Lactose permease of *Escherichia coli*: Properties of mutants defective in substrate translocation
(galactoside:H\(^+\)-cotransport/substrate binding/lacY gene/\(\alpha\)-helix)

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ABSTRACT Mutants of lactose permease of *Escherichia coli* with amino acid changes (Gly-24 → Glu; Gly-24 → Arg; Pro-28 → Ser; Gly-24, Pro-28 → Glu-Ser and Gly-24, Pro-28 → Arg-Ser) within a putative membrane-spanning \(\alpha\)-helix (Phe-Gly-Leu-Phe-Phe-Phe-Phe-Tyr-Phe-Phe-Ile-Met-Gly-Ala-Tyr-Phe-Pro-Phe-Pro-Ile) are incorporated into the cytoplasmic membrane. The mutant proteins retain the ability to bind galactosides, and the affinity for several substrates is actually increased. However, the rate of active transport is decreased to 0.01% of the wild-type rate in the mutants carrying Arg-24 or Arg-24, Ser-28. Kinetic analysis demonstrates that the two mutants require 10 min to cause occupied binding sites for galactoside and H\(^+\) to change their exposure from the periplasm to the cytoplasm as compared to 50 ms in the wild type. The effect is less pronounced when these sites are unoccupied.

The translocation of solutes across biological membranes by integral proteins is considered to occur in at least four steps. First, the solute binds to the transporter at binding sites exposed to one side. Second, a conformational transition of the substrate–protein complex leads to the exposure of the binding sites to the other side of the membrane. In a third step, the solutes dissociate. Finally, a second conformational change of the unloaded transporter restores the original orientation of the binding sites. For most systems, the evidence for this cyclic transport scheme rests on kinetic experiments, whereas the molecular mechanisms remain largely unknown. In particular, the lack of information on the three-dimensional structure and the internal dynamics of these proteins has impeded progress in the understanding of the putative conformational changes.

One of the most extensively studied solute transporters is the lactose permease catalyzing galactoside:H\(^+\)-cotransport across the cytoplasmic membrane of *Escherichia coli* (1–4). Within the framework of the translocation cycle, described above, kinetic experiments have led to a detailed model of how the transporter brings about the facilitated diffusion of galactoside and proton in the absence of an electrochemical proton gradient, \(\Delta\psi\)\(_H\), and which kinetic parameters are altered during active transport in the presence of \(\Delta\psi\)\(_H\) (refs. 4–6; J.K.W., unpublished data). Lactose permease is a monomeric protein (7, 8) of known sequence (9, 10). Current models for the folding of the protein envision a highly compact structure of a ring of some 10 hydrophobic or amphipathic \(\alpha\)-helices and a core of more hydrophilic sequences (5, 8, 11). Only minor parts are exposed to the aqueous phase (2, 3, 11, 12). The binding sites for galactoside and proton are likely located in the center of the protein (13) so that concerted motions of peptide regions in both the core and the surrounding palisades of \(\alpha\)-helices may make the binding sites of the cosubstrates alternately accessible to either side of the membrane.

If substrate recognition and the conformational change involved in translocation are distinct events in transporter function it may be possible to differentially affect these steps by mutation. Mutants that are defective in substrate binding but retain the ability to undergo the conformational change(s) normally required for translocation cannot be characterized at present because the conformational change as such cannot be measured. On the other hand, mutants that retain the ability to bind substrate but are defective in translocation can be defined if a binding assay is available. Such mutants are the subject of this report.

MATERIALS AND METHODS

Isolation and Characterization of Mutants. The lacY\(^+\) carrying hybrid plasmid pTE18 (14) was mutagenized with NH\(_2\)OH for 30 min at 70°C (15). Transformants of strain T28RT (14) were selected on nutrient agar plates containing 10 \(\mu\)g of tetracycline per ml. Lac\(^-\) colonies were detected by replica plating onto mineral salts agar plates containing 1.4 mM lactose. Growth of cells, membrane preparations, methods for measuring substrate binding and transport, and immunological techniques have been described (6, 14, 16–19).

