arg-302 in membrane-spanning segments of the lactose carrier has been emphasized by the work of Kaback and his collaborators [1,7–10]. Because of the close proximity in the α-helix between His-322 and Glu-325 they are probably salt bridged. The change of each of the three residues individually to a neutral amino acid results in almost complete loss of the ability to accumulate sugars and a defect in proton cotransport. The conversion of another charged amino acid Lys-319 to a neutral residue changes the sugar specificity [11].

The purpose of this paper is to further explore the possible role of charged amino acids in membrane spanning regions of the carrier. We describe experiments involving second site revertants which provide support for the view that Asp-237 is closely associated with Lys-358.

Materials and Methods

Materials. T7 polymerase was used for double stranded sequencing utilizing the kit provided by Pharmacia. Lactose, Melibiose, TDG, ONPG, and TMG were from Sigma. Deoxyadenosine 5′-[α-^{35}S]triphosphate and [D-*glucose*-1-^{14}C]lactose were from Amersham International. [*Me*-^{14}C]TMG was from New England Nuclear. [^{3}H]Melibiose was a generous gift of Dr. Gérard Le Blanc [12]. All radioactive sugars were purified by descending paper chromatography (Whatman No. 1 paper) using propanol/water (3 : 1, v/v). Bacteriological media were from Difco. Other chemicals were obtained from usual sources and were of the highest quality commercially available.

Bacterial strains and plasmids. All strains are *Escherichia coli* K-12. The genotypes of these strains along with the plasmids they carry are given in Table I. Nucleotide changes found in the mutants are given in Table II. The plasmid, pMB050-W4680, was a gift from Dr. Michael Malamy. This plasmid is similar to pMB040 which has been described [16]. Plasmid pRAF-S11 is an invertase-constitutive derivative of the raffinose-positive

TABLE I

Genotypes for plasmids and E. coli K-12 strains

	Genotype chromosome/F′-factor/plasmid	Source or reference
Strain		
DP90C Nal	*Δ(lac-pro)thi nal A/−/−*	Hobson et al. [13]
DP90CY	DP90CNal/*lacI^{Q}Z^{+}Y^{+}A^{+}/−*	Hobson et al. [13]
AE43	DP90CNal/*lacI^{Q}Z^{+}Y^{-}A^{+}/−*	Hobson et al. [13]
020	DP90CNal/*lac^{Q}Z^{+}Y^{-}A^{+}/−*	Hobson et al. [13]
DW1	*lacI^{+}Δ(ZY)melΔ (AB)strA/−/−*	Wilson and Wilson [14]
DW2	*lacI^{+}Δ(ZY)melA^{+}ΔB strA/−/−*	Wilson and Wilson [14]
DW10Y^{-}	DW2/*lacZ^{+}Y^{-}/−*	King and Wilson [15]
TG1	*Δ (lac-pro), supE, thi*, hsdD5/F′ *traD36, proA^{+}b^{+} lacI^{Q}, zΔM15/$^{-}$*	Amersham Corp.
Plasmids		
pMB050-W4680	*−/−/lacO^{+}P^{UV5}Δ(Z)Y^{+}Δ(A)* amp^{r}	Malamy et al. [16]
pSCK1	*−/−/lacO^{+}P^{UV5}Z^{-}Y^{suc+}Δ(A)* amp^{r}	King and Wilson [15]
pRAF-S11	*−/−/Raf ABCD*	King and Wilson [15]
pDP90CY	*−/−/lacO^{+}P^{UV5}Z^{+}Y^{+}Δ(A)* amp^{r}	King and Wilson [15]
pAE43-D237N	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(D237N)}$Δ(A) amp^{r}*	this work
pAE43-D237N-K358Q	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(D237N-K358Q)}$Δ(A) amp^{r}*	this work
p020-K358T	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(K358T)}$Δ(A) amp^{r}*	this work
p020-K358T-D237N	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(K358T-D237N)}$Δ(A) amp^{r}*	this work
p020-K358T-D237G	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(K358T-D237G)}$Δ(A) amp^{r}*	this work
p020-K358T-D237Y	*−/−/lacO^{+}P^{UV5}Z^{+}Y$^{(K358T-D237Y)}$Δ(A) amp^{r}*	this work
pDW10Y^{-}	*−/−/lacO^{+}P^{UV5}Z^{+}Y^{-}Δ(A) amp^{r}*	King and Wilson [15]
pBR322	*−/−/amp^{r} tet^{r}*	Bolivar et al. [17]
pBRY	*−/−/lacΔ(IOPZ)Y^{+}Δ(A) tet^{r}*	King and Wilson [15]
pBRY-K358T	*−/−/lacΔ(IOPZ)Y$^{(K358T)}$Δ(A) tet^{r}*	this work
pTE18	*−/−/lacO^{+}P^{+}Δ(Z)Y^{+} amp^{r} tet^{r}*	this work
pTKY-D237G	*−/−/lacO^{+}P^{+}Δ(Z)Y$^{(D237G)}$ amp^{r} tet^{r}*	this work

