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Biochimica et Biophysica Acta 1278 (1996) 111–118

BBA Biochimica et Biophysica Acta

Physiological evidence for an interaction between Glu-325 and His-322 in the lactose carrier of *Escherichia coli*

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Received 22 June 1995; accepted 11 August 1995

Abstract

Site-directed mutagenesis and second-site suppressor analysis have proven to be useful approaches to examine the role of charged amino acids in the structure and function of the lactose carrier of *Escherichia coli*. A lactose carrier mutant Glu-325 → Ser failed to ferment melibiose and showed white clones on melibiose MacConkey indicator plates. Several red revertants were isolated from these plates. Two of these revertants showed a double mutation, the original mutation (Glu-325 → Ser) plus His-322 → Asp. Seven revertants showed a second site mutation His-322 → Asn. Although the second site revertants failed to accumulate sugars they do show more rapid uptake of melibiose into cells containing α-galactosidase than the original mutant Glu-325 → Ser. The complete loss of transport activity due to the removal of the negative charge at 325 can be partially compensated for by the introduction of a new negative charge at 322. A site-directed double mutant His-322 → Asn/Glu-325 → Asn showed a greater rate of lactose uptake (V_{max}) than either of the single mutants His-322 → Asn or Glu-325 → Asn. It was concluded that there is some type of physiological interaction (possibly a salt bridge) between His-322 and Glu-325.

Keywords: Lactose carrier; Cation cotransport; Salt bridge

1. Introduction

Active uptake of galactosides across the cytoplasmic membrane by *Escherichia coli* occurs in symport (cotransport) with protons via the lactose carrier with a stoichiometry of unity [1,2]. Charged amino acid residues in membrane-spanning helices have been shown to have important functional roles in the transport process (for a review see [3]). Substituting neutral amino acid residues for charged residues in membrane-spanning regions resulted in negligible sugar accumulation activity. The possibility of several salt bridges between the intramembrane charged residues has been suggested. For example, when the mutant DW2/pLys-358 → Thr was streaked on melibiose MacConkey indicator plates it appeared as white clones and after several days melibiose positive revertants (red clones) were isolated [4]. All of these revertants proved to contain a second site mutation converting Asp-237 to a neutral amino acid (Asn, Gly or Tyr). In a reverse type of experiment the mutant Asp-237 → Ala grew poorly on melibiose minimal plates. Faster growing revertants contained a second site mutation of Lys-358 → Gln. Sahin-Toth et al. [5] have shown that substitution of Cys for either Asp-237 or Lys-358 results in an inactive carrier while the double mutant Cys-237/Cys-358 has activity. Thus, the substitution of a neutral amino acid for either charged residue is detrimental as it results in an uncompensated charge while the double neutral replacement gives activity. It was proposed that Asp-237 forms a salt bridge with Lys-358 [4]. When the positions of the Asp-237 and the Lys-358 were reversed good transport activity was observed [5–7] suggesting that this pair may play a structural role rather than a functional role in the carrier protein.

Loss of sugar accumulation activity resulted when Asp-240 was changed to Ala [8]. A second-site revertant was isolated in which Lys-319 was changed to Gln. This cell showed good sugar accumulation. When either Asp-240 or Lys-319 were substituted by a neutral amino acid (Ala or Cys), transport activity was very low [5,7] while substitution by neutral amino acids in both positions (such as

Abbreviations: TMG, methyl β-D-thiogalactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; TDG, galactosyl β-D-thiogalactopyranoside; LB, Luria-Bertani broth.

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SSDI 0005-2736(95)00209-X

Cys-240/Cys-319) resulted in good activity. This suggested that Asp-240 and Lys-319 interact to form a salt bridge [8]. Exchanging the positive and negative charges (Lys-240/Asp-319) resulted in loss of lactose transport [5] suggesting an important functional role for this charged pair.

