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Galactoside-dependent proton transport by mutants of the *Escherichia coli* lactose carrier: substitution of tyrosine for histidine-322 and of leucine for serine-306

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The *lacY* genes from two *Escherichia coli* mutants, MAB20 and AA22, have been cloned in a multicopy plasmid by a novel 'sucrose marker exchange' method. Characterization showed that the plasmids express a lactose carrier with poor affinity for lactose. Neither mutant carried out concentrative uptake with methyl β-D-galactopyranoside, lactose, or melibiose as the substrate. Nor did the mutants catalyze counterflow or exchange with methyl β-D-galactopyranoside. Both mutants did, however, retain the capacity to carry out facilitated diffusion with lactose or melibiose. DNA sequencing revealed that MAB20 (histidine-322 to tyrosine) and AA22 (serine-306 to leucine) have amino acid substitutions within the putative 'charge-relay' domain thought to be responsible for proton transport. Galactoside-dependent H^+ transport was readily measured in both mutants. We conclude, therefore, that the presence of a histidine residue at position 322 of the lactose carrier is not obligatory for H^+ transport per se.

Introduction

The *Escherichia coli* lactose carrier is a transmembrane protein encoded by the *lacY* gene. The primary structure of the lactose carrier protein has been inferred from the DNA sequence [1]. Based primarily upon spectroscopic results and upon analysis of hydropathy, the tentative secondary structure is predicted to comprise 12 or 14 membrane spanning α-helices [2–4]. The lactose carrier has been purified and reconstituted into proteoliposomes [5], such that the activity of the carrier is retained [6] even when reconstituted with only a single carrier molecule per proteoliposome, suggesting that the carrier functions as a monomer [7]. Therefore, the activity of the lactose carrier, catalysis of tightly coupled β-galactoside/H^+ symport, must be accounted for without involvement of proteins other than the 417 amino acid polypeptide encoded by the *lacY* gene. The mechanism by which the lactose carrier captures and transfers the free energy released from a proton moving down its electrochemical gradient to a galactoside molecule moving up its chemical gradient is of general interest. One approach to understanding the cotransport phenomenon is to study defective mutants.

Valuable insight into the molecular basis for the coupling of protonmotive force to galactoside transport has been gained by studying mutants of the lactose carrier which fail to accumulate substrates against a concentration gradient in the normal way. Wong et al. [8] and West and Wilson [9] studied lactose carrier mutants which did not appear to effectively use the protonmotive force to carry out active substrate accumulation. Downhill transport appeared to occur without concomitant proton translocation. Mutants displaying this characteristic were said to be 'uncoupled'. Kaback and co-workers have used oligonucleotide-directed mutagenesis to construct a series of lactose carrier mutants which have the 'uncoupled' phenotype [10,11]. Their studies implicate histidine-322, glutamate-325, and arginine-302 as critical residues in a putative charge-relay mechanism which is thought to be responsible for the transmembrane movement of protons coupled to galactoside translocation. Point mutations affecting these residues are thought to interfere, therefore, with reactions involving protonation or deprotonation of the lactose carrier [10,11].

It is not established, however, that mutations affecting proton interactions with the lactose carrier should

Abbreviations: TDG, β-D-galactopyranosyl 1-thio-β-D-galactospyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside; TMG, methyl β-D-thiogalactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; XG, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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necessarily be distinct from mutations affecting galactoside interactions with the carrier. Indeed, the precise relationship between these sites is unclear – both in terms of their relative locations within the carrier and in terms of possible thermodynamic linkage of the binding reactions at these sites. In this communication we report the cloning, DNA sequencing, and characterization of two substrate recognition mutants with poor apparent affinity for lactose. These mutations were found on F′-factors in the *E. coli* strains, AA22 and MAB20, from the collection of Müller-Hill [12], and were characterized as strains that grew on high but not low concentrations of lactose [13,14]. The results described in this communication suggest that the distinction between sugar recognition and proton recognition is less clear than previously imagined, for these mutations, His-322 → Tyr in MAB20 and Ser-306 → Leu in AA22, lie squarely within the putative 'charge-relay', a region thought to be the proton conduit of the lactose carrier. Inasmuch as the MAB20 mutant (His-322 → Tyr) is shown to mediate galactoside-dependent proton translocation, critical details of the proposed 'charge-relay' mechanism [15] should be reconsidered.

Materials and Methods

Materials. Klenow fragment, T4 DNA ligase, all restriction enzymes, and DNA sequencing reagents were from New England Biolabs. Calf intestinal phosphatase was from Boehringer Mannheim. 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 [16]. 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. The plasmid, pMB050-W4680, was a gift from Dr. Michael Malamy. This plasmid is similar to pMBO40 which has been described [17]. Plasmid pRAF-S11 is an invertase-constitutive derivative of the raffinose positive plasmid from strain DS25-91 from Schmid and Schmitt [18]. Strain DW10ΔY was constructed by transferring the F′*lacZ*$^{+}$*ΔY* (from Langridge [19]) into DW2.

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), tetracycline (10 μg/ml), or streptomycin (100 μg/ml).

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. [12]
DP90CY	DP90CNal/*lacI^{Q}Z^{+}Y^{+}A^{+}/-*	Hobson et al. [12]
AA22	DP90CNal/*lacI^{Q}Z^{+}Y^{-}A^{+}/-*	Hobson et al. [12]
MAB20	DP90CNal/*lacI^{Q}Z^{+}Y^{-}A^{+}/-*	
DW1	*lacI^{+}Δ(ZY) mel Δ(AB) strA/-/-*	Wilson and Wilson
DW2	*lacI^{+}Δ(ZY) mel A^{+}ΔB strA/-/-*	
DW2RA	*lacI^{+}Δ(ZY)melA^{+}ΔB strA/RecA::Tn10/-/-*	This work
2.00R	*lacI^{+}Z^{+}ΔY/-/-*	Jacob and Monod [32]
DW2IQ	DW2/-/pSG825	This work
DW10ΔY	DW2/*lacZ^{+}Δ(Y)/-*	This work
Plasmids		
pMBO50-W4680	*/-/lacO^{+}P^{UV5}Δ(Z)Y^{+}Δ(A) amp^{r}*	Michael Malamy
pSCK1	*/-/lacO^{+}P^{UV5}Z^{-}Y$^{lactose+}$Δ(A) amp^{r}*	This work
pDP90CY	*-/-/lacO^{+}P^{UV5}Z^{+}Y^{+}Δ(A) amp^{r}*	This work
pAA22-S306L	*-/-/lacO^{+}P^{UV5}Z^{+}Y$^{(S306L)}$Δ(A) amp^{r}*	This work
pMAB20-H322Y	*-/-/lacO^{+}P^{UV5}Z^{+}Y$^{(H322Y)}$Δ(A) amp^{r}*	This work
pDW10ΔY	*-/-/lacO^{+}P^{UV5}Z^{+}Δ(YA) amp^{r}*	This work
pSG825	*-/-/lacI^{Q}Δ(ZY) amp^{r}*	Steven Goff
pBR322	*-/-/amp^{r} tet^{r}*	Bolivar et al. [33]
pBRY	*-/-/lacΔ(I OPZ)Y^{+}Δ(A) tet^{r}*	This work
pBRY-S306L	*-/-/lacΔ(I OPZ)Y$^{(S306L)}$Δ(A) tet^{r}*	This work
pBRY-H322Y	*-/-/lacΔ(I OPZ)Y$^{(H322Y)}$Δ(A) tet^{r}*	This work
pRAF-S11	*-/-/Raf ABCD*	This work

Cloning the lac Y by homologous recombination. The plasmid, pMB050-W4680 (*lacΔZY*$^+$), was used to rescue mutant *lacY* gene from the F′-factor carried by *E. coli* strain AA22. When pMB050-W4680 was placed in this strain by transformation, homologous recombination occurred with the F′-factor such that it was possible to obtain plasmids of the genotype *lacZ*$^+$ *Y*^{mut}*amp*^{R}. The procedure follows.