TABLE II

The nucleotide changes and amino acid changes of mutants

Mutant	Nucleotide change (sense strand)	Amino acid substitution
p020-K358T	A1178 → C	Lys-358 to Thr
pAE43-D237N	G814 → A	Asp-237 to Asn
p020-K358T-D237N	A1178 → C	Lys-358 to Thr
	G814 → A	Asp-237 to Asn
p020-K358T-D237G	A1178 → C	Lys-358 to Thr
	A815 → G	Asp-237 to Gly
p020-K358T-D237Y	A1178 → C	Lys-358 to Thr
	G814 → T	Asp-237 to Tyr
pAE43-D237N-K358Q	G814 → A	Asp-237 to Asn
	A1177 → C	Lys-358 to Gln

plasmid from strain DS25-91 from Schmid and Schmitt [18]. The plasmid pDW10Y$^-$ contains a *lacY* gene with an *IS*1 insertion after base 170, resulting in complete loss of transport activity.

Growth conditions and media. Cell growth on liquid or solid media was always at 37°C. When used, the antibiotics were present at the following concentrations: ampicillin (100 μg/ml) or tetracycline (10 μg/ml) or streptomycin (100 μg/ml).

Oligonucleotide-directed mutagenesis. The *lacY* on a 2300-base pair EcoRI fragment taken from pTE18 was isolated by electrophoresis through 1% SeaPlaque agarose and then placed in the dephosphorylated EcoRI site of phage M13mp18 by ligation carried out in molten agarose according to the method of Struhl [19] except that the ligation buffer was as described elsewhere [20]. The mutagenic oligonucleotide 5′-CCTGCACCTAC-GaTGTTTTTGA-3′ was used to create the Asp-237 → Gly substitution in the lactose carrier (mutation in lower case bold). Oligonucleotide-directed mutagenesis was carried out in the host strain, TG1, by the method of Eckstein [21] as implemented commercially (Amersham Corp.) with exonuclease III. The mutant was rescued from the M13mp18 replicative form by restricting with EcoRI followed by isolation of *lacY* and ligation (as described above) into the dephosphorylated EcoRI site of pBR322. The ligated DNA was initially introduced into TG1, and transformants were selected as clones resistant to ampicillin and tetracycline. Plasmid DNA was isolated from several clones. A clone with the *lacY* insert oriented as in pTE18 was identified by restriction mapping with AvaI. That the *lacY* from pTKY-D237G contained no unexpected mutations was confirmed by placing the *lacY* insert back into the EcoRI site of M13mp18 for DNA sequencing by the method of Sanger et a. [22].

Cloning the lacY by homologous recombination. The plasmid, PSCK1, used in these studies is a sucrose-positive (*lacY*^{suc}) derivative of pMB050-W4680 (*lacΔZY*$^+$ *Amp*^{r}) that has been used to clone *lacY* mutants found on an F-factor [15,23]. The mutants 020 and AE43 were transformed by pSCK1 and spread on LB plates plus ampicillin. The transformed cells were mated to DW2 (*lacI*$^+$*ΔZYrpsL*) and spread on LB plates plus XG, ampicillin and streptomycin. The transfer of the ampicillin-resistant gene by conjugation involves the formation of a cointegrate between F-factor and plasmid, a process requiring recombination across the *lacZY* region. Six blue clones were picked pooled together and plasmid DNA isolated. This mixed plasmid DNA should contain plasmids of the original pSCK1 phenotype (*lacZ*$^-$ *Y*$^{suc+}$) as well as examples of the recombinant *lacZ*$^+$ *Y*$^{suc-}$ phenotype. Screening for the recombinant was performed by placing the plasmids in the genetic background of DW2/pRAF-S11 (constitutive for invertase) by transformation. The sucrose-negative recombinants (white colonies) could be readily distinguished from the sucrose-positive, non-recombinants (red colonies) MacConkey agar containing 2% sucrose and ampicillin. Further evidence of recombination was obtained by confirming that the sucrose-negative clones were *lacZ*$^+$ (blue on XG agar) and retained the pRAF-S11 (fermentation-positive on MacConkey agar containing 1% raffinose). We found that all sucrose-negative cells were *lacZ*$^+$ and raf$^+$.

Subcloning lacY in pBR322. The 2200 base pair PvuII restriction fragments from pDP90CY and p020-K358T were placed in the dephosphorylated ScaI site of pBR322 to construct the plasmids, pBRY and pBRY-K358T. In these constructs, the lactose carrier is expressed constitutively (though at low levels) from the *amp* promoter.

Plasmid DNA purification and sequencing. Double stranded plasmid DNA was extracted from cells and purified by the method of Qiagen (Studio City, CA) following the directions of the manufacturer. The entire *lacY* gene was sequenced by the method of Sanger et al. [22] using the T7 sequencing kit from Pharmacia (Piscataway, NJ). Appropriately spaced oligonucleotides complementary to the *lacY* coding strand were used in order to synthesize the second strand which was labeled with [α-^{35}S]dATP (> 600 Ci/mmol).