Replacement of Lys-319 with Asn results in the inability of the mutant to ferment melibiose on MacConkey indicator plates [9]. Melibiose-positive revertants isolated from such indicator plates revealed two types of second site revertants. The first type of revertants showed a replacement of Asp-240 by either a Val or a Gly whereas the second type showed a replacement of Glu-269 by Asn. Furthermore, the Glu-269 → Asn mutant, which grew slowly on melibiose minimal plates, exhibited fast growing revertants in which a second site (Lys-319) was changed to Asn [9]. This study strongly suggested that Lys-319 interacts with both Asp-240 and Glu-269, with a more crucial role for the Lys-319, Glu-269 pair. Site-directed pyrene eximer fluorescence studies of the lactose carrier revealed that Glu-269 is in close proximity to His-322 [10]. Recent studies of the mutants at positions 269 and 322 showed that $T1^+$ quenched the excimer fluorescence which was reversed by thiomethylgalactoside. This implied that Glu-269 and His-322 are in or near the sugar binding site [11].

Because of the close proximity of Glu-325 to His-322 in a putative α-helical membrane-spanning region of the carrier it was proposed that they were ion paired [12]. The present study provides physiological evidence to support this view. In one type of experiment second site revertants of Glu-325 → Ser showed mutations at His-322 (Asn and Glu). In another type of experiment the double mutant Asn-322/Asn-325 was constructed and compared with the single mutations Asn-322 and Asn-325. The double mutant was shown to have a greater activity (V_{max}) for lactose transport than either of the single mutants. These studies suggest that when the charge is removed from one member of the pair the remaining uncompensated charge is detrimental to the function and that removal of both charges results in a carrier with somewhat more activity than either single mutant.

2. Materials and methods

2.1. Materials

Biological media were purchased from Difco. IPTG was obtained from Boehringer Mannheim. Lactose, melibiose, and TMG were from Sigma. [*methyl*-^{14}C]TMG was from Du Pont-New England Nuclear, [*glucose*-1-^{14}C]lactose was from Amersham, and [^{3}H]melibiose was generously provided by Dr. G. Le Blanc (Department de Biologie du CEA, Villefranche-sur-Mer, France). Radiolabeled sugars were purified using Whatman No. 3MM chromatography paper and a mixed solvent phase of three parts of 1-propanol to one part of water. Reagents for DNA manipulation and sequencing were purchased from New England Biolabs.

2.2. Bacterial strains and plasmids

All strains are *E. coli* K12. The genotypes of the strains and plasmids are detailed in Table 1. LB medium was employed to grow cells in all experiments. Ampicillin (0.1 mg/ml) or tetracycline (0.01 mg/ml) was added to the medium when necessary, and IPTG (0.5 mM) was used to induce the lactose operon. The plasmids pASN322 [14]

Table 1
Genotype of *E. coli* strains and plasmids

	Genotype (chromosome/F′-factor/plasmid)	Source
Strains		
DW1	*lacI*$^+$ *Δ(ZY)mel Δ(AB) rpsL/−/−*	[13]
DW2	*lacI*$^+$ *Δ(ZY)melA*$^+$ *ΔB rpsL/−/−*	[13]
DW2 (pcn)	*lacI*$^+$ *Δ(ZY)melA*$^+$ *ΔB rpsL pcn*::Tn*10/−/−*	[8]
DW2/F′	*lacI*$^+$ *Δ(ZY)melA*$^+$ *ΔB rpsL/F′lacI*^{Q}*Z*$^+$*Y*$^-$*/−*	[8]
DW2 (pcn)/F′	*lacI*$^+$ *Δ(ZY)melA*$^+$ *ΔB rpsL pcn*::Tn*10/F′I*^{Q}*Z*$^+$ *ΔY*::cat*/−*	this work
TG1 *Δ*	*(lac-pro)supE/F′proA*$^+$*B*$^+$ *lacI*^{Q}*ZΔ*M15*/−*	Amersham
Plasmids		
pACYC184	*−/−/cap*^{r} *tet*^{r}	New England Biolabs
pKK223-3	*−/−/amp*^{r}	Pharmacia
pACYC-Y	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*$^+$ *Δ(A) tet*^{r}	[14]
pKK-Y	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*$^+$ *Δ(A) amp*^{r}	this work
pS325	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(Glu-325 → Ser) *Δ(A) tet*^{r}	[15]
pN325	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(Glu325 → Asn) *Δ(A) amp*^{r}	this work
pN322	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(His322 → Asn) *Δ(A) amp*^{r}	[14]
pD322	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(His322 → Asp) *Δ(A) tet*^{r}	this work
pS325/N322	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(Glu325 → Ser; His322 → Asn) *Δ(A) tet*^{r}	this work
pS325/D322	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(Glu325 → Ser; His322 → Asp) *Δ(A) tet*^{r}	this work
pN325/N322	*−/−/lac Δ(I)O*$^+$*P*$^+$ *Δ(Z)Y*(Glu325 → Asn; His322 → Asn) *Δ(A) amp*^{r}	this work