The mutants in Y\(^{599}\), Y\(^{17}\), and Y\(^{106}\) all map at the 5′ end of the gene, as determined by sequencing the 5′ end up to the Ava I site (9) and the Lac\(^-\) phenotype of recombinants at the Ava I site carrying the mutant 5′ end and the wild-type 3′ end of the gene (20). The double mutations Y\(^{599}\) and Y\(^{106}\) were obtained by oligonucleotide-directed mutagenesis of the Ava I fragment containing the 5′ end of the Y\(^{599}\) gene using the oligonucleotides 5′ GAAATAGGGCTCTCATGATAA 3′ for Y\(^{599}\) and 5′ GAAGTAGGCTCTCATGATAA 3′ for Y\(^{106}\) according to Kunkel (21).

RESULTS

Mutant Isolation. In vitro mutagenesis of the lacY gene coding for the lactose permease yielded 144 mutants unable to grow on 1.4 mM lactose. Among 44 of these mutants, 3 were found in which lactose permease retained the ability to bind the high-affinity model substrate p-nitrophenyl \(\alpha\)-D-galactopyranoside (NpaGal) but was strongly reduced in the binding.

Abbreviations: NpaGal, p-nitrophenyl \(\alpha\)-D-galactopyranoside; GalGal, \(\beta\)-D-galactopyranose; DodGal, dodecyl \(\beta\)-D-galactopyranoside; DodOMalt, dodecyl \(\beta\)-D-maltoside; RSO, right-side-out; ISO, inside-out; EMB, esoin/methylene blue.
ability to transport o-nitrophenyl β-d-galactopyranoside. The mutations were designated Y05, Y17, and Y106. Y05 caused a Lac phenotype on eosin/methylene blue (EMB) indicator plates and on mineral salts (M9) plates containing elevated levels of lactose (Table 1). Y17 and Y106 gave rise to a weakly positive Lac phenotype on EMB plates and a positive phenotype on M9 plates containing 55 mM lactose, a relatively high concentration. In addition, Table 1 lists the mutant genes YP03 and YP01 that were obtained by oligonucleotide-directed mutagenesis. These mutants have a completely negative Lac phenotype.

**DNA Sequence.** Table 1 lists the relevant codons of the wild-type gene and the nucleotide and amino acid changes in the mutants. Y05 showed three transitions leading to a Thr → Ile change at codon 7, to a Met → Ile change at codon 11, and a Gly → Arg change at codon 24. For the substrate-binding positive, translocation negative phenotype of this mutant, only the Gly → Arg change is relevant. This was shown by the isolation of a Lac+ revertion of Y05. This reversion (Y098R) had a nucleotide change from AGA → GGA at codon 24, thus restoring the wild-type glycine at this position, but retained the two isoleucine codons at numbers 7 and 11. The properties of the product of Y098R were indistinguishable from the wild type in terms of transport and substrate binding (20).

The Y17 and Y106 genes had transitions corresponding to a Pro → Ser change at codon 28 and a Gly → Glu change at codon 24, respectively.

In addition, the double mutants YP03 and YP01 were constructed. YP03 combines the change in Y17 and the relevant change in Y05, therefore coding for a permease with Arg-24, Ser-28. YP01 combines the nucleotide changes of Y17 and Y106 and thus codes for glu-24, ser-28.

**Immunoblots and Substrate Binding.** Incorporation of the mutant permeases into the cell membrane was demonstrated by binding of antibodies against the synthetic carboxy-terminal peptide in immunoblots of membrane proteins separated by NaDodSO4/polyacrylamide gel electrophoresis (18, 19). All five mutants incorporated the same amount of lacY gene product into the membrane, whereas membranes from strain T215 used as a background were completely deficient (see ref 14, Table 2). Therefore, the mutant polypeptide chains were translated in the correct reading frame and incorporated to similar levels into the membrane.