Isolation of melibiose-positive revertants. DW2/p020-K358T failed to ferment melibiose and thus appeared white after growth for 18 h on melibiose MacConkey agar. However, with continued incubation at 37°C for 48 more hours, a few small red papillae would occasionally appear on a plate containing several hundred white clones. A red papillus (one chosen per plate to ensure independence) would be picked and purified by restreaking. The purified revertant clones were fermentation-positive and grew bright red on melibiose MacConkey agar. Plasmid DNA isolated from each of several independent mutants was placed in *E. coli* DW2 by transformation. In about half the cases (eleven) the transformants were found to be red on melibiose Mac-

Conkey agar, strongly suggesting the involvement of the plasmid-borne *lacY* in the revertant phenotype. The *lacY* from such plasmids was sequenced to determine the mutant locus.

In the isolation of melibiose-positive revertants from DW2/pAE43-D237N we were unable to use the previous method because this cell gave red clones on melibiose-MacConkey plates. However, it was possible to isolate revertants on melibiose minimal plates with a very low sugar concentration (0.01%) plus ampicillin. When cells were spread on such plates it was possible to pick rare clones which grew in 2 days to a diameter of 0.2 mm. These clones were restreaked on minimal plates to purify and plasmid DNA was isolated. DW2 was transformed with such DNA and plated on 0.01% melibiose minimal plates (plus ampicillin). In 12 cases the melibiose-positive phenotype was found to be specified by the plasmid and the *lacY* gene from such plasmids was sequenced.

Sugar transport assays. Cells were grown overnight in LB medium containing the appropriate antibiotic. The next morning the cells were diluted 50-fold into the same medium and grown at 37°C for 3–4 doublings. Cells were harvested by centrifugation and washed once with an equal volume of 100 mM potassium phosphate buffer (pH 7.0). The cells were resuspended in the same buffer and used at the concentration indicated in the figure legends. Transport assays were carried out at 25°C in 100 mM potassium phosphate buffer (pH 7.0). Cells were incubated in the presence of radioactive sugar; samples were periodically removed, and vacuum filtered through 0.65 μm pore size filters (Sartorius). After washing with 5 ml of buffer, the filters were dissolved in either 4 ml of Liquiscint (National Diagnostics) for ^{14}C-labeled compounds or 4 ml of Liquiscint plus 10% water for [^{3}H]melibiose; radioactivity was quantitated in a scintillation spectrophotometer.

Counterflow experiments were carried out in 100 mM potassium phosphate buffer (pH 7.0) essentially as described by Wong and Wilson [24]. The cells were first preloaded with nonradioactive sugars for 30 min at room temperature in 100 mM potassium phosphate buffer (pH 7.0) containing 30 mM KN_3 and then centrifuged. Counterflow was initiated by resuspending the pellet in 2.1 ml of buffer containing 30 mM KN_3, and 0.1 mM [^{14}C]TMG. Reactions were terminated by filtering a 0.3 ml sample at the times indicated in the figure.

Proton transport. The pH electrode was used to measure the galactoside-dependent H^+ uptake into *E. coli* using the method of West [25]. The cells were grown to early log phase in 400 ml 1% tryptone/M63. The cells were washed twice in 120 mM KCl and then resuspended in 120 mM KCl-30 mM KSCN at a concentration of about $1 \cdot 10^{11}$ cells/ml and placed on ice until use. The cells were made anaerobic by bubbling with nitrogen prior to initiating the pH recordings which were carried out at room temperature. The cells (2.3 ml) were placed in a 3 ml plastic vial with the lid cut to fit over an inserted pH electrode. There was a small vent in the lid for the introduction of nitrogen and a second vent for the addition of substrate. N_2 was passed through

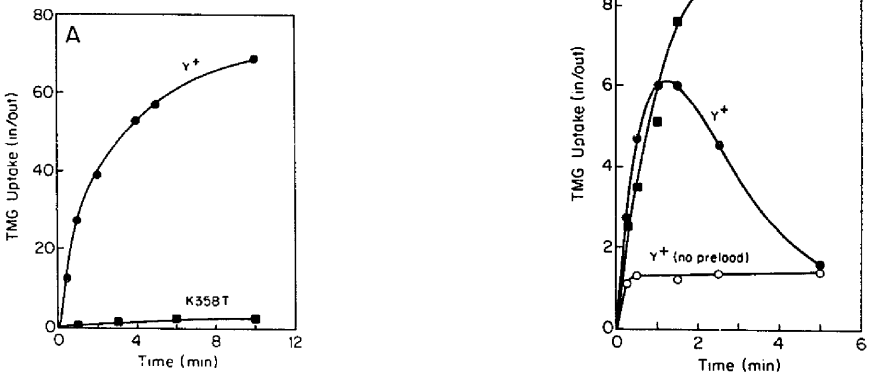

Fig. 1. TMG transport by parent and K358T. DW2 carrying a plasmid with the normal Y gene, pDP90CY (●), or with the mutant, p020-K358T (■), were grown in LB medium and harvested as indicated in Materials and Methods. Panel A: cells exposed to 0.1 mM TMG and the accumulation measured. Panel B: counterflow was measured as follows: azide treated cells were preloaded (● or ■) with 10 mM melibiose or not preloaded (○) and cells centrifuged. After discarding the supernatant the pellet was rapidly resuspended with buffer containing 0.1 mM radioactive TMG. Samples were taken at the indicated times and filtered as described in Materials and Methods.