and pE325S [15] were kindly provided by Dr. Brooker (University of Minnesota, St. Paul, MN).

2.3. Oligonucleotide-directed mutagenesis

Single-stranded plasmid with a *lacY* insert (pKK-Y) was used as a template for mutagenesis using Transformer™ kit from Clontech according to the directions. Glu-325 was converted to Asn (pN325) using mutagenic primer 5′-GCATATGTTT**a**AcGTACCGTTCC-3′ (mutation in lowercase bold). Construction of pN322/N325 was carried out using single-stranded pN325 as a template and mutagenic primer 5′-TCAAACATAT**t**CAGCGTTTTC-3′ (mutation in lowercase bold). This primer was kindly provided by Dr. Steven King. Single-stranded M13 DNA with a *lacY* insert was used as a template to convert His-322 to Asp using mutagenic primer 5′-CGGTACT-TCAAACATAT**c**CAGCGTTTTGAG-3′ according to the method of Sayers et al. [16]. The mutations were confirmed by completely sequencing the *lacY* gene by the method of Sanger et al. [17].

2.4. Subcloning

The plasmid pN322 was constructed by removing the *Eco*RI-*Esp*I fragment of PKK-Y and replacing it with the same restriction fragment (containing N322) from pAsn322.

2.5. Selection of revertants

DW2/S325 ferments melibiose very poorly forming white colonies on melibiose MacConkey plates. Red Revertants appeared after several days of incubation at 37°C, from which plasmids were isolated. Transformation of the plasmid back into the host cells transferred the red phenotype, indicating the new mutation was on the plasmids. The mutations on these plasmids were identified by complete sequencing.

2.6. Measurement of sugar transport

Cells were grown in LB medium (with or without 0.5 mM IPTG or 1 mM α-methyl galactoside) to mid-log phase and centrifuged. The cells were washed with M63 medium, and resuspended in the same buffer. Radioactive sugar (0.1 ml) was added to 0.9 ml of cell suspension (0.45 mg of protein/ml) at room temperature. Samples (0.2 ml) were removed at several time points after the mixing, and filtered over a 0.65 μm pore size membrane filter (Sartorius). The cells were washed with 4 ml of the buffer to remove external sugar. The radioactivity on the filter was determined in a liquid scintillation counter. Values from cells containing vector plasmid (pACYC184 or pKK223) with no *lacY* insert were subtracted from the data.

2.7. Kinetic analysis

Lactose transport by DW2 (pcn)/F′$I^QZ^+\Delta Y$ containing the normal and mutant plasmids was performed with various concentrations (0.1–5 mM) of the sugar. In this cell, lactose is metabolized by β-galactosidase, and transport occurs down the sugar concentration gradient. To measure initial transport rates radioactive sugar was mixed with the cell suspension and samples (0.1 ml) were removed at 30 s. Each point was taken in triplicate. Two such experiments were carried out for each sugar concentration. K_m and V_{max} were determined by double-reciprocal plot.

2.8. Proton transport assay

Sugar-induced proton uptake was measured using a pH electrode according to the method of West [18] as modified by Wilson et al. [19]. Cells (100 ml) grown to mid-log phase were washed with 120 mM KCl and resuspended to a density of 3 mg of protein/ml in 120 mM KCl, 30 mM KSCN. The cell suspension (2.5 ml) was placed in a small vial with a lid which had holes for a pH electrode and injection of nitrogen and sugar solution. N_2 was continuously passed over the cell suspension, and cells were mixed with a magnetic stirrer. An anaerobic solution (25 μl) of 500 mM lactose or 1 M melibiose was injected into the cell suspension to induce proton uptake. The pH of the extracellular medium was monitored using a combined pH electrode (Radiometer GK 231-C).