The strain, AA22/pMBO50-W4680, was constructed by transformation and picked as a blue, ampicillin-resistant clone from XG agar containing 0.5 mM IPTG. Through homologous recombination in the *lac* operon, pMBO50-W4680 will sometimes become integrated into the F′-factors which also contain the *lac* operon. Thus, occasionally both ampicillin resistance (from pMBO50-W4680) and *lacZ* (from the F′-factor) can be transferred to a *Δlac* strain by mating. Therefore, AA22/pMBO50-W4680 was mated to DW2 and plated on XG/IPTG agar containing streptomycin (kills the male) and ampicillin (kills the female). Blue clones found on these plates must contain *lacZ* and be simultaneously resistant to both antibiotics. Presumably such colonies arise via transfer of the cointegrate to DW2. Through a second recombination event, free plasmid DNA is regenerated. Such doubly recombinant plasmid DNA was prepared directly from 6 to 12 blue clones which were gathered together in a single Eppendorf centrifuge tube. This plasmid DNA is non-clonal, comprising in theory the phenotypes Z$^-$Y$^+$, Z$^-$Y^{mut}, Z$^+$Y$^+$, and Z$^+$Y^{mut}.

To identify Y^{mut} recombinants, we initially screened for Z$^+$ recombinants, reasoning that recombination across the *Z* gene would frequently be accompanied by recombination across *lacY*. Therefore, DW2 was transformed with the nonclonal plasmid DNA and transformants were selected on XG agar containing ampicillin. Blue clones were picked, purified, and then screened individually for defects in the lactose carrier. Fermentation assays failed to distinguish any *lacY* gene recombinants (all clones were fermentation positive). Transport assays, both [^{14}C]TMG and β-ONPG, were used to unambiguously identify clones which contained the defective *lacY*. A transport negative clone was chosen and the plasmid DNA isolated from that strain is designated pAA22-S306L.

Cloning by the sucrose marker exchange method. The *lacY* gene from MAB20, DW10ΔY, and DP90C/F′I^Q Z$^+$Y$^+$ were cloned by a similar method that is useful in particular for cloning *lacY* mutants which have no distinctive phenotype on agar plates. The method uses pSCK1, a plasmid structurally analagous to pMBO50, except that the former contains a mutation in both the *lacZ* and the *lacY*. These mutations make the plasmid β-galactosidase negative and sucrose transport positive (King, S.C. and Wilson, T.H., manuscript in preparation). It is therefore easy to screen for recombinants which are β-galactosidase-positive (blue on XG agar) and sucrose-negative (white on sucrose MacConkey agar). The details of this method follow.

The strains DP90C/F′I^{Q}Z$^+$Y$^+$, MAB20, and DW10ΔY, were mated to DW2/pSCK1 with subsequent counterselection on YT agar containing XG, IPTG and ampicillin. A blue clone from each mating was purified and mated to DW2PurE::Tn10 with selection on YT agar containing XG, IPTG, ampicillin (kills the female) and tetracycline (kills the male). Several blue clones were picked and plasmid DNA was prepared directly from colonies. As described above, this plasmid DNA is non-clonal and should contain plasmids of the original pSCK1 (*lacZ*$^-$ *Y*$^{sucrose+}$) phenotype as well as examples of the recombinant *lacZ*$^+$ *Y*$^{sucrose-}$ phenotype. Screening for the recombinants was performed by placing the plasmids in the genetic background of *E. coli* DW2/pRAF-S11 by transformation (with a limiting amount of DNA such that 50 to 300 colonies per plate were obtained). Because pRAF-S11 expresses an invertase (sucrase) activity constitutively, pSCK1 was readily distinguished from recombinants as only the former fermented (red colonies) on MacConkey agar containing 2% sucrose and ampicillin. The non-fermenting (white) clones were considered to be recombinants which have rescued *lacY* from the F-factor (and therefore have lost *lacY*$^{sucrose+}$). Further evidence of recombination was obtained by confirming: (i) that the sucrose-negative clones were *lacZ*$^+$ (blue on XG agar) and (ii) that the sucrose-negative clones retained pRAF-S11 (fermentation positive on MacConkey agar containing 1% raffinose). The simultaneous presence of β-galactosidase activity and raffinose fermentation together with the absence of sucrose fermentation was taken as strong evidence for homologous recombination across the *lacY* gene with concomitant rescue of *lacY*^{mut} from the F′-factor. Our experience to date is that clones isolated by this procedure which fail to ferment sucrose are always *lacZ*$^+$ and *raf*$^+$.

Subcloning and DNA sequencing. The replicative form of M13mp18 was cut with *SmaI* and was dephosphorylated with calf intestinal phosphatase as described [20] except that the phosphatase was inactivated by heating to 70°C for 40 min in the presence of 10 mM trinitriloacetic acid, a zinc chelator, which was added from a 10× stock solution (pH 7.8). The dephosphorylated DNA was placed in TE buffer by passing it through a spun column [20].

lacY was obtained on a *PvuII* restriction fragment from pAA22-S306L or pMAB20-H322Y. Ten units of *PvuII* were used to digest the DNA prepared by alkaline lysis from 0.75 ml of overnight culture in LB medium containing ampicillin. The restriction fragments were separated by electrophoresis through 1% SeaPlaque low melting agarose (FMC). The mini-gel was run at 8 V/cm at 4°C in buffer containing 50 mM

Tris acetate (pH 8.3 with NaOH) and ethidium bromide (0.25 μg/ml). The buffer was replenished after approximately 1.5 h. The 2200 base pair fragment (fourth largest) which exhibits anomalously high mobility under these conditions, was excised from the gel, and the agarose was melted at 70°C. Eight microliters of the melted agarose (cooled to 37°C) was added to 30 ng (2 μl) of dephosphorylated M13mp18 (*SmaI* digested). T4 DNA ligase (400 units) in 10 μl of buffer (100 mM Tris, 20 mM $MgCl_2$, 10% polyethylene glycol 8000, 2 mM Na_2ATP, 2 mM dithiothreitol, adjusted to pH 7.6) was added. The ligation mixture was incubated overnight at 16°C.

The entire *lacY* gene was sequenced by the method of Sanger et al. [21] using protocols provided by New England Biolabs (Beverly, MA). 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). Apart from the mutations described here, the sequencing data were in accord with the published *lacY* sequence [1].