At saturating concentrations of NpaGal all five mutants showed the same number, n, of binding sites per mg of cell envelope protein (Table 2). However, compared to the wild type (Kt = 22 µM, see ref. 24) the affinity was increased by a factor of 3–4 for the products of Y05, Y17, Y106, and YP01 and a factor of nearly 10 for YP01. This result is illustrated in Fig. 1A for membranes containing the mutant proteins YP01 and YP01. The affinity of the products of Y05 and YP01 also was increased for lactose, for which Kd (Y05) = 0.63 mM and Kd (YP01) = 0.95 mM versus Kd (Y17) = 14 mM, but only slightly increased for β-d-galactosyl 1-thio-β-d-galactopyranoside (GalSgal), for which Kd (YP01) = 44 µM and Kd (YP01) = 26 µM versus Kd (Y17) = 76 µM, as shown by competition experiments. Therefore, folding of the mutant proteins led to a basically correct assembly of the sugar binding site that recognizes some substrates with an increased affinity.

**Transport.** Wild-type permease gave rise to an initial rate of uptake of 46.5 nmol of GalSgal per min/mg of cell protein at a concentration of 10 µM, resulting in a 17-fold accumulation within 1 min (Table 2). Influx of this substrate has been shown to occur with a maximum rate, kcat = 20 sec⁻1, and a half-saturation constant, Kt = 80 µM (6).

Mutant proteins Y17 and Y106 evinced reduced rates of uptake at 4–5% of the wild-type rate, and transport was clearly active (9-fold accumulation in 20 min). A residual transport activity of these mutants was consistent with their Lac phenotype at high lactose concentrations. The combination of these mutations in the YP01 product led to a further decrease in transport rate (by a factor of 20; a factor of 1000 below the wild-type rate) and reduced the accumulation to a factor of merely 2 in 20 min (Table 2).

The products of the Y05 and YP01 genes were studied in more detail because their defect in transport was most severe (Table 2 and Fig. 1B). Y05 transported GalSgal with a half-saturation constant, Kt, = 22 µM, and a maximum velocity, kcat = 0.11 min⁻1, lower by a factor of 11,000 than in the wild type at 1200 min⁻1. Within 20 min the substrate was accumulated 1.7-fold. The combination of Arg-24 and Ser-28 in the YP01 product did not lead to a further significant decrease in the rate of transport (Kt = 18 µM, kcat = 0.09 min⁻1, smaller by a factor of 13,000 than the wild type, 1.8-fold accumulation in 20 min). These low rates of transport are considered to be characteristic for the mutant permease.

**Table 1. Properties of lacY mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>lacY genotype on plasmid</th>
<th>EMB lactose</th>
<th>M9 lactose</th>
<th>Reversion frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.4 mM</td>
<td>55 mM</td>
<td></td>
</tr>
<tr>
<td>T52RT</td>
<td>pTE18</td>
<td>Y*</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
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<td>pTE18-59</td>
<td>Y05</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>T307</td>
<td>pTE18-17</td>
<td>Y05</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>T308</td>
<td>pTE18-106</td>
<td>Y06</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T215</td>
<td>pGM21</td>
<td>Y*</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>T217</td>
<td>pGM21-59</td>
<td>Y05</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Y05</td>
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<td>T4JP</td>
<td>pJAS9</td>
<td>Y06</td>
<td>+</td>
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<tr>
<td>T6JP</td>
<td>pJAS11</td>
<td>Y06</td>
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<tr>
<td>T303</td>
<td>pPO3</td>
<td>Y06</td>
<td>+</td>
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<tr>
<td>T304</td>
<td>pPO4</td>
<td>Y06</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Strain T52RT [lacI+ lacZ+/F lacPZ+ Y-lacΔ1/Δ(Z) Y*] carries the lacY* gene as part of a 2300-base-pair (bp) fragment inserted into the EcoRI site of plasmid pBR322 (14). Strains T212, T307, and T308 carry the mutant plasmids pTE18-59, pTE18-17, and pTE18-106, respectively, but are otherwise isogenous to strain T52RT. Strain T215 served as background for all other strains listed. It has the identical chromosomal permease (lacI3 lacZ+) as strain T52RT—i.e., it is derived from strain T217 through T304 all carry hybrid plasmids of pACYC184 (23) and the 2300-bp fragment with either the Y* gene (pGM21, see ref. 14) or various mutant genes (strains T219 through T304). pGM21-59 was obtained by transfer of the 2300-bp insert from pTE18-59 to pACYC184; derivation of hybrid plasmids pJPR1 through pPO4 is described in the text. Orientation of the insert in the vector is always the same as in pGM21. For further markers on the chromosome, F factors, and plasmids, see ref. 14. The Lac phenotype was determined on EMB lactose plates or mineral salts (M9) lactose plates in the presence of 10 µg of tetracycline per ml. ND, not determined.
molecules rather than revertants because the reversion frequency of these mutants was on the order of $10^{-6}$ to $10^{-7}$ (Table 1).