2.9. Immunochemical assay

The amount of wild-type and mutant lactose carrier protein in the membrane was measured by the method of Lolkema et al. [20] using a polyclonal antibody produced against the C-terminal decapeptide of the lactose carrier.

3. Results

3.1. Isolation of second site revertants

The *lacY* mutant Glu-325 → Ser is known to show a severe defect in lactose and melibiose transport [21]. When a plasmid containing the D325S mutation was placed in strain DW2 (*ΔlacZY melA*$^+$*ΔB*) the strain failed to ferment melibiose and showed white clones on melibiose MacConkey indicator plates. After incubation of such plates for 3–4 days at 37°C occasional red mutant clones arose. These were purified, the plasmid DNA isolated, and DW2 transformed. In about 50% of the cases the red phenotype was transferred to the transformed cells indicating a mutation was carried on the plasmid. The lac Y gene on the plasmid was sequenced. In two cases the original mutation (S325) was present plus a second site mutation of His-322

→ Asp. In seven cases the second site mutation was His-322 → Asn.

3.2. Fermentation studies of revertants

The fermentation of melibiose or lactose by each of the mutants was studied on MacConkey indicator plates (Table 2). Clones containing S325 or D322 were white on melibiose MacConkey plates indicating lack of melibiose uptake. The two second site revertants were red indicating good sugar transport.

3.3. Sugar transport properties of revertants

The uptake of radioactive melibiose was measured in cells of DW2 containing individual plasmids (Fig. 1). The cells were grown in the presence of α-methylgalactoside to induce the enzyme α-galactosidase. Such cells will metabolize melibiose as it enters the cell so that the internal concentration of sugar remains low. Therefore the entry of sugar is thermodynamically 'downhill' and accumulation of melibiose does not occur. In this experiment the external melibiose concentration was 0.1 mM. All of the mutants showed less than 10% of the activity of the wild type. The uptake of S325 was almost zero; Asp-322 was very low and the double mutant S325/D322 was distinctly higher than either of the single mutants. The double mutant S325/N322 was higher than S325.

Lactose entry was measured in cells of DW2/F′$I^QZ^+Y^-$ (Fig. 2). In such a cell β-galactosidase was induced by growth in the presence of IPTG. Lactose enters such cells on the carrier and is metabolized. Such entry is 'downhill' and does not involve accumulation. The mutants showed no transport of lactose.

Accumulation of melibiose, lactose and TMG was measured in the host strain DW1 (Table 3). The mutants showed little or no accumulation of any of the three sugars.

3.4. Immunological assay for revertant transport proteins

The amount of transport protein in the membrane was assayed by an immunoblot assay [20]. There was a reduced quantity of lactose carrier of pS325 and pS325/N322 in the membrane while the amounts of the pD322 and pS325/D322 were approximately normal (Table 4).

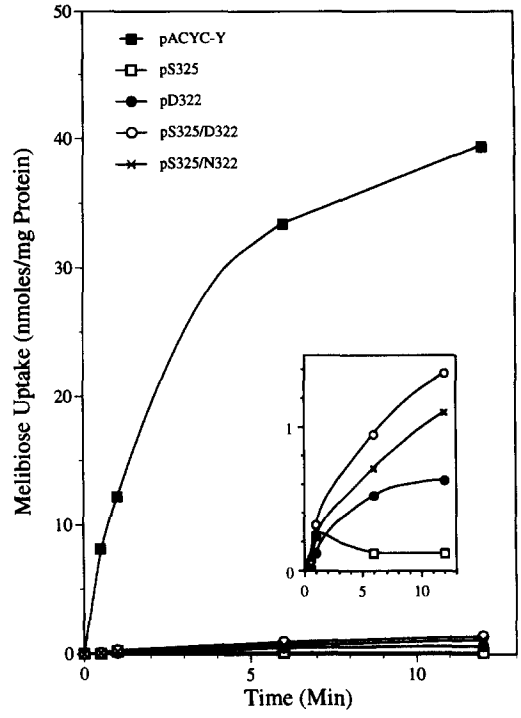

Fig. 1. Melibiose downhill transport. DW2/F′$I^QZ^+Y^-$ containing the appropriate plasmid was grown in LB plus α-methylgalactoside. Under these conditions α-galactosidase is induced and melibiose entering the cell is metabolized. Thus the internal concentration of sugar is maintained very low and sugar entry is thermodynamically downhill. Washed cells were exposed to 0.1 mM [^{3}H]melibiose and filtered at the times indicated. The data represent the averages of three independent experiments.