The 2200 base pair *PvuII* restriction fragments from pDP90CY, pMAB20-H322Y, and pAA22-S306L were also placed in the dephosphorylated *ScaI* site of pBR322 to construct the plasmids, pBRY, pBRY-Y322, and pBRY-L306 respectively. In these constructs, the lactose carrier is expressed constitutively (though at low levels) from the *amp* promoter, and clones could be identified by lactose fermentation on MacConkey agar when the plasmids were placed in the genetic background of *E. coli* 2.00R.

Transport assays. Cells from an overnight culture (1% Tryptone/M63 containing an appropriate antibiotic) were diluted 50-fold into the same medium. After 3 to 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-labeled compounds; radioactivity was quantitated in a scintillation spectrometer. β-ONPG transport was measured colorimetrically as previously described [22].

En rance counterflow. Counterflow experiments were carried out in 100 mM potassium phosphate buffer (pH 7.0) essentially as described by Wong and Wilson [23]. 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 figures.

Efflux. Cells were grown to early logarithmic phase in LB medium containing ampicillin, and the cells were washed in 100 mM potassium phosphate (pH 7.5) and resuspended in the same buffer. Strains containing plasmids encoding the normal lactose carrier were loaded overnight at 4°C in 2 mM [^{14}C]TMG (2 μCi/ml) while cells harboring plasmids encoding mutant forms of the lactose carrier were preloaded with 8 mM [^{14}C]TMG (4 μCi/ml). These incubations contained $5.3 \cdot 10^{10}$ cells/ml. Efflux was initiated by diluting 10-μl aliquots 400-fold into 100 mM potassium phosphate buffer (pH 7.5). Efflux was terminated by adding 0.25 volumes of 100 mM $HgCl_2$ followed by rapid filtration. The filters were washed with 3 ml of 100 mM potassium phosphate, pH 7.0 containing 5 mM $HgCl_2$. 'Trans'-stimulation of efflux was assayed by including unlabeled substrate in the diluent at the concentrations indicated in the figure legends.

Proton transport. The pH electrode was used to measure the galactoside-dependent H^+ uptake into *E. coli* using the method of West [24] as modified by Wilson et al. [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 to $1.5 \cdot 10^{11}$ cells/ml and held on ice until use. The cells were diluted 5-fold with 120 mM KCl, 30 mM KSCN and made anaerobic by bubbling with nitrogen prior to initiating the pH recordings which were carried out at room temperature. 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 nmoles of anaerobic HCl at the end of each record.

Results

Sugar transport

Both mutants exhibited a severe defect in uphill sugar transport. Figs. 1A and 1B show that under the conditions studied, neither TMG nor melibiose was significantly accumulated by lactose carriers specified on pAA22-S306L or pMAB20-H322Y. Lactose accumulation studies could not be carried out with these cells since the plasmid contained the *lacZ* gene (coding for β-galactosidase). However, lactose accumulation could be assayed in cells containing a different plasmid construction which did not contain the *lacZ* gene. Fig. 1C shows that lactose was not accumulated by cells containing the plasmids pBRY-S306L and pBRY-H322Y.

In order to study downhill galactoside transport it was necessary to [illegible] plasmid containing the *lacZ* gene

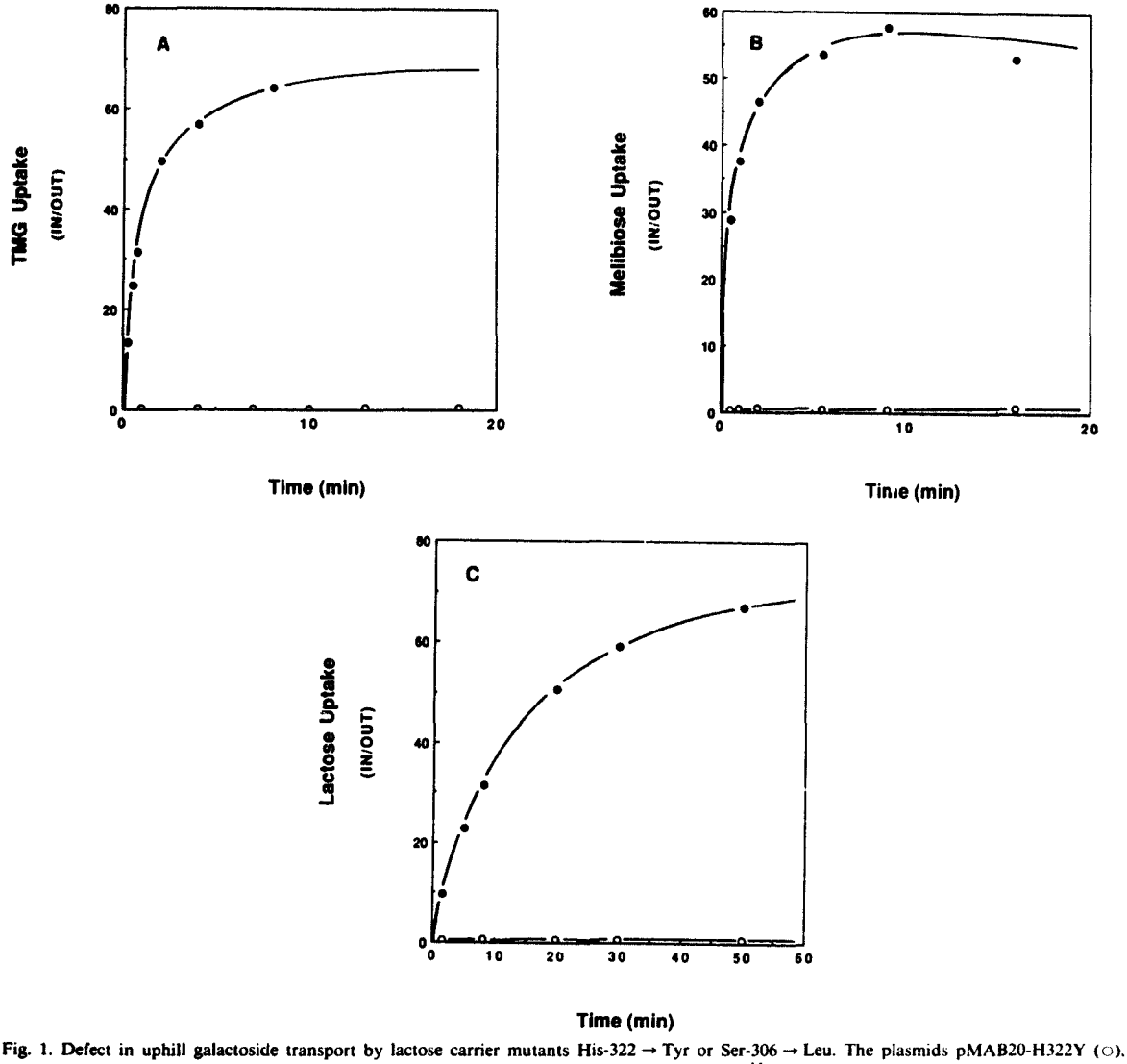

Fig. 1. Defect in uphill galactoside transport by lactose carrier mutants His-322 → Tyr or Ser-306 → Leu. The plasmids pMAB20-H322Y (○), pAA22-S306L (○), and pDP90CY (●) were placed in the *E. coli* DW2RA background for [^{14}C]TMG transport (Panel A), or in the DW1 background for [^{3}H]melibiose transport (Panel B). The plasmids pBRY-H322Y (○), pBRY-S306L (○), and pBRY (●) were placed in the DW2 background for [^{14}C]lactose transport (Panel C). The cells were grown and harvested as indicated in Materials and Methods. Cells ($3 \cdot 10^{9}$/ml) were incubated in buffer containing 0.1 mM radioactive sugar. The data are expressed as the ratio of the intracellular to extracellular substrate concentration.

