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Mechanism of enhanced melibiose transport rate catalyzed by an *Escherichia coli* lactose carrier mutant with leucine substituted for serine-306. The pH-dependence of melibiose efflux

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The mechanism of melibiose transport was studied in cells containing plasmid pAA22 which expresses the mutant lactose carrier (serine-306 to leucine) cloned from *Escherichia coli* AA22. These studies were of interest because several lines of evidence suggested that the AA22 mutation conferred novel properties upon the lactose carrier, decreasing turnover with several β-galactoside substrates, increasing turnover with melibiose, and abolishing active accumulation even though equilibration occurred via symport with H^+. Although severely defective in active melibiose accumulation, the present study indicates that in cells poisoned with azide the AA22 carrier does in fact equilibrate melibiose across the membrane more rapidly than the normal lactose carrier. Similarly, melibiose efflux from cells preloaded with melibiose was more rapidly catalyzed by the AA22 carrier than by the normal carrier (pH 7.0). Furthermore, although external H^+ did reduce net melibiose efflux to a rate slower than seen in equilibrium exchange, a lower than normal pH was required to achieve this effect. Therefore, at pH 7.0, the AA22 carrier (but not the normal carrier) catalyzed net efflux at a rate approaching that for the exchange process (which was pH-resistant in both the mutant and the parent). At pH 8.0 both the AA22 carrier and the normal carrier catalyzed net melibiose efflux at a rate indentical to the equilibrium exchange rate. We suggest (i) that the sensitivity of melibiose efflux to external pH indicates that during efflux the AA22 carrier interacts with protons in a manner similar to the normal carrier (i.e., sugar is cotransported with H^+) and hence the absence of accumulation is not explained by internal leak via a binary carrier-melibiose complex; and (ii) that the modest increase in rate constants for melibiose exit reflect small changes in activation energy (1 kcal/mol) consistent with a steric mechanism possibly involving van der Waals contacts.

Introduction

The *Escherichia coli* lactose carrier is an H^+/galactoside cotransport protein encoded by the *lacY* gene. The carrier has been solubilized [1], purified [2], and reconstituted in functional form [2] such that the carrier probably functions as a monomer [3]. It has been predicted that the secondary structure comprises 12 or 14 transmembrane α-helices [4–6]. The lactose carrier captures energy from protons moving down their electrochemical gradient in order to drive active translocation of lactose or other galactoside substrates moving up a chemical gradient [7].

One approach to developing a better understanding of energy transduction mechanisms is to study mutants which transport substrates but fail to concentrate them. One such mutant, AA22, was isolated by Hobson et al. who selected for reduced ability to transport lactose [8]. Further study revealed that the AA22 mutant had interesting properties. It could grow on minimal medium containing 100 mM lactose, but not on medium containing 5 mM lactose, suggesting that the mutation affected affinity for lactose [9]. In contrast, the mutant lactose carrier expressed by AA22 transported melibiose at a rate greater than that of the normal carrier. Wilson et al. [10] found that whereas the *lac* operon with *lac* Y^+ under control of *lac* I^Q could not be induced by

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

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melibiose, the *lac* operon containing *lac* Y^{AA22} could be induced by melibiose. Comparison of the initial rates of melibiose-dependent H^+ transport measured with the pH electrode also suggested that the mutant carrier from AA22 transported protons and melibiose across the membrane more rapidly than the normal carrier [11].

Thus, a remarkable feature of the AA22 mutant was that it failed to accumulate melibiose against a concentration gradient even though proton translocation occurred concomitantly with downhill sugar transport. Interestingly, DNA sequencing revealed that the mutation in AA22 changed serine 306 → leucine [11]. This was significant because molecular modeling [12] suggested that Ser-306 could be quite near the important 'charge-relay' domain postulated to be critical to either H^+ transport [13,14] or energy transduction [15].

In this communication, we present further evidence to support the idea that lactose carrier mutant AA22 mediates proton-coupled melibiose transport at a rate faster than the parent. The results imply (i) that the absence of active melibiose accumulation is not due to internal leakage of melibiose via a binary melibiose-carrier complex and (ii) that enhanced carrier turnover observed with the mutant is attributable to rapid formation and/or equilibration of the ternary melibiose-carrier-H^+ complex between forms oriented either inward or outward.