3.5. Sugar stimulated proton transport of revertants

Proton uptake in response to sugar addition was measured. Cells were first made anaerobic to remove that proton pumping of the respiratory chain. Anaerobic sugar was then added to the cells and the pH was measured. Melibiose stimulated proton uptake by D322 and S325/D322 at about one third of the rate of the parent

Table 2
Fermentation of revertants on indicator MacConkey plates

Strains	Melibiose	Lactose
pACYC184	white	white
pACYC-Y	red	red
pS325	white	white
pD322	white	red center
pS325/D322	red	red center
pS325/N322	red center	red center

Fermentation studies were carried out on 30 mM lactose or 15 mM melibiose MacConkey plates in *E. coli* strain DW2/F′$I^QZ^+Y^-$ containing plasmid.

Table 3
Sugar accumulation by revertants

Sample	Concentration of sugar in/out at 12 min		
	melibiose	lactose	TMG
pACYC-Y	78	86	67
pS325	0.8	0.4	0.1
pD322	1.1	1.6	1.2
pS325/N322	2.0	0.6	0.1
pS325/D322	1.1	0.9	0.4

DW1 was used as the host strain for the expression of the lacY containing plasmids. Washed cells were exposed to 0.1 mM sugar (1 uc/ml).

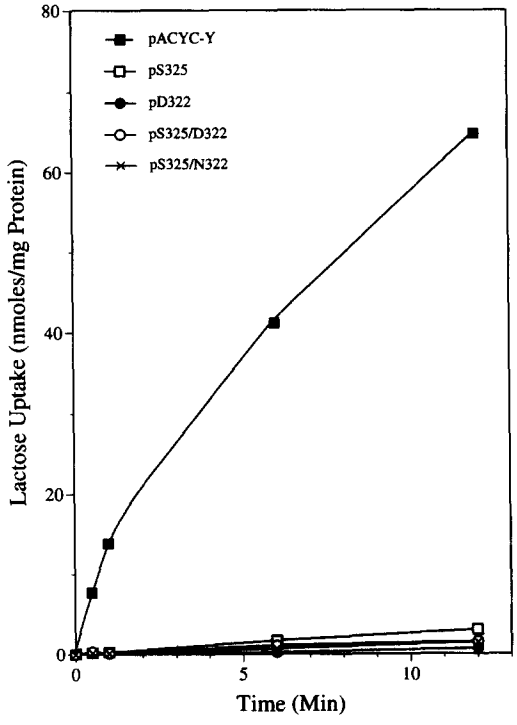

Fig. 2. Lactose downhill transport. DW2/F'$I^{Q}Z^{+}Y^{-}$ containing the appropriate plasmid was grown in LB plus 0.5 mM IPTG. Under these conditions β-galactosidase is induced and lactose entering the cell is metabolized. Washed cells were exposed to 0.1 mM [^{14}C]lactose and filtered at the times indicated. The data represent the averages of three independent experiments.

(Fig. 3A). pS325 and S325/N322 showed no proton uptake. None of the mutants showed proton uptake in response to lactose addition (Fig. 3B). Addition of thiodigalactoside (TDG) caused a large proton uptake by the double mutant S325/D322 (greater than normal) and D322 showed proton uptake about one third of normal (Fig. 3C). No uptake was observed with S325 and S325/N322.

Table 4
Immunochemical assay of revertants

Strains	Percent of wild type (pACYC-Y)
pS325	47
pD322	101
pS325/N322	36
pS325/D322	119

3.6. The N322/N325 double mutant

When it was found that the second site revertant S325/N322 showed more activity than S325 alone a possible interaction between the glutamic acid at 325 and the histidine at 322 was suggested. It was considered of interest, therefore, to construct another double mutant with a neutral residue substituted for Glu-325 and for His-322. The double mutant N322/N325 as well as the single mutants N325 and N322 were constructed.

