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Aromatic Stacking in the Sugar Binding Site of the Lactose Permease†

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ABSTRACT: Major determinants for substrate recognition by the lactose permease of *Escherichia coli* are at the interface between helices IV (Glu126, Ala122), V (Arg144, Cys148), and VIII (Glu269). We demonstrate here that Trp151, one turn of helix V removed from Cys148, also plays an important role in substrate binding probably by aromatic stacking with the galactopyranosyl ring. Mutants with Phe or Tyr in place of Trp151 catalyze active lactose transport with time courses nearly the same as wild type. In addition, apparent K_m values for lactose transport in the Phe or Tyr mutants are only 6- or 3-fold higher than wild type, respectively, with a comparable V_{max}. Surprisingly, however, binding of high-affinity galactoside analogues is severely compromised in the mutants; the affinity of mutant Trp151→Phe or Trp151→Tyr is diminished by factors of at least 50 or 20, respectively. The results demonstrate that Trp151 is an important component of the binding site, probably orienting the galactopyranosyl ring so that important H-bond interactions with side chains in helices IV, V, and VIII can be realized. The results are discussed in the context of a current model for the binding site.

Like many membrane transport proteins comprising the Major Facilitator Superfamily (MFS)[1] (*1*), the lactose permease of *Escherichia coli* (LacY) transduces free energy stored in an electrochemical H^+ gradient into a concentration gradient of D-galactopyranosides and vice versa (galactoside/H^+ symport) (*2*). LacY is a 12-transmembrane−helix bundle with the N and C termini on the cytoplasmic face of the membrane (*3*−*5*), and it is physiologically (*6*) and structurally a monomer in the membrane (*7*−*9*). Analysis of an extensive library of mutants, Cys-replacement mutants in particular (*10*), with various site-directed biophysical and biochemical techniques has led to the formulation of a tertiary structure model (*11*), as well as a hypothesis for the mechanism of lactose/H^+ symport (*12*).

LacY is selective for disaccharides containing a D-galactopyranosyl ring, as well as D-galactose (*13*−*15*), but does not interact with D-glucopyranosides or D-glucose (*15*−*17*). The specificity of LacY is directed toward the galactopyranosyl ring of the substrate, and although C-4 OH is by far most important for specificity, the C-2, C-3, and C-6 OH groups are also important for binding (C-4 OH ≫ C-3 OH ≥ C-6 OH > C-2 OH) (*15*, *17*).

The major determinants for the substrate binding site are located at the interface between helices IV, V, and VIII. Glu126 (helix IV) and Arg144 (helix V), which are charge-paired, are irreplaceable with respect to substrate binding; a carboxyl group at position 126 and a guanidino group at position 144 are absolute requirements, and the side chains are located at the interface between helices IV and V, respectively (Figure 1) (*18*−*22*). Although Cys148 (helix V) is not irreplaceable, it interacts weakly and hydrophobically with the galactopyranosyl moiety of LacY substrates (*16*, *23*−*26*), and Ala122 (helix IV), another nonessential residue, abuts the nongalactopyranosyl moiety of the disaccharide substrates (*22*, *27*, *28*). Thus, alkylation of mutant A122C or replacement with Phe or Tyr abrogates disaccharide binding and transforms LacY into a galactose-specific symporter (*22*). This conclusion receives strong support from recent experiments (*28*) demonstrating that 1-methanethiosulfonyl-β-D-galactopyranosyl derivatives are affinity inactivators for mutant A122C. Finally, recent experiments utilizing carbodiimide labeling in the absence and presence of ligand in conjunction with mass spectroscopy indicate that Glu269 (helix VIII) interacts directly with the galactopyranosyl ring (Weinglass, A. B., Whitelegge, J. P., Hu, Y., Faull, K. F., and Kaback, H. R., submitted for pubication).