Materials and Methods

Materials. Bacteriological media were from Difco. Melibiose was from Sigma. [^{3}H]Melibiose was a generous gift of Dr. Gérard Le Blanc [16], and was purified by descending paper chromatography (Whatman No. 1 paper) using propanol/water (3 : 1, v/v). Other chemical were obtained from usual sources and were of the highest quality commercially available.

Strains. The genetic background for cells used in all experiments was *E. coli* DW1 which has the relevant genotype *lac* I^+ $\Delta(ZY)$ *mel* $\Delta(AB)$ *strA* [17]. The plasmid pDP90CY [11] encodes the normal lactose carrier. Plasmid pAA22 is isogenic with pDP90CY except for a point mutation in *lacY* which substitutes leucine for the serine normally specified by codon 306 [11].

Growth of cells. Overnight cultures were grown in LB medium containing ampicillin (100 μg/ml). The overnight cultures were diluted 100-fold into fresh medium containing 1 mM IPTG and grown to approx. $(6–8) \cdot 10^8$ cells/ml.

Preparation of cells for transport. Freshly grown cells were washed one time with 100 mM potassium phosphate (pH 7.5) and resuspended in the same buffer to give $(1.8–2.4) \cdot 10^{10}$ cells/ml (1/30 the original culture volume). The cells were equilibrated to 37 °C in a water bath and treated with EDTA as described by Sarkar et al. [18]. An equal volume of resuspension buffer containing 2 mM EDTA was added. The incubation was continued for exactly 3 min before quenching the EDTA with 100 volumes of 100 mM potassium phosphate/10 mM $MgSO_4$ (pH 7.5). The EDTA-treated cells were harvested by centrifugation, and then resuspended in 3–4 ml of the same buffer to give approx. $6 \cdot 10^9$ cells/ml.

Preloading with sugars. An Eppendorf centrifuge was used to harvest $3.7 \cdot 10^9$ EDTA-treated cells. The supernatant was carefully removed with an aspirator, and the pellet was resuspended with 70 μl of 100 mM potassium phosphate (pH 7.0) containing the appropriate sugar. The solution containing the resuspended cells was then made up to 10 mM $MgSO_4$ and 20 μg/ml DNAase I [19] from 100-fold concentrated stock solutions. Unless otherwise indicated, the cells were then stored overnight at 4 °C. The next day the cells were made up to 30 mM KN_3 (added from a 3 M stock solution) and incubated for 2 h at room temperature before storing on ice for use in experiments. Where cells were additionally treated with CCCP, this was done by adding the KN_3-treated cells to an Eppendorf tube containing sufficient CCCP residue (dried from ethanol) to achieve 50 μM CCCP in the stock cell suspension. Under these conditions, [^{3}H]melibiose was essentially equilibrated with the cytoplasm.

Efflux and exchange experiments. 8 μl of EDTA-treated cells preloaded with 20 mM [^{3}H]melibiose (20 μCi/ml) were diluted 500-fold into medium containing an equal concentration of unlabeled sugar (equilibrium exchange) plus 30 μM CCCP or into sugar-free medium plus 30 μM CCCP for net efflux. At times shown in the figures the reactions were quenched by adding a quarter volume of 100 mM $HgCl_2$. The sample was immediately poured over a suction filter (0.65 μm pore size cellulose nitrate; Sartorius). The tube was rinsed with 5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM $HgCl_2$; this was poured over the filter, and the filter was washed with 5 ml of rinsing solution. The filter was dissolved in 4 ml of Liquiscint (National Diagnostics) and radioactivity was quantitated with a scintillation spectrophotometer. Carrier-independent exit was monitored by treating the preloaded cells with 2 mM $HgCl_2$ (from a 50-fold concentrated stock solution) prior to dilution into efflux or exchange medium already containing 20 mM $HgCl_2$. Data from numerous experiments indicate that in mercury-treated cells (both mutant and parent) carrier-mediated exit and exchange are abolished, and the cells became essentially impermeable to melibiose over the time course followed in these experiments.

Counterflow experiments. To initiate counterflow, 8 μl of EDTA-treated cells preloaded with 20 mM melibiose were diluted 200-fold into 100 mM potassium phosphate containing 30 mM KN_3 and [^{3}H]melibiose such

that the final external melibiose concentration was 0.1 mM (0.6 μCi/ml). At times shown in the figures, the reactions were quenched by adding 2 vols of 100 mM potassium phosphate (pH 7.0) containing 20 mM $HgCl_2$. The samples were processed in the manner described for the efflux and exchange experiments.