Fermentation data in Table 5 indicates that the double mutant N322/N325 shows more activity on the melibiose fermentation plate than the single mutant N325. On the other hand, the double mutant has the same activity as N322 and the normal gene. On the lactose plates the results were similar.

Immunoblot analysis of the amount of the transport protein in the membrane is given in Table 6. Both N325 and N322/N325 show about 70% normal content in the membrane while N322 showed 95%.

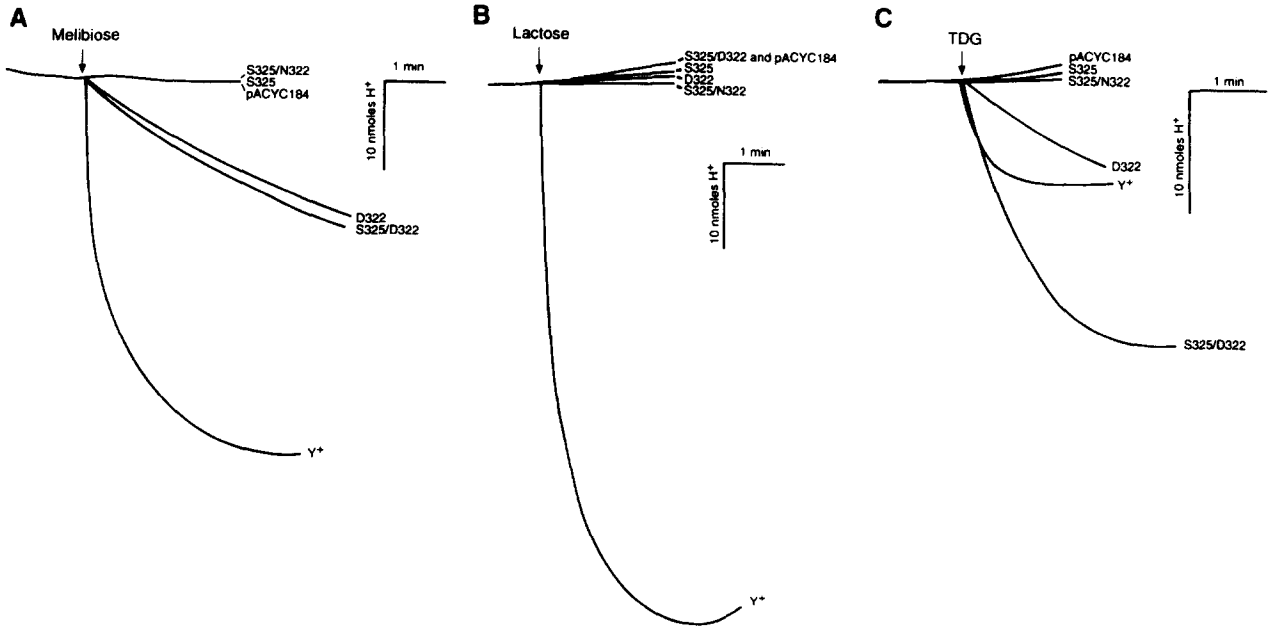

Fig. 3. Proton uptake induced by addition of sugar to anaerobic cells. DW2 containing the appropriate plasmid was grown in LB.

Table 5
Fermentation on MacConkey plates

Strains	Lactose (30 mM)	Melibiose (30 mM)
pKK223	white	white
pKK-Y	red	red
pN322	red	red
pN325	white	white
pN322/N325	red center	red

Lactose fermentation studies were carried out with the plasmid in DW2 (pcn)/F'I^QZ^+ Y. Melibiose studies were in DW2.

Table 6
Immunochemical assay

Strains	Percent of wild type (pKK-Y)
pN322	95
pN325	68
pN322/N325	70

Table 7
Sugar accumulation by lactose carrier mutants

Sample	Concentration of sugar in/out at 10 min		
	melibiose	lactose	TMG
pKK-Y	66	88	55
pN322	0.6	2.8	0.5
pN325	0.4	0.3	0.1
pN322/N325	0.9	0.6	0.2

DW2 was used as host strain for the indicated plasmids.