one vent over the surface of the suspension, which was stirred with a magnetic stirrer. Proton uptake was initiated by the addition of various volumes (10–30 μl) of anaerobic sugar (0.5 to 1 M). The experiments were calibrated by adding 50 nmol of anaerobic HCl at the end of each record. The pH values were recorded with a Linear Instruments recorder. The combined pH electrode was from Radiometer, Copenhagen (GK 2321-C).

Results

A *lacY* mutant (020) was isolated which was resistant to the toxic galactoside thio-*o*-nitrophenyl-β-galactoside [26]. The accumulation of this galactoside via proton cotransport depletes the cell of its protonmotive force and mutants which fail to accumulate the sugar are resistant to its toxic effect on growth. This *lacY* mutation was cloned onto a plasmid (see Materials and Methods) and the DNA sequenced. A single base change was found which changed lysine 358 to threonine.

The mutant carrier expressed from pTKY-K358T was severely defective for accumulation of thiomethylgalactoside and lactose (Figs. 1A and 2). TMG accumulation was 2–3-fold in 10 min compared with 60-fold for the parental carrier expressed from pDP90CY. Although the accumulation by the mutant was very low it was significantly greater than the transport negative control (pDW10Y⁻). A similar defect in lactose accumulation was observed in the mutant K358T (Fig. 2). While the parental strain was able to accumulate lactose 65-fold, the mutant showed 2.5-fold accumulation.

Another assay for transport is the counterflow experiment which involves the preloading of azide treated

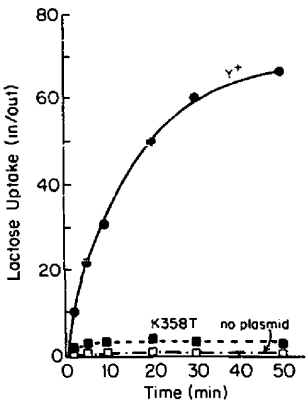

Fig. 2. Accumulation of lactose by parent and K358T. DW2 harboring a plasmid with the normal *lacY* gene, pBRY (●), or with the mutant, pBRY-K358T (■), were grown in LB medium with ampicillin. DW2 (□) was grown in LB medium. Washed cells were exposed to [^{14}C]lactose (0.1 mM) and uptake measured. In these β-galactosidase-negative strains lactose is not metabolized.

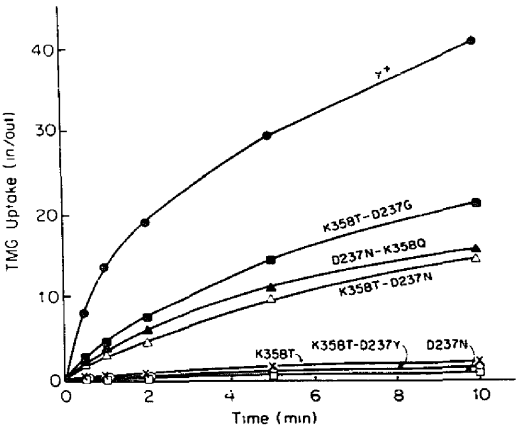

Fig. 3. TMG accumulation by parent and mutants. DW1 was used as the host strain for expression of the normal lactose carrier from pDP90CY (●), two mutant carriers from either p020-K358T (×) or pAE43-D237N (□), and four melibiose-positive revertants from p020-K358T-D237N (△), p020-K358T-D237G (■), p020-K358T-D237Y (○), and pAE43-D237N-K358Q (▲). The cells were grown in LB medium containing ampicillin. Washed cells were exposed to [^{14}C]TMG (0.1 mM), and the transport assays were carried out as described in Materials and Methods.

cells with non-radioactive sugar (10 mM melibiose in this experiment). Such preloaded cells are centrifuged, resuspended into radioactive TMG (0.1 M) and uptake of radioactivity is monitored. The accumulation observed in this counterflow experiment is due to competitive inhibition of exit of radioactive TMG by non-radioactive melibiose. The exit of melibiose on the carrier gradually reduces its inhibition of TMG exit and the radioactive sugar concentration in the cells falls to that in the external medium. The initial rates of counterflow measured for the normal and the Thr-358 mutant were about the same. A fall in the internal concentration of [^{14}C]TMG was observed at about 1 min in cells expressing the normal carrier and had equilibrated across the membrane at 5 min. In the Thr-358 mutant, on the other hand, internal [^{14}C]TMG remained at a higher level for a prolonged period of time. Presumably the exit of melibiose was slower in the mutant, thus sustaining the competition with TMG for exit. Similar results were obtained by preloading the cell with 20 mM non-radioactive TMG and exposing cells to 0.1 mM [^{14}C]TMG (data not shown).