Calculation of intracellular sugar concentration. In several experiments the results are expressed as ratio of intracellular to extracellular sugar concentration (in/out). The internal concentration was calculated based on the correlation between intracellular water content and absorbance (600 nm) in which 1 absorbance unit is equivalent to 0.7 μl intracellular water per ml of cell suspension [20].

Results

Lactose carrier mutant AA22 is severely defective with regard to concentrative uptake of melibiose present at a low (0.2 mM) extracellular concentration (Fig. 1). It should be emphasized that the genetic background of these cells (deletion of the *melA* gene) does not permit metabolism of the melibiose. However, when uptake was studied with a 10-fold higher melibiose concentration (Fig. 2) under conditions in which entry can be observed without accumulation (cells poisoned with azide and CCCP), the sugar equilibrated far more rapidly with the internal space of cells expressing the pAA22 lactose carrier (Ser-306 → Leu) than with the internal space of cells expressing the normal lactose carrier from pDP90CY (half-time 5 s vs. 120 s).

Similarly, net efflux from poisoned cells (pH 7.0) was catalyzed 6-fold more rapidly by the pAA22 carrier than by the normal carrier expressed from pDP90CY (Fig. 3). Efflux appeared to be first-order, and half-times from cells expressing the AA22 or DP90CY carrier were 7 s and 40 s, respectively. Whereas placing 20 mM unlabeled melibiose in the external medium (equilibrium exchange conditions pH 7.0) accelerated [^{3}H]melibiose exit from cells expressing the normal carrier, no significant change was noted for cells expressing the pAA22 carrier at pH 7.0.

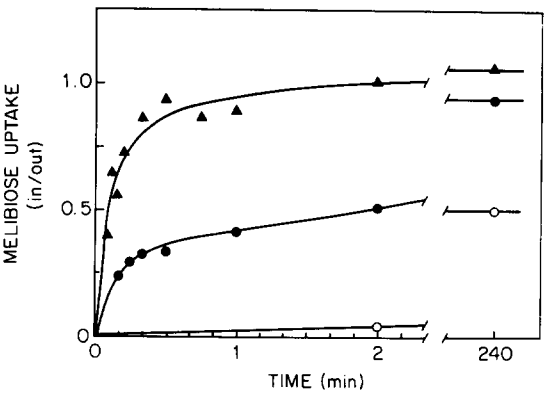

Fig. 2. Equilibration of [^{3}H]melibiose with the cytoplasm of azide-poisoned cells. *E. coli* DW1 harboring plasmids pDP90CY (●) or pAA22 (▲) were grown and treated with EDTA, concentrated to $5.3 \cdot 10^{10}$ cells/ml in 100 mM potassium phosphate/10 mM $MgSO_4$ (pH 7.0) and poisoned with 30 mM KN_3 as indicated in Materials and Methods. Uptake reactions were initiated by adding 10 μl of 4 mM [^{3}H]melibiose (6 μCi/ml) to an equal volume of the cell suspension. After the intervals shown, the reactions were quenched by adding 4 vols of 100 mM potassium phosphate buffer (pH 7.0) containing 20 mM $HgCl_2$. The reaction mixture was filtered, washed twice with buffer containing 5 mM $HgCl_2$ and processed as described in Materials and Methods. Carrier-independent sugar entry (○) was estimated by treating the pDP90CY with 1 mM $HgCl_2$ before measuring transport.

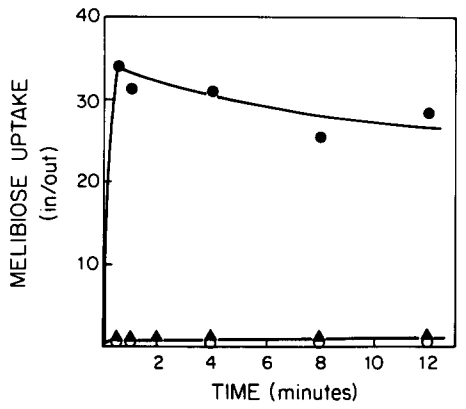

Fig. 1. Defective [^{3}H]melibiose accumulation. *E. coli* DW1 (○) or DW1 harboring plasmids pDP90CY (●) or pAA22 (▲) were grown to mid log phase in LB medium containing 1 mM IPTG, centrifuged, and washed twice with 100 mM potassium phosphate buffer (pH 7.0). Transport was initiated by exposing cells ($3 \cdot 10^9$/ml) to 0.2 mM [^{3}H]melibiose (0.4 μCi/ml). The reactions were terminated at appropriate intervals by filtering 0.1 ml samples and washing with 5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM $HgCl_2$.