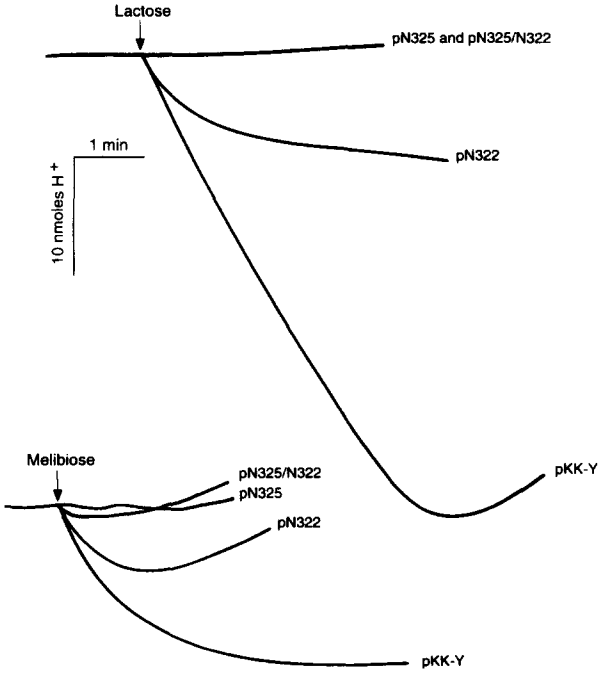

Fig. 4. Proton uptake induced by addition of sugar to anaerobic cells. DW2 containing the appropriate plasmid was grown in LB.

Table 8
Kinetics of lactose transport by site-directed mutants

Strains	K_m (mM)	V_{max} (nmol/mg protein/min)
pKK-Y	1.7	785
pN322	8.5	200
pN325	1.6	9
pN325/N322	33	300

DW2 (pcn)/F'$I^QZ^+\Delta Y$ containing the appropriate plasmid was grown in LB plus 0.5 mM IPTG.

3.7. Sugar and proton transport by double mutant

Accumulation of melibiose and TMG was absent in each of the three mutants (Table 7). Lactose accumulation by N322 was 2.8-fold (10% of the wild type). N322/N325 and N325 failed to accumulate lactose.

Sugar stimulated proton uptake indicated that lactose induced proton uptake in the mutant N322 about one third of normal while N325 and N325/N322 showed no uptake (Fig. 4). Very similar results were obtained with melibiose induced proton uptake.

A kinetic analysis was made of the three mutants and the wild type cells (Table 8). The affinity of N325 for lactose was approximately the same as the normal while the V_{max} was extremely low (confirming the observations of Franco and Brooker [15]). N322 showed a 4-fold higher K_m than normal and about 25% of the V_{max} of normal. The striking result was that although the double mutant N322/N325 had a very poor affinity ($K_m = 33$ mM) it showed a higher V_{max} than either of the single mutants.

4. Discussion

The presence of a charged amino acid residue in the hydrophobic region of a protein is thermodynamically unfavorable. In the case of hydrophobic regions of water soluble proteins Rashin and Honig [22] have summarized the data on 36 proteins and found that most charged residues in the hydrophobic regions are salt bridged to residues of opposite charge. In the periplasmic galactose binding protein of *E. coli* Quiocho [23] found that Lys-10 is salt bridged to Asp-90 in the sugar binding site. Similarly, there are salt bridges between Arg-70 and Glu-68 and between Glu-52 and Arg-49 in the lactose binding site of the S-Lac Lectin crystal structure [24].

In membrane transport proteins there are several examples of salt bridges. In bacteriorhodopsin Asp-85 probably salt bridges with Arg-82 and Asp-96 to Arg-227 [25,26]. Cain and Simoni [27] showed an interaction between Glu-219 and His-245 within the α subunit of the F_1F_0-ATPase in *E. coli*.