A second mutant in which a charged amino acid was changed to a neutral residue came from the collection of Müller-Hill [13]. Strain AE43 was classified as a lactose K_m mutant because it grew on 100 mM lactose but failed to grow on 5 mm sugar. When this *lacY* gene was cloned and sequenced it was found to have a point mutation changing Asp-237 to Asn. This cell showed a

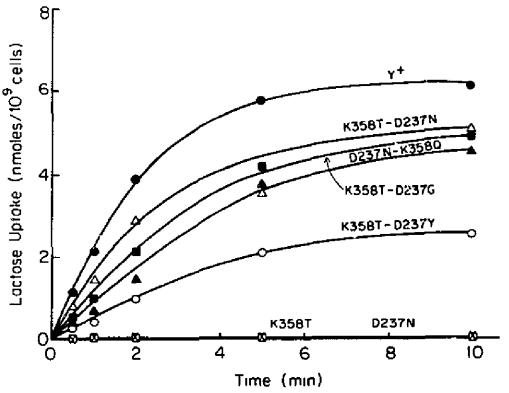

Fig. 4. Lactose uptake by parent and mutants. DW1 contained a plasmid with the normal *lacY* gene, pDP90CY (●), two mutants, p020-K358T (×), pAE43-D237N (□), and four melibiose-positive revertants: p020-K358T-D237N (△), p020-K358T-D237G (■), p020-K358T-D237Y (○), and pAE43-D237N-K358Q (▲). Cells were exposed to 0.1 mM [^{14}C]lactose and uptake measured as described in Materials and Methods. Note that these cells contain β-galactosidase so that [^{14}C]lactose is metabolized by the cell.

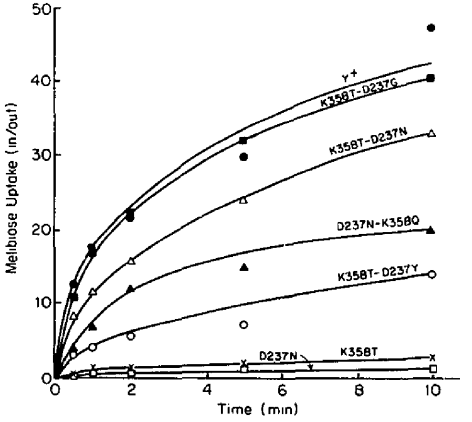

Fig. 5. Melibiose accumulation by parent and mutants. DW1 contained a plasmid with the normal *lacY* gene, pDP90CY (●), two mutants, p020-K358T (×), pAE43-D237N (□), and four melibiose-positive revertants: p020-K358T-D237N (△), p020-K358T-D237G (■), p020-K358T-D237Y (○), and pAE43-D237N-K358Q (▲). Cells were exposed to 0.1 mM [^{3}H]melibiose and uptake measured as described in Materials and Methods. Note that these cells do not contain α-galactosidase so that melibiose is not metabolized.

severe defect in the transport of TMG (Fig. 3) lactose (Fig. 4) and melibiose (Fig. 5).

Another mutation affecting codon 237 in the *lacY* gene was produced by site-directed mutagenesis and changed Asp-237 to the neutral amino acid, Gly. Cells expressing the mutant lactose carrier from pTKY-D237G showed 5% of the normal ability to accumulate TMG (Fig. 6A) and 10% of normal accumulation of melibiose (Fig. 6B).

Melibiose-positive revertants

Second-site revertants were isolated from pK358T. It was observed that DW2/pK358T gave white clones on Melibiose MacConkey indicator plates in 18 h at 37°C. After further incubation of an additional day at 37°C an occasional white clone showed a red papillus (perhaps 1 or 2 per plate). Cells from such papillae were restreaked on the same type of plate to purify the revertants which grew as bright red colonies. The plasmid DNA from such revertants was isolated and placed back into DW2. In a number of cases the transfer of the plasmid resulted in the simultaneous transfer of the melibiose-positive phenotype. Eleven such melibiose positive revertants were isolated from p020-K358T. In all cases Asp-237 was changed to a neutral amino acid; 8 showed a change to Asn, one to Gly and two to Tyr.

The melibiose transport for each of these double mutants is shown in Fig. 5. The three revertants accu-