and expressing β-galactosidase activity in excess of transport rate. Table II shows that large amounts of β-galactosidase were produced in cells induced with IPTG. The transport rate of cells containing the plasmid with a normal *lacY* gene (pDP90CY) was about 0.1 nmol ONPG entry per min per 10^{7} cells (Fig. 2). Since the β-galactosidase activity was 30-times larger (Table II), carrier mediated entry of β-ONPG is rate limiting. Therefore, in *lacZ* containing strains exposed to β-ONPG, the rate of *o*-nitrophenol production was taken as a measure of sugar entry. Both pMAB20-H322Y and pAA22-S306L encode a lactose carrier which is highly defective with regard to the downhill transport of β-ONPG (Fig. 2). The normal lactose carrier (pDP90CY) transports as much β-ONPG in 10 s as the mutants do over the course of 20 min.

Entrance counterflow experiments were performed using cells preloaded either with 20 mM TMG (Fig. 3A) or 10 mM melibiose (Fig. 3B). Neither pMAB20-H322Y nor pAA22-S306L specify a carrier which exhibited the

TABLE II

Activity of the cloned β-galctosidase

Strain	1 mM IPTG	β-Galactosidase	
		nmol/min per 10^7 cells	Fold induction[a]
DW2IQ	+	0	
DW2IQ	−	0	
DW2IQ/ pDP90CY	+	3.12	11
DW2IQ/ pDP90CY	−	0.28	
DW2IQ/ pAA22-S306L	+	3.38	9
DW2IQ/ pAA22-S306L	−	0.37	
DW2IQ/ pMAB20-H322Y	+	3.47	14
DW2IQ/ pMAB20-H322Y	−	0.24	

[a] In strains containing only a chromosomal *lacI*$^+$, the β-galactoidase is expressed constitutively. In such strains, β-galactosidase activity is comparable to the fully induced cases shown in this table.

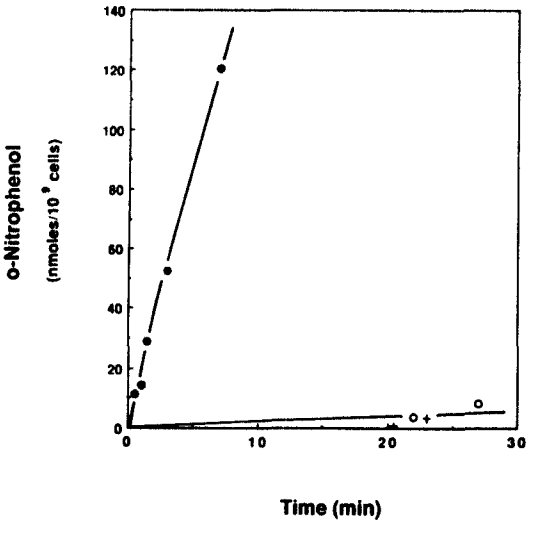

Fig. 2. Defect in downhill β-ONPG transport by lactose carrier mutants His-32 → Tyr or Ser-306 → Leu. The plasmids pMAB20-H322Y (+), pAA22-S306L (○), or pDP90CY (●) were placed in the *E. coli* DW2RA background. The cells were grown and harvested as indicated in Materials and Methods. The β-ONPG (1 mM) was exposed to $3.5 \cdot 10^8$ cells/ml and the reactions were quenched at the indicated times by adding two volumes of 0.6 M Na_2CO_3. Transport was quantitated by determining the *o*-nitrophenol concentration in a spectrophotometer (420 nm).

counterflow phenomenon under the conditions studied here. As expected, the normal carrier encoded on pDP90CY carried out counterflow. This transient accumulation of TMG failed to occur in cells that were not preloaded, indicating that the cells were adequately poisoned.

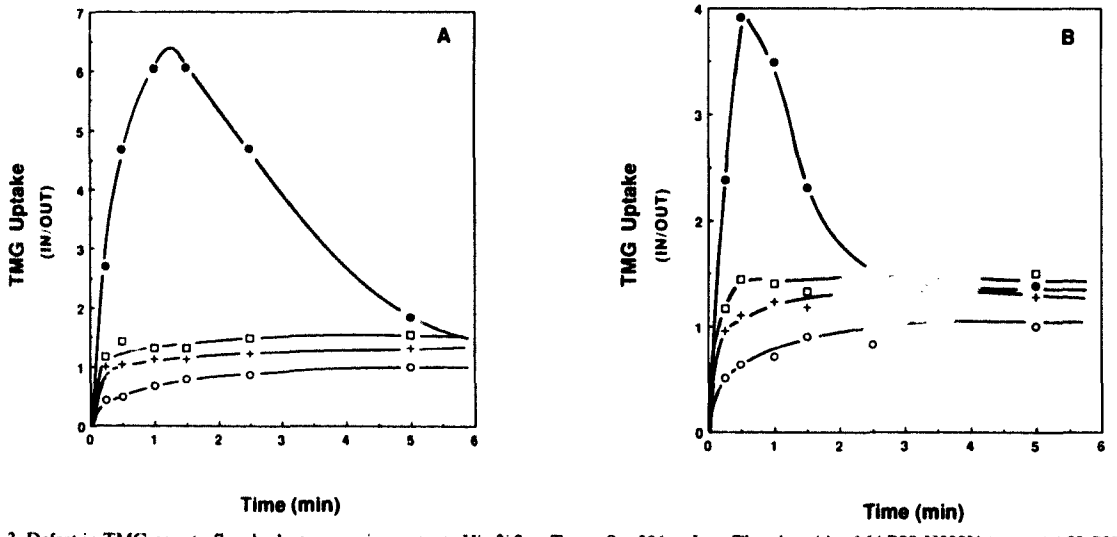

Fig. 3. Defect in TMG counterflow by lactose carrier mutants His-32 → Tyr or Ser-306 → Leu. The plasmids pMAB20-H322Y (+), pAA22-S306L (○), or pDP90CY (●) were placed in the *E. coli* DW1 background. The cells were poisoned with azide and preloaded with either 20 mM TMG (Panel A) or 10 mM melibiose (Panel B) as indicated in Materials and Methods. TMG uptake by the pDP90CY carrier was also tested in cells which were poisoned but not preloaded with sugar (□). Cells ($1.3 \cdot 10^9$/ml) were resuspended in buffer containing 0.1 mM [^{14}C]TMG. The data are expressed as the ratio of intracellular to extracellular [^{14}C]TMG concentration.