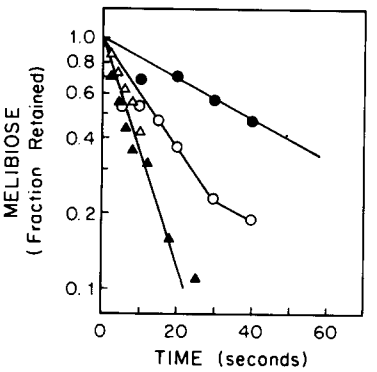

Fig. 3. Melibiose efflux and equilibrium exchange at neutral pH. EDTA = treated cells were preloaded with 20 mM [^{3}H]melibiose (20 μCi/ml) and poisoned with 30 mM KN_3 and 50 μM CCCP as indicated in Materials and Methods. Exchange (open symbols) was initiated by diluting cells 500-fold into 100 mM potassium phosphate (pH 7.0) containing 30 μM CCCP and 20 mM melibiose. Efflux (solid symbols) was initiated by dilution into the same buffer without melibiose. The parental plasmids, pDP90CY (● or ○) and the mutant plasmid, pAA22 (▲ or △) were held in the genetic background of *E. coli* DW1.

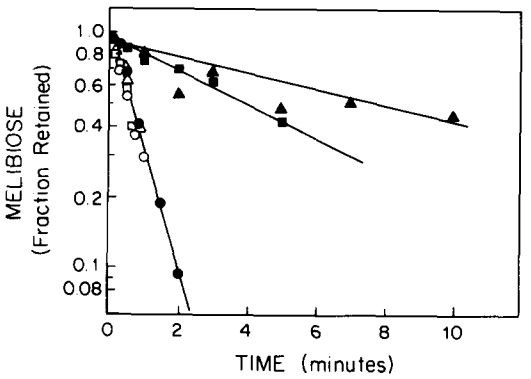

Fig. 4. Effect of proton concentration on melibiose efflux or equilibrium exchange in the normal lactose carrier expressed from pDP90CY. EDTA-treated cells were preloaded with 20 mM [^{3}H]melibiose (20 μCi/ml) and then poisoned with 30 mM KN_3 and 50 μM CCCP as described in Materials and Methods. Efflux (▲, □, ●) was initiated by diluting the cells 500-fold into 100 mM potassium phosphate buffer pH 5.6 (triangles), pH 6.5 (squares), or pH 8.0 (circles) containing 30 μM CCCP. Equilibrium exchange reactions (△, □, ○) were carried out analogously except that the diluting buffer contained 20 mM melibiose.

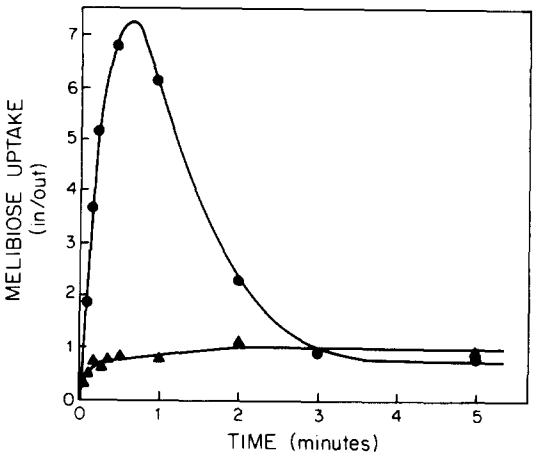

Fig. 6. Comparison of counterflow catalyzed by the parental and mutant lactose carriers. EDTA-treated cells were preloaded with 20 mM melibiose. Counterflow was initiated by diluting the preloaded cells into medium containing [^{3}H]melibiose such that the final concentration was 0.1 mM (0.6 μCi/ml). The parental plasmid pDP90CY (●) and the mutant plasmid pAA22 (▲) were held in the genetic background of *E. coli* DW1.