Next, efflux and exchange by the mutant permeases $Y^{p3}$ and $Y^{p03}$ were investigated. In poisoned cells loaded with 10 $\mu$M [3H]GalSGal, efflux was first order with half-times of 36 min (Fig. 1C, D and E) compared to a $t_{1/2} < 0.5$ min in the wild type (not shown). Initial rates of efflux (2–3 pmol/min per mg of cell protein at 10 $\mu$M [3H]GalSGal) in the mutants were somewhat smaller than the rates of active transport (Table 2). When loaded cells were diluted into medium containing 125 $\mu$M unlabeled GalSGal, the half-times for efflux under conditions of galactoside exchange decreased to 22 min and 16 min for $Y^{p3}$ (■) and $Y^{p03}$ (○), respectively. The loss of GalSGal from carrier-deficient Y- cells by passive diffusion could be distinguished by its slower rate ($t_{1/2} = 121$ min) and by the lack of stimulation by external galactoside ($A, t_{1/2} = 121$ min). These experiments demonstrate that the $Y^{p3}$ and $Y^{p03}$ gene products catalyze efflux and exchange of galactoside at greatly reduced rates compared to those of the wild-type permease, suggesting that the conformational change of the loaded transporter is severely impaired in both directions. This leaves the rate of the conformational change of the unloaded transporter undetermined. The measurement of the rate of the in ↔ out isomerization of the $Y^{p03}$ gene product was attempted by rapid binding experiments.

### Table 2. Active transport and substrate binding

<table>
<thead>
<tr>
<th>Strain</th>
<th>lacY genotype on plasmid</th>
<th>Uptake at 10 $\mu$M [3H]GalSGal</th>
</tr>
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<tbody>
<tr>
<td>T217</td>
<td>$Y^{p3}$</td>
<td>cpm/min per mg cell protein</td>
</tr>
<tr>
<td>T219</td>
<td>$Y^{p03}$</td>
<td>nmol/min per mg cell protein</td>
</tr>
<tr>
<td>T303</td>
<td>$Y^{p03}$</td>
<td>Fold accumulation*</td>
</tr>
<tr>
<td>T4JP</td>
<td>$Y^{p3}$</td>
<td></td>
</tr>
<tr>
<td>T6JP</td>
<td>$Y^{p03}$</td>
<td></td>
</tr>
<tr>
<td>T304</td>
<td>$Y^{p03}$</td>
<td></td>
</tr>
<tr>
<td>T215</td>
<td>—</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Binding of NpaGal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n_{50} \times 10^{-3}$ nmol/ml</td>
</tr>
</tbody>
</table>

*Accumulation after 1 min in strain T217 and after 20 min in the other strains. Uptake was measured in 50 mM K$_2$PO$_4$/10 mM glycerol, pH 6.3, under aeration. Strain T217 was induced with 0.1 mM isopropyl-β-D-thiogalactoside; all other strains were grown with 0.5 mM inducer.

1$\mu$mol of NpaGal binding sites at saturation in 50 mM K$_2$PO$_4$/M MgSO$_4$, pH 6.3, in a cell envelope preparation obtained by sonication.

2Dissociation constant for NpaGal.