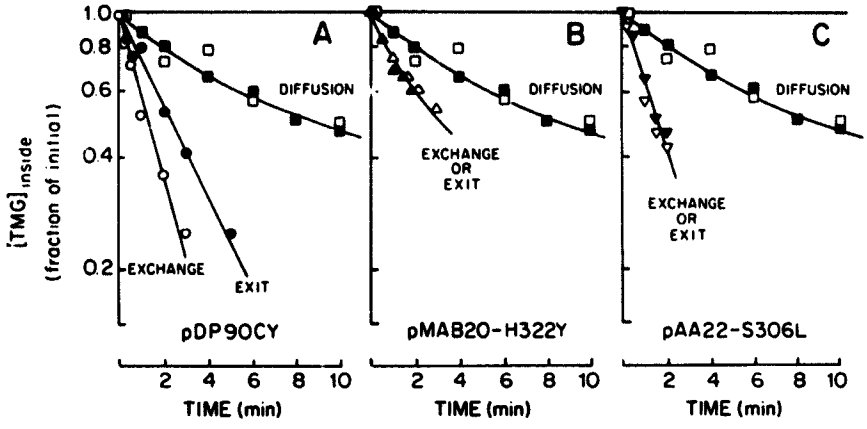

Fig. 4. TMG exit and exchange by the lactose carrier mutants His-322 → Tyr or Ser-306 → Leu. Cells preloaded with [^{14}C]TMG were diluted 400-fold into buffer with (open symbols) or without (solid symbols) 5 mM TMG. The radioactivity remaining in the cells at the indicated times was measured. All plasmids were placed in the *E. coli* DW2RA background. Panel A: pDP90CY (○ or ●). Panel B: pMAB20-H322Y (△ or ▲). Panel C: pAA22-S306L (▽ or ▼). In each panel, exit which was not mediated by the lactose carrier ('diffusion') was assessed with cells containing the plasmid pDW10ΔY (□ or ■) which does not express the lactose carrier.

Though they fail to show counterflow, both mutants catalyze net [^{14}C]TMG efflux (Fig. 4), for substrate exit from these cells was faster than that from cells carrying pDW10ΔY (*lacY* deletion). Unlabeled TMG in the extracellular solution failed to accelerate [^{14}C]TMG efflux on the lactose carriers specified by pMAB20-H322Y or pAA22-S306L. In contrast, external substrate did accelerate TMG efflux from cells expressing the parental lactose carrier from pDP90CY. We infer that the reactions leading to reorientation of the ternary complex between the inward/outward orientations are more rate limiting in either mutant than in the parental lactose carrier (under the conditions of Fig. 4).

In spite of the severely defective accumulation and counterflow, carrier activity was clearly apparent when downhill entry was examined. A qualitative measure of sugar entry at high sugar concentrations is given by the degree of fermentation observed on indicator plates (Fig. 5). Cells (with *lacZ* gene) containing a plasmid with no *lacY* gene fail to ferment lactose and colonies are white on the indicator plate. Positive lactose fermentation (red color) was observed for the cell with the

	MacConkey Agar Phenotype	
Plasmid	30 mM Melibiose	30 mM Lactose
pBR322	White ○	White ○
pBRY	Red Center ◉	Red ●
pBRY-S306L	Red ●	Red ●
pBRY-H322Y	Red ●	Red Center ◉

Fig. 5. Fermentation of lactose and melibiose by lactose carrier mutants His-322 → Tyr or Ser-306 → Leu. The plasmids pBR322, pBRY, pBRY-S306L, and pBRY-H322Y were placed in the *E. coli* 2.00R background (*lacI*$^+$*Z*$^+$*ΔY*) for lactose fermentation and in the DW2 background (*lacΔZYmelA*$^+$*ΔB*) for melibiose fermentation. The fermentation assays were carried out on MacConkey agar containing tetracycline and the indicated sugars (30 mM). The agar was incubated at 37°C for 15 h before recording the indicated phenotypes. Cells which fermented the sugar gave rise to red clones (indicated in the figure as solid circles). Non-fermenting clones were white (open circles).

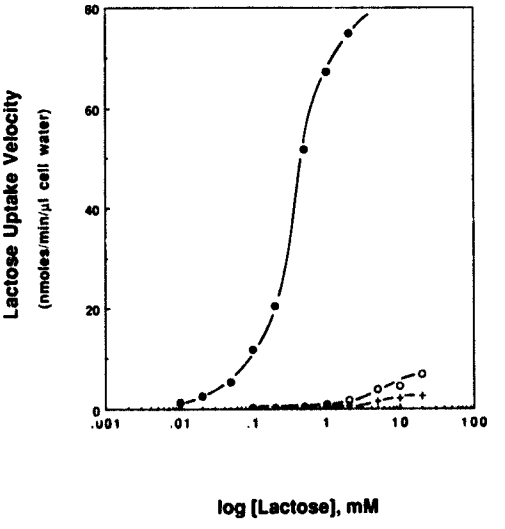

Fig. 6. Concentration dependence for downhill lactose transport by lactose carrier mutants Ser-306 → Leu or His-322 → Tyr. The plasmids pDP90CY (●), pAA22-S306L (○), or pMAB20-H322Y (+), were placed in the *E. coli* DW2RA background. Each point represents the slope obtained by least-squares fit to five data points from the linear portion of the time course for uptake at each [^{14}C]lactose concentration. The data have been corrected for the basal uptake rate obtained in parallel experiments carried out with a strain carrying the plasmid, pDW10ΔY, which expresses β-galactosidase, but not the lactose carrier.

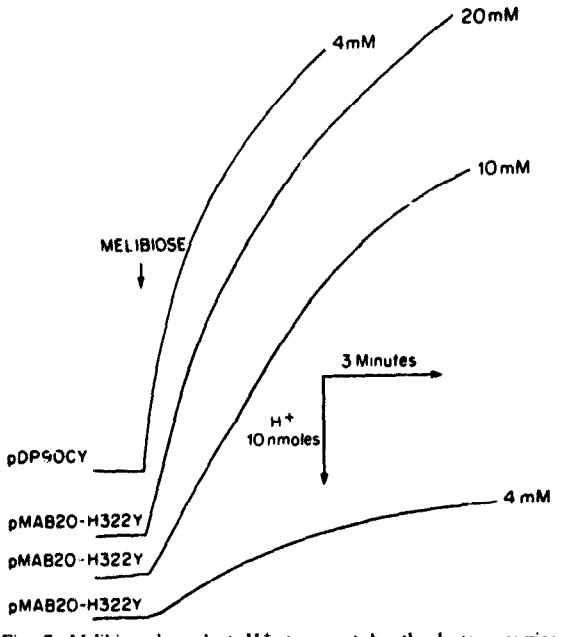

Fig. 7. Melibiose-dependent H^+ transport by the lactose carrier mutant with the His-322 → Tyr. The plasmids, pMAB20-H322Y and pDP90C, were placed in the *E. coli* DW1 background. The cells were made anaerobic as described in Materials and Methods. Anaerobic melibiose was added (at arrow) from a 1 M stock solution to achieve the indicated final concentrations. Upward deflections are in the alkaline direction.