Although it has been suggested (see ref *22*) that Cys148 interacts with the hydrophobic face of the galactopyranosyl ring, in this configuration the C-4 OH (which is the most important OH group in the ring) cannot interact with Arg144, its postulated H-bond partner, nor can the C-6 OH interact with Glu126 (see ref *19*). However, by positioning the

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[1] Abbreviations: MFS, Major Facilitator Superfamily; LacY, lactose permease; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; NEM, *N*-ethylmaleimide; KP_i, potassium phosphate.

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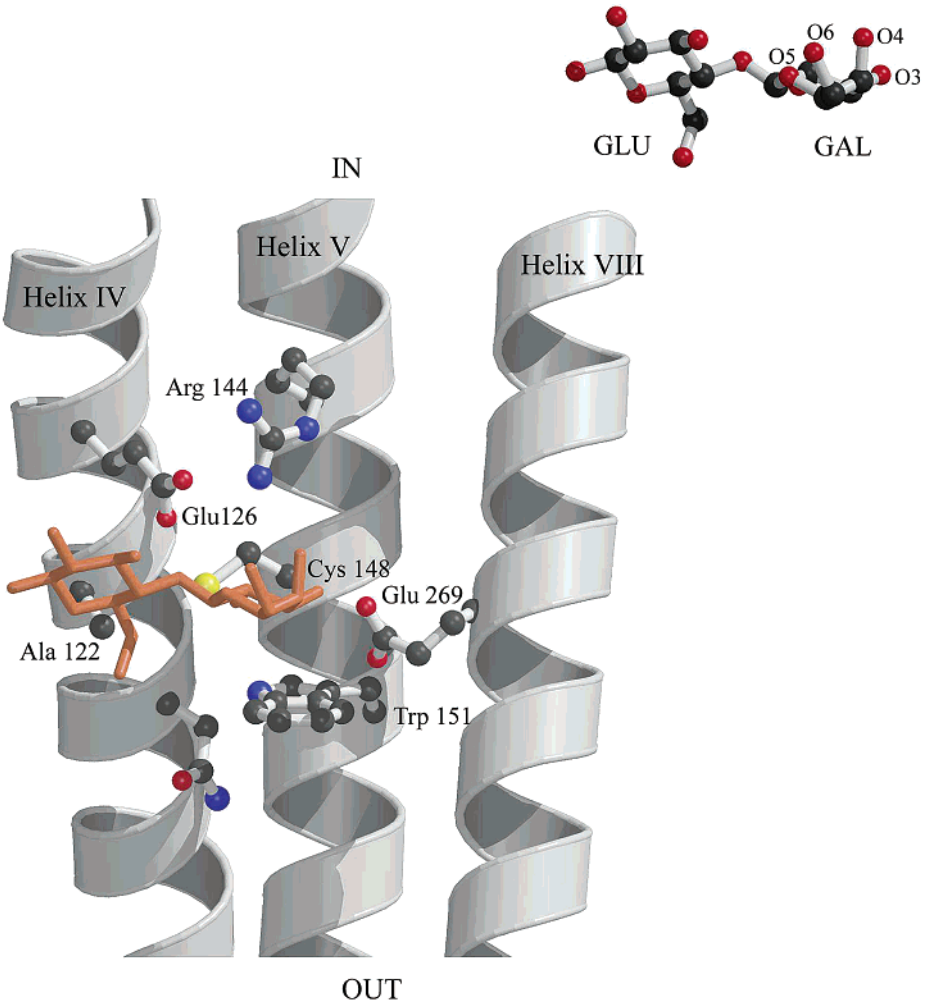

FIGURE 1: Proposed binding site in LacY showing helices IV, V, and VIII with bound lactose. The galactopyranosyl ring, which contains all the determinants for specificity, is shown in a β-1,4 linkage with D-glucose. The C-4 OH is most important with respect to specificity (*17*). Cys148 (helix V) interacts weakly and hydrophobically with the galactopyranosyl ring, and Ala122 is in close proximity to the glucopyranosyl moiety (*22*). Data presented here indicate that Trp151 stacks with the hydrophobic face of the galactopyranosyl ring, placing it at a right angle with helix IV and abutting Cys148 near the 1 position. In this orientation, the C-4 OH can H-bond directly with either NH_1 or NH_2 of Arg144, and the C-6 OH can H-bond with Glu126. Since the C-3 OH is close to Glu269 (helix VIII) but at an angle, it is a reasonable suggestion that a water molecule may mediate this interaction.