For both normal and the Ser-306 carrier, net efflux (but not equilibrium exchange) was pH-dependent over the range 5.6 to 8.0 (Figs. 3, 4 and 5). As a result, the rates of equilibrium exchange and net efflux of melibiose were equivalent at pH 8.0 but efflux became far slower than exchange as pH was lowered to 5.6. It was also apparent that the maximum rate of carrier turnover was 6.5-fold faster for the Leu-306 carrier compared to the parent (pH 8.0).

Although the behavior of the AA22 mutant was qualitatively similar to the parent, the sensitivity of the Leu-306 carrier to inhibition by external protons appeared to be lower than that of the normal carrier as judged from the effect of pH on efflux. Comparison of Figs. 3, 4, and 5 shows that lower pH values were required with the Leu-306 carrier in order to retard net efflux relative to exchange. Interestingly, these experiments also revealed a slight inhibition of the exchange reaction catalyzed by the mutant when pH was decreased to 5.6 (Fig. 5). This effect was absent from the parental carrier (Fig. 4).

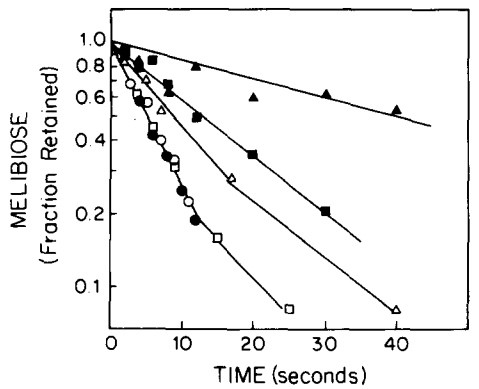

Fig. 5. Effect of proton concentration on melibiose efflux or equilibrium exchange in the mutant lactose carrier expressed from pAA22. EDTA-treated cells were preloaded with 20 mM [^{3}H]melibiose (20 μCi/ml) and then poisoned with 30 mM KN_3 and 50 μM CCCP as described in Materials and Methods. Efflux (▲, ■, ●) was initiated by diluting the cells 500-fold into 100 mM potassium phosphate buffer pH 5.6 (triangles), pH 6.5 (squares), or pH 8.0 (circles) containing 30 μM CCCP. Equilibrium exchange reactions (△, □, ○) were carried out analogously except that the diluting buffer contained 20 mM melibiose.

Although the parental cell catalyzed vigorous counterflow of 0.1 mM [^{3}H]melibiose against 20 mM unlabeled melibiose, the mutant did not (Fig. 6). This result could imply a strong preference (at neutral pH and low external sugar concentration) for dissolution of the externally oriented binary carrier-proton (CH) complex. This would lead to net efflux instead of reformation of the ternary complex as would be required for exchange and counterflow activity [21]. However, counterflow is a complex phenomenon and defects manifest at the inner aspect of the membrane should be considered (see Discussion).

Discussion

E. coli AA22 is one example of the class of lactose carrier mutants which have been called Y^{-K} [9] or K_m mutants [10]. Most notably, this class of mutant fails to grow on a low lactose concentration (5 mM), whereas a much higher concentration (100 mM) will support growth [9,10]. These mutants do not appear to carry out active accumulation of non-metabolized substrates such as TMG [10], and downhill transport of the hydrolyzable substrates (e.g., β-ONPG and lactose) is also severely defective [10,11].

TABLE I

Summary of transport data

n.a., not applicable (TMG not metabolizable); n.d., not determined.

	Melibiose	TMG	Lactose	References
Accumulation (in aerobic cells)	none	none	n.d.	11
Sugar-dependent H^+ entry	increased	normal	decreased	11
Fermentation (on indicator plates) [a]	increased	n.a.	normal	11
Exit rate at pH 7	increased 6-fold	normal	n.d.	11, here
Counterflow (azide-treated cells)	none	none	n.d.	11, here

[a] Sugar concentration = 30 mM.

Melibiose entry. A characteristic of the AA22 mutant which distinguished it from other mutants in the Y^{-K} family was that it appeared to ferment the α-galactoside melibiose even more vigorously than the parental strain [10] (see Table I for summary of properties). Similarly, uptake of 2 mM [^{3}H]melibiose by the AA22 carrier was faster than that catalyzed by the normal carrier when cells were poisoned (Fig. 2). These data confirm that the AA22 carrier retains the capacity to transport melibiose across the membrane at rates comparable to the parent. What factors, then may be held to account for the observation (Fig. 1) that a low melibiose concentration (0.2 mM) cannot be significantly accumulated?