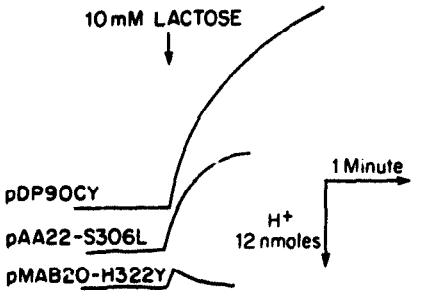

Fig. 9. Lactose-dependent H^+ transport by the lactose carrier mutants the Ser-306 → Leu or His-322 → Tyr. The plasmids, pAA22-S306L, pMAB20-H322Y, or pDP90CY, were placed in the *E. coli* DW1 background. The cells were made anaerobic as described in Materials and Methods. Anaerobic lactose was added (at arrow) from a 0.5 M stock solution. Upward deflections are in the alkaline direction.

parental *lacY* gene and also with the two mutants, the His-322 → Tyr mutant showing less color than the parent or the Ser-306 → Leu mutant. Wher melibiose fermentation was measured, the two mutants showed results that were somewhat more positive than the parent. Thus, entry rates for lactose and melibiose (when present at high concentrations) into cells containing the two mutant *lacY* genes were quite significant.

A quantitative measure of downhill lactose entry was carried out in cells harboring the two mutant plasmids

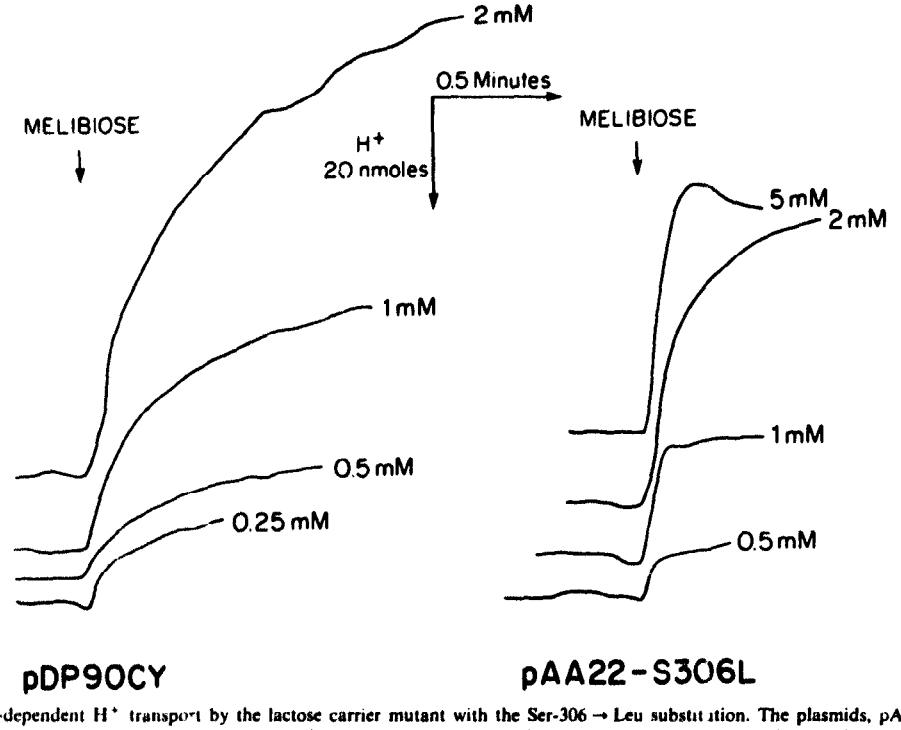

Fig. 8. Melibiose-dependent H^+ transport by the lactose carrier mutant with the Ser-306 → Leu substitution. The plasmids, pAA22-S306L and pDP90C, were placed in the *E. coli* DW1 background. The cells were made anaerobic as described in Materials and Methods. Anaerobic melibiose was added (at arrow) from a 1 M stock solution to achieve the indicated final concentrations. Upward deflections are in the alkaline direction.

(Fig. 6). The V_{max} as well as the apparent affinity for lactose transport on the carriers expressed by pAA22-S306L (K_T = 4.3 mM; V_{max} = 5.9 nmol/min per μl cell water) and pMAB20-H322Y (K_T = 5.6 mM; V_{max} = 2.6 nmol/min per μl cell water) were greatly reduced compared to the parent, pDP90CY (K_T = 0.6 mM; V_{max} = 94 nmol/min per μl cell water). The poor affinity and reduced maximum velocity for lactose uptake are consistent with the principal observation which served to distinguish the Y^{-K} mutants from other Y^- mutants, namely that the former grow on minimal medium, when the lactose concentration is raised to 100 mM [13], while *lacY*$^-$ mutants cannot.

Proton uptake

The next question was whether galactoside transport by cells expressing the mutant lactose carriers was associated with proton movement. Measurements of pH were carried out by the method of West [24] to determine whether proton entry was stimulated by addition of sugar as is observed with normal galactoside/H^+ cotransport. Addition of 20 mM melibiose to cells harboring pMAB20-H322Y resulted in proton uptake that was similar to that observed with the addition of 4 mM sugar to the cell expressing a normal carrier (Fig. 7). The V_{max} values for the two carriers may be similar. The apparent affinity of the mutant carrier for melibiose was quite defective, for adding 4 mM sugar to the mutant resulted in very much less proton uptake than that observed with the same concentration of sugar added to the cell expressing the normal carrier. In the Ser-306 → Leu mutant, on the other hand, melibiose-dependent proton uptake appeared slightly more rapid than in the parent (Fig. 8). This confirms the previous observation that melibiose fermentation was more vigorous in AA22 than in the parent [14]. Proton transport on the pAA22-S306L carrier was saturated over the range of concentration studied (0.5 to 5 mM) indicating that the K_T was quite low (less than 0.5 mM). Proton uptake stimulated by lactose (10 mM) was quite apparent in cells containing pAA22-S306L, but was severely defective for pMAB20-H322Y (Fig. 9). TMG-stimulated proton uptake was observed in cells harboring pAA22-S306L but not pMAB20-H322Y (Fig. 10).

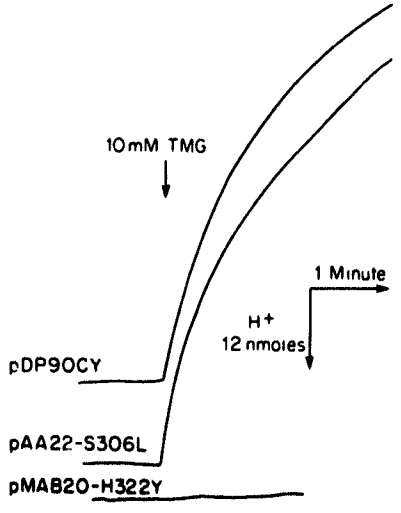

Fig. 10. TMG-dependent H^+ transport by the lactose carrier mutants Ser-306 → Leu or His-322 → Tyr. The plasmids pAA22-S306L, pMAB20-H322Y, or pDP90CY, were placed in the *E. coli* DW1 background. The cells were made anaerobic as described in Materials and Methods. Anaerobic TMG was added (at arrow) from a 1 M stock solution. Upward deflections were in the alkaline direction.