3Number of permease molecules in the identical membrane preparations used for NpaGal binding determined by quantitative immunoblotting (19).

4Membranes from strain T217 were employed as a standard.

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**Fig. 1.** Binding and transport of galactosides in mutants T219 and T303. (A) Binding of NpaGal to total membrane preparations obtained by repeated sonication of cells. The degree of binding $P$ in nmol of NpaGal per mg of membrane protein is plotted against $1/[\text{NpaGal}]$, with the concentration of free (0) NpaGal in $\mu$M. The plots were evaluated for the number of binding sites, $n$, and the dissociation constant, $K_d$ (see Table 2). (B) Transport in EDTA-treated cells (1 mg of cell protein per ml) at 25°C in well-aerated 50 mM K$_2$PO$_4$/2 mM MgSO$_4$, pH 6.3, containing 10 mM glycerol (volume, 1 ml). Permease-mediated uptake of [6,6-$\alpha$H$_2$]GalGalG (1.9 TBq/mol) was determined as the difference in radioactivity in samples incubated without and with 0.5 $\mu$mol unlabelled NpaGal taken at time points up to 20 min. Uptake was halted by addition of 0.35 M KCl/2 mM HgCl$_2$. The rate of active transport, in pmol of GalSGal per min/mg of cell protein, was plotted against $v/[\text{GalSGal}]$, yielding $V_{max}$ values of 19.1 pmol/min per mg for strain T219 and 16.7 pmol/min per mg for strain T303. The half-saturation constants, $K_s$, were 22 and 18 $\mu$M, respectively. (C) Efflux of GalSGal in cells treated with EDTA and washed in 50 mM K$_2$PO$_4$/pH 6.3/2 mM MgSO$_4$/10 $\mu$M CIP/2CICN$_2$/10 mM Na$_2$S$_2$. Cell suspensions (45 mg of protein per ml) were made 10 $\mu$M in [6,6-$\alpha$H$_2$]GalGalG (75 TBq/mol) and 20 $\mu$g/ml in DMSO and incubated 18 hr at 4°C. After an additional 3 hr at 25°C, a 20-$\mu$l suspension was diluted into 2 ml of 50 mM K$_2$PO$_4$/pH 6.3/2 mM MgSO$_4$ without (open symbols) or with (closed symbols) 125 $\mu$M unlabeled GalSGal. Efflux was terminated by addition of 0.35 M KCl/2 mM HgCl$_2$. Background radioactivity corresponding to infinite time was determined either by immediately diluting cells after addition of labeled GalSGal or by incubating (3 hr, 25°C) loaded cells after a 1:100 dilution with 0.5 mg of sodium cholate per ml and a drop of toluene. For clarity, the curves are set off from each other; similar internal levels of GalSGal were achieved after incubation overnight in each case.
translocating conformational changes. The Pro-28 → Ser exchange may have a similar effect by removing a bend in the α-helix. Interestingly, the double mutation Glu-24, Ser-28 affects transport more severely than the single mutations, whereas this effect is not observed when the Ser-28 is combined with Arg-24.

Because the introduction of a positively or negatively charged amino acid residue into a highly hydrophobic domain of the protein close to the N terminus allows assembly into the membrane, this region does not serve a similar function as the signal sequence in secreted proteins. In general, introduction of charged residues into the hydrophobic part of signal sequences blocks synthesis or export of a given protein at an early stage in secretion (28, 29). More relevant for the mode of insertion of permease are models that propose that incorporation occurs in loops or helical hairpins (see ref. 30 for review). Furthermore, thermodynamic considerations suggest that the introduction of a charged residue in an otherwise hydrophobic α-helix will reduce the free energy of insertion by only a few kcal/mol (31).

The N terminus of lactose permease is exposed at the cytoplasmic face of the cell membrane (3). It is involved in the binding of factor IIIαc of the phosphotransferase system because the YP39 gene product is defective in the binding of factor IIIαc (32). This defect has been traced to either one or both of the isoleucine residues (Ile-7 and Ile-11) present in YP39 in addition to the Gly-24 → Arg change, because the YF39 product in which the wild-type Gly-24 is restored but Ile-7 and Ile-11 are retained is still unable to bind factor IIIαc (C. P. Broeckhuizen and P. W. Postma, University of Amsterdam, personal communication).