Thermodynamics hold that for any mechanism of the sort depicted in Fig. 7 the sugar chemical gradient ($-\Delta\mu_S$) at equilibrium should be exactly equal to the protonmotive force [7] (i.e., the kinetic parameters of transport cannot affect the final state). However, this result applies strictly to ideal carriers (which operate with complete efficiency) and is not an inevitable endpoint for real carriers (i.e., thermodynamic limits need not be realized in practice). Real carriers (especially mutants) are subject to problems related to efficiency and problems related to kinetics. Efficiency may be compromised either (i) by 'internal resistance' (work required to mediate carrier turnover per se) or (ii) by two kinds of 'leak' pathway, the 'external leak' involving diffusion of cosubstrates through the lipid bilayer and the 'internal leak' involving isomerization of binary intermediates (CS or CH, see Fig. 7) of the transport mechanism.

In this regard, it is important that direct measurement of melibiose-dependent H^+ transport (using the pH meter) in the AA22 mutant showed proton transport to be quite vigorous compared to the parental strain [11]. Although it is conceivable that the absence of melibiose accumulation (Fig. 1) may to some extent be attributed to 'uncoupling' (i.e., sugar transport via reorientation of the binary CS complex), the predominant sugar-dependent alkalinization of the medium places a severe limit on the extent to which such a mechanism could contribute to the absence of concentrative uptake (i.e., partial 'uncoupling' cannot completely abolish melibiose accumulation at equilibrium).

If 'uncoupling' cannot account for the absence of accumulation, then to what extent might this defect be attributed to kinetic causes? The work of Page [22] as well as Wright and Seckler [23] (who studied the ML307-22 and 54-51 mutants, respectively) establishes the propensity of kinetic effects to mimic the 'uncoupled' phenotype. In the present study, results obtained from efflux, exchange, and counterflow experiments suggest that rather than being 'uncoupled' the AA22 carrier manifests a kinetic defect possibly involving its interaction with H^+.

Efflux and equilibrium exchange. Melibiose efflux catalyzed by the AA22 carrier was 6-fold faster than that catalyzed by the parental carrier at neutral pH (Fig. 3). Apparently, two effects of the Ser-306 → Leu substitution contribute to this observation. The first effect is related to an apparent decrease in the affinity of the outwardly oriented carrier for H^+. The second effect is related to an apparent increase in the rate of the melibiose exchange reaction (i.e., formation and/or isomerization of the ternary H^+-carrier-melibiose complex from inward to outward facing forms).

The Ser-306 → Leu substitution altered the pH-dependence for net efflux in a manner consistent with decreased apparent affinity for H^+. As a result, the AA22 carrier catalyzes net efflux at a rate comparable to the exchange rate at neutral pH (Fig. 3). Under the same conditions, the normal carrier experiences considerable inhibition by external protons and hence net efflux, which involves carrier deprotonation, is slow relative to exchange (Fig. 3).

The literature contains no consensus regarding the mechanism by which external protons inhibit efflux. Three separate phenomena may contribute to the inhibitory effect of external H^+. The first phenomenon is an effect which has been emphasized by Kaback and coworkers [21,24,25]. External H^+ limits efflux-driven carrier turnover by a simple product inhibition mechanism unrelated to events at the inner aspect of the plasma membrane. The second phenomenon is an effect of the plasma membrane. The second phenomenon is an effect which stems from the reasoning of Wright et al. [7] that the carrier should partition among intermediates of the transport cycle in a manner consistent with the laws of mass action. Accordingly, external protons cause accumulation of the CH complex (external) at the expense of internal CH′ (via the C ↔ C′ isomerization) thereby slowing efflux by slowing formation of CSH′ (Fig. 7). The third phenomenon, described by Page et al. [26], involves the effect of H^+ binding to a site (not the

symport site) having pK_a 6.3. When occupied by a proton, this site decrease the carrier turnover number.

Irrespective of whether there is agreement concerning the lactose carrier mechanism, the Ser-306 → Leu substitution appears to increase the melibiose exchange rate. Exchange almost certainly involves formation and isomerization of a ternary complex, and it is also clear that exchange bypasses reactions that are rate-limiting for net efflux. Thus, the observation that the AA22 carrier catalyzed melibiose exchange 6.5-fold faster (pH 8.0) than the parental carrier (compare Figs. 4 and 5), suggests that there is rapid formation and isomerization of the ternary melibiose-carrier-H^+ complex. According to one simple model, equilibrium exchange involves only reactions 1, 2 and 3 (Fig. 7). Hence, the Ser-306 → Leu substitution facilitates one of these reactions which limit melibiose exchange catalyzed by the normal carrier to a rate about 6-fold slower than the mutant.