Discussion

The lactose carrier mutants, AA22 (Ser-306 → Leu) and MAB20 (His-322 → Tyr), which were studied in this communication were first described several years ago by Hobson et al. [12]. The characteristic which distinguished these mutants from literally hundreds of other *lacY*$^-$ mutations [13] was that growth on minimal salts depended on the presence of 100 mM lactose, whereas all other *lacY*$^-$ mutants failed to grow even with this high lactose concentration. Thus, it was presumed that these mutations must have affected the sugar recognition site and hence the affinity of lactose for these carriers. Accordingly, the mutants displaying this behavior were placed in a class which became known as Y^{-K} [13] or K_m [14] mutants. Indeed, transport studies confirmed the earlier observations that these two mutants show a poor affinity for lactose. On the other hand, DNA sequencing revealed the unexpected finding that these K_m mutants have amino acid substitutions which lie within the putative 'charge relay' domain [11,15], which is thought to be responsible for proton recognition.

Kaback and his colleagues have provided abundant evidence that His-322, Glu-325, and Arg-302 are important in coupling proton movement to lactose accumulation [10,11]. When His-322 was converted to arginine lactose accumulation, counterflow, exit, exchange, and proton transport were lost while downhill entry of 25 mM lactose (in fermentation tests) was present [26]. Identical properties were attributed to the mutant in which Arg-302 was converted to leucine [27]. Conversion of Glu-325 to alanine abolished lactose accumulation, exit, and proton transport, but not exchange, counterflow or fermentation of 25 mM lactose [15]. Modeling of the three dimensional structure of the carrier suggested that the three amino acids may be close to one another [11,27]. These observations have led Kaback et al. to suggest a charge-relay system as a mechanism for proton translocation through the carrier [15]. According to the hypothesis: (i) His-322 is necessary for protonation of the lactose carrier, and a defect

at this position abolishes all modes of galactoside transport proceeding through the ternary galactoside/carrier/H^+ complex (i.e., active uptake, efflux, counterflow, and exchange reactions); (ii) Glu-325 is on the deprotonation pathway, and a defect at this position abolishes those modes of galactoside transport requiring dissolution of the binary H^+/carrier complex (i.e., only exchange and a 'non-dissipative counterflow' phenomenon are retained); (iii) finally, there is a mechanistic implication that His-322 and Glu-325 form an essential ion pair, and that the pathway of the proton through the lactose carrier is similar to that found in the catalytic triad of the serine proteinases [15].

The two K_m mutants showed features common to the 'charge relay' mutants: Amino acid substitutions in the region of His-322 and Arg-302 as well as some of the physiological properties of these uncoupled mutants. Mutant MAB20 was found to have His-322 changed to tyrosine and in AA22 the nearby Ser-306 [11,27] was changed to leucine. Thus both sugar recognition and proton recognition might be altered simultaneously by a single amino acid substitution. Certain properties of the two mutants described here are similar to those observed by the Kaback laboratory for mutants at His-322 and Arg-302. Accumulation and counterflow are defective in all cases, while assays of fermentation remained positive. Like the His-322 → Arg mutation of the charge-relay domain [26], MAB20 (His-322 → Tyr) fails to show accumulation or counterflow (Figs. 2 and 4). Similarly, the leucine substitutions for either Arg-302 [27] or for Ser-306 (AA22) also abolish counterflow as well as accumulation (Figs. 2 and 4).

The most striking difference between the properties of His-322 → Tyr and those of the His-322 → Arg mutant is in exit and in proton cotransport with sugar entry. No proton cotransport with lactose was observed by Püttner et al. [26] in cells containing the His-322 → Arg substitution (melibiose was not tested). In contrast, strong proton uptake was observed in the His-322 → Tyr mutant when melibiose was added (Fig. 7). Although the affinity for melibiose in these proton uptake experiments appeared to be lower than that observed for the parent, the maximum rate for the mutant appeared to be the same order of magnitude as for the parental strain. The second mutant (Ser-306 → Leu) showed melibiose-dependent H^+ uptake which was as good or better than that of the parent. In addition, this latter mutant showed proton uptake with high concentrations of lactose and TMG. Both mutants catalyzed net TMG efflux (Fig. 4).

Our studies with the MAB20 mutation (His-322 → Tyr) show that while His-322 may play an important role in catalyzing *efficient* proton/galactoside symport, its absence causes no *fatal* defect in the capacity of the lactose carrier to catalyze galactoside-dependent proton transport per se. This observation is incompatible with an obligatory role for His-322 in proton translocation by the carrier in H^+/sugar cotransport.

A critical and presently unanswered question is whether the proton transport seen with MAB20 proceeds through the vicinity of position 322, perhaps aided by hydrogen bonding to nearby residues and/or to water molecules. However, it is clear from site-directed mutagenesis that the phenylalanine-322 substitution carries out both lactose-dependent and melibiose-dependent proton transport. These data indicate that the tyrosine hydroxyl group at position 322 plays no critical role in the proton transport carried out by the mutant MAB20 (King, S.C. and Wilson, T.H., unpublished data).

Another point of interest is that the results with mutant AA22 suggest that the activity of the lactose carrier is conceivably as sensitive to the volume of amino acid side chains as it is to chemical properties of the side-chains when the mutations happen to be located in the domain of the putative charge relay system. Kaback and co-workers have shown that serine-306, though probably near histidine-322 and glutamate-325 [11,27], does not complete the catalytic triad expected by analogy to the mechanism of serine proteinases. It was found that the amino acid substitution, Ser-306 → Ala, left the lactose carrier completely functional [27]. The chemical properties of alanine of course forbid its participation in reactions calling for hydrogen bond formation. The results reported here indicate that the chemically conservative amino acid substitution in the AA22 mutant which creates Leu-306 rather than Ala-306 has devastating effects on the K_T and V_{max} of the carrier when using lactose as a substrate (Fig. 6). The point that bears emphasis is that the difference between the Ala-306 and Leu-306 mutations is unlikely to be explained by differences in side-chain chemistry. Most likely a steric mechanism accounts for the defect in AA22, and therefore the importance of adverse steric interactions in the vicinity of the charge-relay domain must be entertained. On the other hand, it might be argued that the sensitivity of this region to structural perturbation may simply reflect an underlying role for this region in mediating critical conformational changes intrinsic to the mechanism of cotransport.