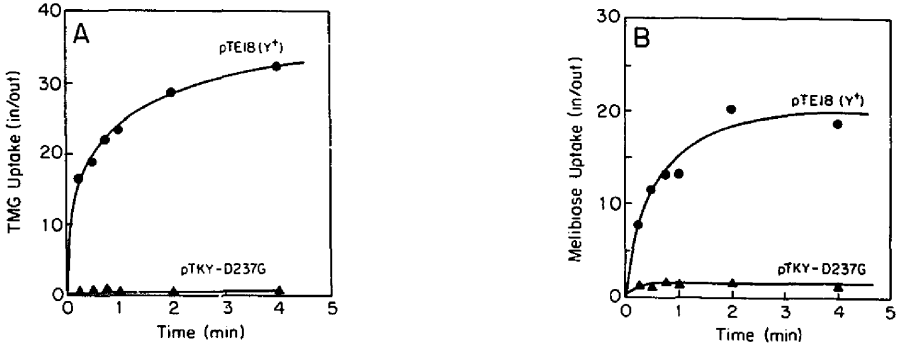

Fig. 6. TMG and melibiose accumulation by parent and D237G. DW1 contained a plasmid with the normal *lacY* gene, pTE18 (●), and the mutant pTKY-D237G (▲). Panel A: cells exposed to 0.1 mM [^{14}C]TMG. Panel B: cells exposed to 0.1 mM [^{3}H]melibiose. The cells were grown in LB containing ampicillin and tetracycline. Transport assays were as described by Materials and Methods.

TABLE III

Metabolism of melibiose by K358T and D237N and their revertants

	Fermentation on MacConkey [a] (20 mM melibiose)	Growth on melibiose (0.01%) minimal	
		2 days	6 days
DW2/DP90CY	red	0.3 mm	1.5 mm
DW2/pDW10Y⁻	white	0	0
DW2/p020-K358T	white	0	0.01 mm
DW2/pAE43-D237N	red center	0	0.01 mm
DW2/p020-K358T-D237N	red center	0.2 mm	1.5 mm
DW2/p020-K358T-D237Y	red center	0.1 mm	0.5 mm
DW2/p020-K358T-D237G	red center	0.2 mm	0.75 mm
DW2/pAE43-D237N-K358Q	red center	0.2 mm	1.5 mm

[a] Red indicates strong fermentation of melibiose, red center indicates weak fermentation and white indicates no fermentation.

mulated melibiose to internal concentrations 15–22-times that in the external medium. In contrast, cells with the single mutation (pK358T) showed only a 4-fold accumulation. Growth of the revertants on minimal plates with 0.01% melibiose as carbon source was far greater than cells expressing the mutant lactose carrier from p020-K358T (Table III).

The second-site reversions also affected the transport of β-galactosides. Lactose entry rate in the revertants was 30–60% the rate of the parental strain. TMG transport was 50% of normal in cells expressing the carrier from p020-K358T-D237G, 30% of normal in cells expressing the carrier from p020-K358T-D237N, while in cells expressing the carrier from p020-K358T-D237Y transport remained highly defective. Thus like other mutations affecting charged residues within the putative transmembrane domain [7], the observed effect is sugar specific.

Melibiose-positive revertants were also isolated from cells expressing the mutant lactose carrier from pAE43-D237N. The previous technique for isolation of revertants could not be used as this cell fermented melibiose and lactose sufficiently well to show red clones on the plate. On the other hand the cells failed to grow on minimal plates containing a very low concentration of melibiose (0.01%), and occasional revertants would arise on such plates after several days' growth at 37°C. Plasmid DNA isolated from these revertants was placed in DW2 by transformation. In 12 cases the transformed cells grew well on the melibiose minimal plates.

DNA sequencing revealed that 10 of the revertants represented direct genotypic reversion to wild type (Asp-237), whereas in the remaining two revertants a second mutation was found to have changed Lys-358 to the neutral amino acid, Gln. This second site mutation allowed cells expressing the revertant lactose carrier from pAE43-D237N-K358Q to accumulate radioactive galactosides. After 10 min melibiose accumulation (Fig. 5) was about 40% of normal, while TMG accumulation was about 25% of normal (Fig. 3). Similarly, lactose transport (downhill) was about 70% of normal in the revertant (Fig. 4).

Proton uptake on addition of TMG was measured in all mutants and compared with the normal lactose carrier (Fig. 7). A small volume of anaerobic TMG was added to a suspension of anaerobic cells and the pH of the external medium was monitored. Proton uptake by cotransport with sugar was associated with an alkalinization of the external medium. The original mutant carriers expressed from either p020-K358T and pAE43-D237N showed very little proton uptake with 4 mM TMG. On the other hand, all of the revertants showed distinctly greater TMG-dependent proton uptake al-

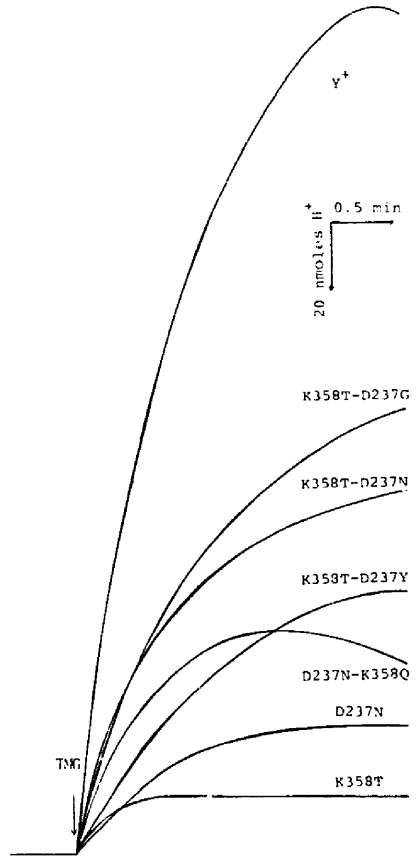

Fig. 7. TMG-induced proton uptake by parent and mutants. Each plasmid was held in the strain DW1.The pH was monitored of an anaerobic suspension of cells (2.5 ml). Anaerobic TMG (20 μl of 500 μM) was added at the arrow. The final sugar concentration was 4 mM. On these records up is alkaline.