Melibiose counterflow. Counterflow is a complex phenomenon and several factors may contribute to the defect (Fig. 6) observed with the mutant *. First, the initial rate of counterflow is proportional to the rate constant for isomerization of the ternary CSH complex from the outward to the inward facing form. The results of equilibrium exchange experiments at high melibiose concentration (20 mM) suggest that this rate constant is at least as large in the mutant as in the parent and should not account for any defect in the initial rate. However, the initial rate of counterflow is also inversely proportional to the dissociation constant of the outwardly oriented carrier for the labeled substrate. Thus, any defect in initial rate would have to be accounted for by a decrease in affinity of the sugar for the externally oriented carrier.

Second, the accumulation ratio achieved in counterflow is influenced by several factors, including the rate constant for isomerization of the unloaded carrier from the inward to outward facing form, the rate constant for isomerization (to the outward facing form) of the ternary

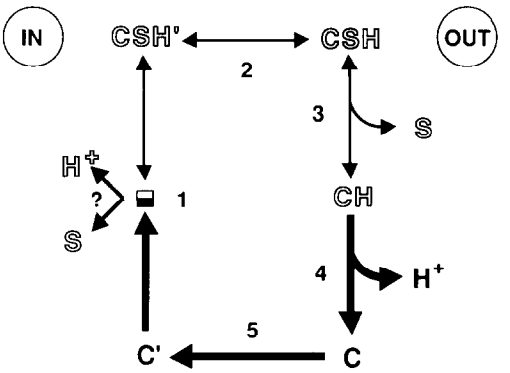

Fig. 7. A simple model of the melibiose efflux and exchange processes catalyzed by the *Escherichia coli* lactose carrier. The melibiose efflux reaction comprises a minimum of five steps: (1) binding of the proton and melibiose (order unspecified) to the inwardly oriented carrier (C′), (2) isomerization of the ternary complex from the inward (CSH′) to the outward (CSH) oriented conformation, (3) dissociation of melibiose (S) from the ternary complex, (4) dissociation of the symported proton from the carrier-proton (CH) complex, and (5) isomerization of the outwardly oriented carrier (C) to the inwardly oriented conformation (C′) which completes the full cycle required for net efflux. Equilibrium exchange is presumed to require only reactions 1, 2, and 3 and unlike net efflux is resistant to changes in external pH since the process occurs without proton dissociation. Intermediates unique to the efflux reactions (represented with black lettering) interconvert via equilibria represented by bold arrows. The subset of intermediates required for the exchange reaction (represented by outline lettering) interconvert via the equilibria represented by thinner arrows. This view of the exit and exchange processes is essentially that of Kaczorowski and Kaback [21].

complex formed with unlabeled substrate at inner aspect of the membrane, the affinity of inwardly oriented carrier for the unlabeled sugar, and the affinity of the inwardly oriented carrier for H^+. The results of influx, efflux, and equilibrium exchange experiments are consistent with the view that the rate constants for isomerization (in to out) for either the free carrier or the ternary complex with melibiose are at least as rapid in the AA22 mutant as in the parent (Figs. 2, 4 and 5). Thus, the absence of [^{3}H]melibiose accumulation in counterflow may be due to a decrease in the internal affinity for either melibiose or H^+. Indeed, the rapid approach to equilibrium for melibiose uptake in poisoned cells (Fig. 2) could be consistent with a lower level of product inhibition at the inner aspect of the membrane in the AA22 mutant. Possibly, the apparent decrease in affinity of the mutant carrier for H^+ (which decreases the product inhibition at the external surface during efflux, Figs. 3 and 5) is also manifest at the inner aspect of the membrane during counterflow such that accumulation of [^{3}H]melibiose is not observed (due to the smaller than normal β'' value **).

Substrate specific effects. The Ser-306 → Leu substitution affects lactose transport in a very different manner.