Although the rate of influx in the mutants is very low, approaching in the YP39 and YP30 gene products 10⁻⁴ times that of the wild type, there is nevertheless a measurable substrate accumulation above mere equilibration. Therefore, the mutants are apparently still able to couple the flux of proton and galactoside and their properties must still be described by the same two-state model

\[
\begin{align*}
&\text{CHG}_{\text{out}} \xrightarrow{k^+} \text{CHG}_{\text{in}} \\
&\text{C}_{\text{out}} \xrightarrow{k^-} \text{C}_{\text{in}}
\end{align*}
\]

In the wild type and in the absence of an electrochemical proton gradient, \(\Delta \mu_{\text{H}^+}\), the rate constants characterizing influx, \(k^+_c\) and \(k^-_c\) for the inward movement of the loaded (c) and the outward movement of the unloaded transporter (o), respectively, as well as the rate constants, \(k^+_s\) and \(k^-_s\), characterizing efflux are all of the order of 1200 min⁻¹. Therefore, in the absence of substrate, the distribution of substrate binding sites between the two sides of the membrane is about equal. In the presence of either one or both of the components of \(\Delta \mu_{\text{H}^+}\), \(\Delta \text{pH}\) (outside acid), and \(\Delta \Psi\) (outside positive), the distribution of binding sites is shifted becoming high on the outside and low on the inside (6). The question is, how the kinetic properties of the mutants can be explained within this kinetic framework.

Because in the YP39 and YP30 gene products influx, efflux, and exchange are affected, it is concluded that the rate constants of the loaded carrier, \(k^+_c\) and \(k^-_c\), are severely reduced. This leaves the rate of the conformational change of the unloaded transporter (\(k^+_s\) and \(k^-_s\)) and thus the binding site distribution undetermined. If the catalytic constant of influx for the YP30 mutant (0.09 min⁻¹) is the result of a decrease of \(k^+_c\) and \(k^-_c\), then it should be possible to measure the percentage of binding sites exposed to inside or outside by comparing substrate binding to membrane vesicles of RSO
and ISO orientation. As shown in Fig. 2, within 0.4 min all binding sites appear to be accessible in vesicles of both orientations. This result suggests that within the limited temporal resolution of this experiment the conformational change of the unloaded transporter exposes the binding site to either face of the membrane. Thus, the $k_d$ step appears to be considerably faster than 0.3 min$^{-1}$. In summary, the experiments lead to the conclusion that the transporter is not "frozen" in one conformation if substrate is absent but reorients very slowly in its presence.

The present findings bear on another problem. We had previously identified a variable change in the apparent affinity of the wild-type permease for galactosides that is dependent on $\Delta \mu_H$. (5, 24). For example, at $\Delta \mu_H = 0$, lactose has a low affinity for the permease, $K_d = 14$ mM. In the presence of $\Delta \mu_H$, the $K_i$ for active transport is 0.1 mM, yielding $K_d/K_i = 140$. For substrates such as GalSGal and NpeGal, the change in the apparent affinity is small with $K_d/K_i = 1-3$. The kinetic terms this effect can be explained either by a $\Delta \mu_H$-induced increase in $k_i^*$ (mobility effect) or by a decrease in the binding constant, $K_d$ (affinity effect). The $Y^{59}$ and $Y^{P02}$ mutations mimic the change in the affinity for lactose because $K_d(\Delta \mu_H = 0) = 0.6-0.9$ mM. Thus, these mutations may cause the protein to assume a conformation in the absence of $\Delta \mu_H$ similar to that of the wild-type permease at $\Delta \mu_H < 0$. Thus, a $\Delta \mu_H$-induced change in the affinity of the wild-type carrier for some galactosides is probably due to a decrease in $K_d$ and not to an increase in $k_i^*$.

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