In the lactose carrier evidence has been provided for several ion pairs. For example a Lys-358 → Thr mutant

failed to ferment melibiose and gave rise to white clones on melibiose MacConkey indicator plates in 18 h at 37°C. After further incubation of 1–2 days at 37°C small red clones appeared. When these red revertants were purified and sequenced they showed second site mutations of Asp-237 converted to Asn, Gly or Tyr. [4]. In the reverse experiment Asp-237 → Asn showed poor activity and a second site mutant showed Lys-358 → Gln. Sahin-Toth et al. [5] have shown that substitution of Ala or Cys for Asp-237 or Lys-358 leads to loss of activity while the double mutant Cys-237/Cys-358 has activity. In addition they found that reversing the positions of the two charged residues gave good activity. These data are consistent with the view that there is an interaction (probably a salt bridge) between Lys-258 and Asp-237. Analysis of suppressor mutations [9] indicate that Lys-319 may interact with Glu-269 or Asp-240. In addition, Sahin-Toth et al. [5] showed that when cysteine was substituted for either Asp-240 or Lys-319 loss of activity was observed but neutral substitution at both positions (Cys-240/Cys-319) gave transport activity.

Considerable evidence has accumulated indicating the functional importance of His-322 [12,14,28–34] and Glu-325 [12,15,32–35]. If they were located in an α-helical region, Glu-325 and His-322 would be expected to be rather close together. Carisco et al. [12] therefore suggested that they might be ion paired. Jung et al. [10] converted both charged residues to cysteines and added a fluorescent reagent (pyrene-maleimide). This exhibited excimer fluorescence indicating that the two residues were within about 3.5 Å of each other. This result would be expected if the two residues were in an α-helical region.

The present work provides physiological experiments to support the view that His-322 interacts with Glu-325. The starting mutant Glu-325 → Ser failed to ferment melibiose (white clones on MacConkey indicator plates). Several red revertants were isolated. Two of these showed a second site mutation His-322 → Asp and seven showed the second site mutation His-322 → Asn. This suggested an interaction between His-322 and Glu-325. In an additional experiment the double mutant His-322 → Asn/Glu-325 → Asn was constructed by site directed mutagenesis. This double mutant showed a greater V_{max} for lactose transport than either of the single mutants His-322 → Asn or Glu-325 → Asn.

An additional point of interest was the appearance of aspartic acid substituted for His-322 as a second site mutation from Glu-325 → Ser. Thus the loss of a negative charge at position 325 can be compensated for by a new negative charge at an adjacent location. This double mutant with a negative charge at position 322 shows quite poor activity compared with the wild type but significantly higher activity than the starting mutation Glu-325 → Ser. For example, the Ser-325 mutant is white on melibiose MacConkey plates while the double mutant is red indicating an improved melibiose transport.

The postulated role of Glu-325 in proton transport [12,15] is supported by the fact that the mutant with a charge substituted for His-322 (S325/D322) is capable of sugar-proton cotransport. Although sugar accumulation does not take place melibiose stimulated proton uptake is present (30% of normal). Furthermore, it is striking that the double mutant S325/D322 shows very high TDG stimulated proton entry. Thus, a carboxyl-group at position 322 can substitute for the loss of a carboxyl-group at position 325 in proton cotransport. It is interesting to note that a negative charge in this region is more important than an intact His-322. These experiments emphasize the importance of Glu-325 in the physiology of sugar transport, probably in proton binding and transport.

Site-directed pyrene eximer fluorescence studies [10] and EPR spectroscopy [36] suggest that His-322 is close to Glu-269. Thus, His-322 may salt bridge with Glu-269. A second example of a positive residue interacting with two different negative residues is the case of Lys-319 interacting with both Glu-269 and Asp-240 [9]. We postulate that at one phase of the transport cycle Lys-319 interacts with Glu-269 [9] and His-322 interacts with Glu-325; at another phase of the cycle His-322 interacts with Glu-269 [10,36] and Lys-319 interacts with Asp-240 [8,9]. Such alterations in salt bridges may be involved in the conformational changes that occur during the transport cycle.

Acknowledgements

We wish to thank Dr. Colin Manoil for the strain containing the $F'I^QZ^+\Delta Y$::cat. This work was supported by Grant DK05736 from the National Institutes of Health.

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