* Wright [29] has given a quantitative description of the kinetic parameters which determine the character of the counterflow timecourse catalyzed by cotransporters. Briefly, $V_0 = (C_T)(k_{1+}) \cdot (\alpha')$, where V_0 is the initial rate of counterflow, C_T is the total carrier concentration, k_{1+} is the isomerization rate constant (out to in) for the ternary (CSH) complex involving the labeled sugar (outside), and α' is the ratio of the concentration of the labeled substrate to its dissociation constant (outside). The accumulation ratio (in/out) at the peak of the counterflow time course is given as $G''/G' = A[k_{o-} + (k_{2-})(\gamma'')(\beta'')]/k_{o-}$, where G''/G' is the accumulation ratio of the labeled substrate, A is the magnitude of the proton motive force, k_{o-} is the rate constant for the isomerization (in to out) of the unloaded carrier (C), γ'' is the ratio of the concentration of the unlabeled substrate to its dissociation constant (inside), and β'' is the ratio of the proton concentration to the proton dissociation constant for the inwardly oriented carrier.

** See footnote on this page, left-hand column.

Lactose-dependent H^+ transport (measured with the pH meter) is abnormal, and 'downhill' [^{14}C]lactose entry is severely defective, showing an elevated K_m and low V_{max} [11]. The sugar-independent reactions (steps 4 and 5, Fig. 7) are quite rapid in the mutant as indicated by the rapid entry and exit of melibiose (Figs. 1 and 3). The defect in lactose transport must therefore be in steps 1, 2, or 3 (Fig. 7). Similarly, it has been demonstrated that for certain other mutations in 'charge-relay' domain (His-322 → Tyr and His-322 → Phe) discrimination between lactose (slow exchange) and melibiose (rapid exchange) is at the level of reactions required for exchange, i.e., the formation, isomerization, and dissolution of the ternary complex. Like the AA22 mutation, the His-322 → Tyr substitution appears to destabilize interaction with protons, accelerate melibiose efflux, (King and Wilson, submitted for publication), and exhibits preference for melibiose over lactose as judged from the ability to couple protons to transport of the former but not the latter [15].

Another mutation at position 306 has been reported by Menick et al. [27], who created a Ser-306 → Ala mutant by oligonucleotide-directed mutagenesis. The Ala-306 mutant was normal in all functions tested. Therefore, active transport does not require a hydroxyl function at position-306. This presumably means that Ser-306 does not interact directly with either the symported H^+ or with sugar substrates in the normal carrier. The relatively small change in the chemical properties of the two substitutions at position 306 (Leu vs. Ala) have profoundly different functional consequences to active transport possibly owing to the close proximity of position 306 (as judged from molecular modelling [12]) to the important 'charge-relay' triad, His-322, Glu-325, and Arg-302 [28]. The modest effect of the Leu-306 mutation on the rate constants for melibiose exit reactions measured under various conditions is indeed consistent with a steric mechanism as previously suggested [11] *.

If the effects of the Ser-306 → Leu substitution are explained by indirect steric effects in the 'charge-relay' domain, then the sugar-specific effect of mutation Leu-306 (as well as the Tyr-322 and Phe-322 mutations) could imply either that the 'charge-relay' domain affects sugar binding energy per se or that the sugar effect is secondary to an indirect effect of these mutations on H^+ binding. Alternatively, the energy of carrier conformations adopted during isomerization of the ternary complex may be affected in a manner dependent upon the structure of the bound sugar. This latter possibility is significant given (i) the failure of all known mutants of the 'charge-relay' domain to mediate active transport [15,34], and (ii) the assertion by Tanford [35,36] that conformational changes associated with the ternary complex are key to energy transduction.

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* It follows from Eyring's transition state theory [30–33] that the approx. 6-fold ratio of rate constants for melibiose efflux (pH 8.0) catalyzed by the mutant or the parental carrier reflects a modest 1 kcal/mol difference in free energy of activation as calculated from the formula:

$$\frac{k_{out(N)}}{k_{out(M)}} = \exp\left(\frac{\Delta G^*_M - \Delta G^*_N}{RT}\right)$$

in which the subscripts N and M represent parameters for the normal and mutant, respectively, R is the gas constant, T is the absolute temperature, and ΔG^* represents the activation energy for k_{cat}/K_m for exist data under non-saturating conditions. Consistent with a steric mechanism for the effect of the AA22 mutation, this 1 kcal/mol increment in relative stability of the transition state is on the order of that expected for a change in van der Waals interactions or hydrogen bond angles [27,33].

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