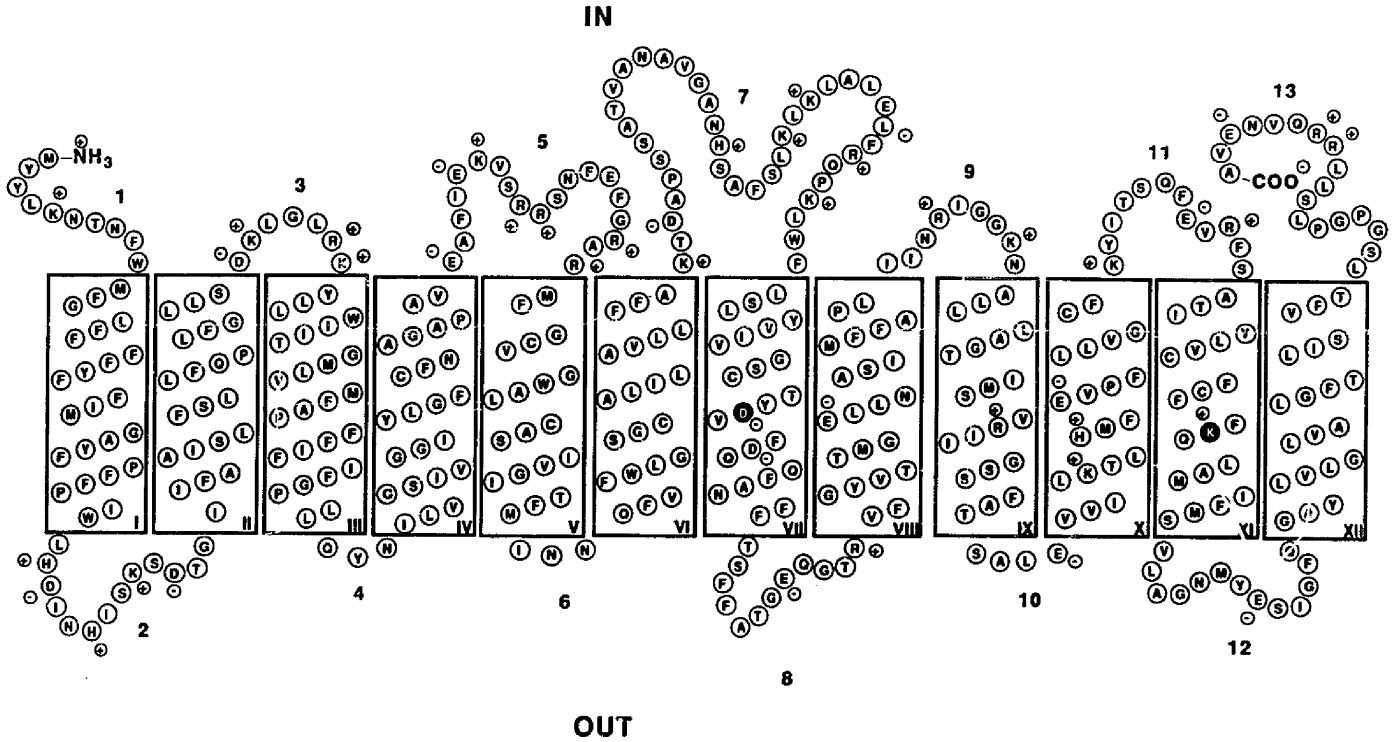

Fig. 8. Proposed model for the lactose carrier. This scheme features 12 transmembrane segments arranged such that 6 of the transmembrane segments are followed by a large cytoplasmic loop that is followed in turn by the remaining 6 transmembrane segments (6-loop-6 model). The model differs from that of Foster et al. [4] in that 13 amino acids from loop 8 (periplasmic) are transferred into the membrane-spanning region, and 13 amino acid residues from the membrane-spanning region are transferred to loop 7 (cytoplasmic). The highlighted residues, Asp-237 and Lys-358, interact with one another in at least a functional capacity and are presumably located within the transmembrane segments VII and XI, respectively.

though none of them showed more than 50% of the activity of the normal carrier (Y^+) expressed from pDP90CY.