The coincident occurrence of both proton and galactoside recognition mutants within quite a limited structural domain suggests that there may be an important relationship between sugar binding and proton binding sites in the lactose carrier of *Escherichia coli*. Indeed, precedence for such a relationship comes from the *E. coli* melibiose carrier for which it was found that mutations affecting sugar recognition simultaneously affect cation selectivity (change in preference for H^+, Na^+, or Li^+) and vice versa [28,29]. Overt changes in cation specificity have not yet been observed in the lactose carrier. If the mechanism of proton transport

involves a hydrogen-bonded chain of any description, then alkali cations are forbidden from participating in the mechanism. On the other hand, the association of altered substrate recognition with changes in pK_a of residues in the charge-relay domain can be easily entertained. For example, Kaback has found that the His-322 → Arg substitution leads not only to a severe defect in lactose recognition but also to complete loss of proton recognition [15]. The substitution, His-322 → Tyr, described here also leads to altered substrate recognition, but proton recognition remains measurable with the alternative substrate, melibiose. Thus, a general principle appears to be emerging that the interaction between the cation and substrate binding sites on cotransporter proteins are such that mutations which modify the interaction of a cotransporter with one of its substrates can simultaneously affect the interaction of the carrier with its cosubstrate. The relationship between cation and sugar recognition on the melibiose and the lactose carriers of *E. coli* is consistent with the hypothesis that there is allosteric linkage between the sites which may be intrinsic to the energy transducing mechanism [30,31], although he data could also be explained if the sugar molecule actually formed part of the cation binding site [29].

Apparently, the mutants described here fail to transduce energy from the protonmotive force. A striking characteristic of the mutants, MAB20 and AA22, is that under all conditions studied, they fail to accumulate substrates against a concentration gradient. Yet, experiments using the pH electrode show clearly that galactoside transport is accompanied by proton transport. It seems likely, therefore, that these mutant carriers proceed through the *same* catalytic cycle as the normal carrier, but with energetics altered such that energy transduction was not observed.

Given that the MAB20 and AA22 mutations lie in the putative 'charge-relay' domain, one might speculate that proton transfer reactions carried out by these mutants are altered such that energy from the protonmotive force is not harvested. In particular, if His-322 does play a major role in proton translocation and free energy transduction, then the importance of this residue may be related to its unique capacity to perform a charge-relay function. Therefore, the substitution of a tyrosine at position 322 could perturb a protonation reaction in the mutant via lost capacity to modulate pK_a values (because electrons cannot be drawn across tyrosine in the same manner as in histidine). Alternatively, a steric mechanism–like that described above for the nearby AA22 (Ser-306 → Leu) mutation–could disturb protonation reactions in the His-322 → Tyr mutant.

On the other hand, it is possible that the defect in either mutant is unrelated to any specific effect on the proton conduction pathway. Tanford [30,31] has suggested that energy transducing transport proteins function because chemical potential is transferred in a concerted manner from a bound form of the driving substrate to the carrier protein itself. The carrier in turn transfers chemical potential to a bound form of the cosubstrate. A reciprocal relationship exists, therefore, between the binding sites for either ligand. The ligand at higher chemical potential in solution becomes the driving substrate. Unfortunately, this leads to an inherent ambiguity in making the inference from transport defects that a mutation is located in either the proton or the galactoside binding domains in the lactose carrier. The question which remains, therefore, is whether to assign mutations (e.g., MAB20 or AA22) which abolish concentrative uptake (but not proton-coupled transport) to a bona fide proton conduit which would in turn cause an indirect defect on apparent galactoside affinity, or alternatively, to assign these loci to a bona fide sugar binding domain, thus causing direct effects of sugar affinity, and allosteric effects on the interaction of H^+ with the proton conduit. These are important mechanistic distinctions which require further study.

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References

1 Büchel D., Gronenborn, B. and Müller-Hill, B. (1980) Nature 283, 541–545.
2 Beyreuther, K., Overath, P. and Wright, K.J. (1982) Hoppe Seyler's Z. Physiol. Chem. 363, 1409–1414.
3 Foster, D.L., Boublik, M. and Kaback, H.R. (1983) J. Biol. Chem. 258, 31–34.
4 Vogel, H., Wright, J.K. and Jähnig, F. (1985) EMBO J. 4, 3625–3631.
5 Newman, M.J. and Wilson, T.H. (1980) J. Biol. Chem. 255, 10583–10586.
6 Newman, M.J., Foster, D.L., Wilson, T.H. and Kaback, H.R. (1981) J. Biol. Chem. 256, 11804–11808.
7 Costello, M.J., Escaig, J., Matsushita, K., Viitanen, P.V., Menick, D.R. and Kaback, H.R. (1987) J. Biol. Chem. 262, 17072–17082.
8 Wong, P.T.S., Kashket, E.R. and Wilson, T.H. (1970) Proc. Natl. Acad. Sci. USA 65, 63–69.
9 West, I.C. and Wilson, T.H. (1973) Biochem. Biophys. Res. Commun. 50, 551–558.
10 Kaback, H.R. (1988) Annu. Rev. Physiol. 50, 243–256.
11 Kaback, H.R. (1987) BioEssays 7, 261–265.
12 Hobson, A.C., Gho, D. and Müller-Hill, B. (1977) J. Bacteriol. 131, 830–838.
13 Mieschendahl, M., Büchel, D., Bocklage, H. and Müller-Hill, B. (1981) Proc. Natl. Acad. Sci. USA 78, 7652–7656.

14 Wilson, T.H., Seto-Young, D., Bedu, S., Putzrath, R.M. and Müller-Hill, B. (1985) Curr. Top. Membr. Transp. 23, 121–133.
15 Carrasco, N., Antes, L.M., Poonian, M.S. and Kaback, H.R. (1986) Biochemistry 25, 4486–4488.
16 Bassilana, M., Pourcher, T. and Le Blanc, G. (1987) J. Biol. Chem. 262, 865–870.
17 Malamy, M.H., Rahaim, P.T., Hoffman, C.S., Baghdoyan, D., O'Connor, M.B. and Miller, J.F. (1985) J. Mol. Biol. 181, 551–555.
18 Schmid, K. and Schmitt, R. (1976) Eur. J. Biochem. 67, 95–104.
19 Langridge, J. (1974) Aust. J. Biol. Sci. 27, 331–340.
20 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor.
21 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
22 Wilson, T.H. and Kusch, M. (1972) Biochim. Biophys. Acta 255, 786–797.
23 Wong, P.T.S. and Wilson, T.H. (1970) Biochim. Biophys. Acta 196, 336–350.
24 West, I.C. (1970) Biochem. Biophys. Res. Commun. 41, 655–661.
25 Wilson, D.M., Tsuchiya, T. and Wilson, T.H. (1986) Methods Enzymol. 125, 377–387.
26 Püttner, I.B., Sarkar, K.H., Poonian, M.S. and Kaback, H.R. (1986) Biochemistry 25, 4483–4485.
27 Menick, D.R., Carrasco, N., Antes, L., Patel, L. and Kaback, H.R. (1987) Biochemistry 26, 6638–6644.
28 Yazyu, H., Shiota, S., Futai, M. and Tsuchia, T. (1985) J. Bacteriol. 162, 933–937.
29 Botfield, M.C. and Wilson, T.H. (1988) J. Biol. Chem. 263, 12909–12915.
30 Tanford, C. (1983) Annu. Rev. Biochem. 52, 379–409.
31 Tanford, C. (1981) Proc. Natl. Acad. Sci. USA 78, 270–273.
32 Jacob, F. and Monod, J. (1961) J. Mol. Biol. 3, 318–356.
33 Bolivar, F., Rodriguez, R.C., Green, P.J., Betlach, M.C., Heynecker, H.L. and Boyer, H.W. (1977) Gene 2, 95–113.