Discussion

The presence of charged amino acid residues in membrane-spanning regions of a membrane protein is thermodynamically unfavorable. However, Honig and Hubbell [27] have pointed out that the transfer of an ion pair from water to a region of low dielectric is not so energetically unfavorable. Indeed such an ion pair making a few additional hydrogen bonds could be extremely stable in the hydrophobic environment of a membrane. In the case of voltage-sensitive ion channels charged residues are a prominent feature of membrane-spanning α-helices and may provide an essential binding site for transported ions or for the 'gating' mechanism. In such channel proteins the positively charged amino acids in amphipathic helices probably line the aqueous channel. It is believed that these positive charges are ion paired with negatively charged residues on adjacent α-helices [28]. Likewise, in bacteriorhodopsin ion pairing may be important. Stern and Khorana [29] have suggested that Arg-227 salt bridges with Asp-96 and Arg-82 with Asp-212.

In the lactose carrier of *E. coli* several charged residues in putative membrane α-helices are important to the substrate recognition and transport processes. When any of the four amino acids Arg-302, Lys-319, His-322, Glu-325 are substituted by neutral amino acids marked loss of activity or altered sugar recognition results [2,7–9,30]. Because of the close proximity of Glu-325 to His-322 it is likely that they are salt bridged. The relationship between other charged residues is not known.

In the present study it was found that a mutation changing Lys-358 to a neutral amino acid, Thr, resulted in a severe loss of activity. This is consistent with its presumed membrane location. A carrier with Asp-237 changed to either Asn or Gly also lost the major fraction of its transport capacity. The striking observation was that all of the 11 melibiose positive revertants of p020-K358T showed a loss of the negative charge at position 237. The Asp residue was substituted by Asn, Tyr or Gly. Conversely, two melibiose-positive revertants of the Asp-237 mutant pAE43-D237N gave a loss of the positive charge at position 358. Such data clearly show an intimate relationship between Lys-358 and Asp-237. One simple hypothesis to account for these observations is that despite their considerable linear separation Asp-237 and Lys-358 are in close physical proximity and salt bridged. Salt bridging between oppositely charged amino acid residues in membrane-spanning regions would greatly reduce the energy required to stabilize two individual charged residues in an hydrophobic environment.

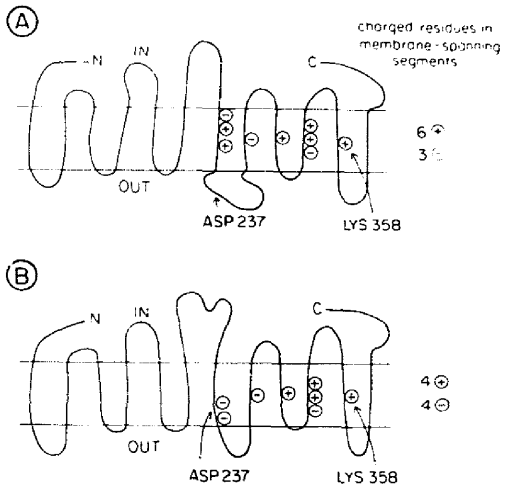

Fig. 9. Schematic representation of the lactose carrier showing the position of charged amino acid residues. A indicates the model of Foster et al. [4]. B indicates the proposed alteration of the above model. Indicated are the positions of Asp-237 and Lys-358.

If this view of salt bridging were correct and Lys-358 were definitely within a membrane-spanning segment, then Asp-237 must also reside in a membrane-spanning region. We therefore propose to move Asp-237 from the periplasmic location suggested by the topological model of Foster, Boublik and Kaback [4] to the membrane-embedded location indicated in Fig. 8. This modification has the consequence that Arg-218, Lys-221, and Glu-215 are removed from the membrane, while Asp-237 and Asp-240 are introduced into the membrane-spanning domain. Thus the model proposed here contains four positively charged and four negatively charged residues within the transmembrane domain (Fig. 9), permitting the possibility that all of the membrane-embedded charges could be salt bridged.

There are two completely independent consequences of this change in the model. The modified scheme enlarges the middle cytoplasmic loop by 13 amino acids making this loop distinctively larger than other cytoplasmic loops, a common feature of many other membrane carriers with 12 transmembrane segments [31]. An additional result of the altered model is that Tyr-236 comes to lie close to Ala-177. Previously we had shown that mutations at either Ala-177 or Tyr-236 resulted in altered sugar recognition for maltose [32] as well as the cosubstrate, H^+ [33]. It would be reasonable for these two residues to be close to one another in the membrane.

Although the data presented here support the idea that Asp-237 and Lys-358 interact with one another, any complete discription of the reversion mechanism has to account for the observation that not all carrier substrates are handed equally well by these double mutants that have a neutral amino acid in both position 237 and position 358. On the one hand, the normal level of melibiose accumulation observed for the K358T-D237G carrier does suggest that neither Asp-237 nor lys-358 is involved in H^+ translocation. On the other hand, the incomplete recovery of TMG transport activity by this same double mutant (about 30% of normal) does indicate that these amino acid residues conspire to affect the architecture of the sugar binding domain (i.e., melibiose appears to fit quite well while TMG fits less well). The K358T-D237Y carrier appears extreme in that although melibiose transport is significantly improved over that by the K358T carrier, the effect of the D237Y mutation to improve TMG transport was negligible. The precise mechanism by which the Asp-237 and Lys-358 residues jointly affect substrate recognition is a topic which requires further study.

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