galactopyranosyl ring at a right angle with helix V so that Cys148 is close to the 1 position (Figure 1), many of the postulated interactions are fulfilled. Furthermore, when the binding site is modeled in this fashion, it becomes clear that Trp151 is in excellent position to stack hydrophobically with the galactopyranosyl moiety of LacY substrates, a common feature of carbohydrate binding sites in over 50 proteins of known structure (see refs *29* and *30*−*34*), as well as maltoporin (*35*, *36*) where aromatic side chains play an important role in the translocation pathway.

On the other hand, previous studies (*37*) in which each Trp residue in LacY was replaced with Phe suggest that none of the Trp residues is essential for activity. Moreover, it was demonstrated that mutant W151F seemingly catalyzes active lactose transport as well as the wild type at a lactose concentration approximating the K_m, thereby suggesting that further studies such as kinetics or ligand binding would be unrevealing. In view of the considerations discussed above, however, studies on Trp151 were reinitiated. Remarkably, despite excellent transport activity in mutants W151F and W151Y with relatively small alterations in kinetic parameters, both mutants exhibit markedly decreased substrate affinity, and mutant W151I exhibits an affinity that is too low to measure accurately. The results support a model for the binding site in which Trp151 plays an important role in ligand binding, interacting hydrophobically with the galactopyranosyl ring and holding it in an orientation that allows H-bond interactions required for specificity.

EXPERIMENTAL PROCEEDURES

Materials. [1-^{14}C]lactose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). *N*-([^{14}C]ethyl)maleimide was purchased from Dupont NEN (Boston, MA). *p*-Nitrophenyl α-D-[6-^{3}H]galactopyranoside (NPG) was the generous gift of Gérard Leblanc (Villefranche-sur-mer, France). Immobilized monomeric avidin was from Pierce (Rockford, IL), and all unlabeled sugars were obtained from Sigma (St. Louis, MO). Oligodeoxynucleotides were synthesized by Sigma-Geneosys (The Woodlands, TX). Restriction endonucleases, T4 DNA ligase, and Vent DNA polymerase were from New England Biolabs (Beverly, MA). All other materials were reagent grade and obtained from commercial sources.

Construction of LacY Mutants. Oligonucleotide-mediated, site-directed mutagenesis by using two-step PCR with pT7-5/cassette *lacY* as template was applied to construct Tyr, Phe, and Ile replacements for Trp151. In addition, pKR35 plasmids encoding LacY mutants W151Y/single-Cys148, W151F/single-Cys148, and W151I/single-Cys148 mutants were generated in a similar manner by using pKR35 encoding single-Cys148 with a C-terminal biotin acceptor domain as template (*22*, *38*−*40*).

Growth of Cells. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A) rpsL,met*$^-$*,thr*$^-$*,recA,hsdM,hsdR/F′,lacI*^{q}*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] containing given mutants was grown in Luria−Bertani broth with 100 mg/L of ampicillin. Overnight cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2−3 h at 37 °C, cells were harvested by centrifugation.

Preparation of Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared by osmotic lysis as described (*41*, *42*), suspended in 100 mM potassium phosphate KP_i (pH 7.5)/10 mM $MgSO_4$ at a protein concentration of about 12 mg/mL, frozen in liquid N_2, and stored at −80 °C until use.

Transport Assays. E. coli T184 containing given LacY mutants were washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and adjusted to an OD_{420} of 10.0 (0.7 mg protein/mL). Transport was carried out with [1-^{14}C]lactose (10 mCi/mmol) at a final concentration of 0.4 mM. Lactose transport in RSO membrane vesicles was assayed in the presence of 20 mM ascorbate/0.2 mM phenazine methosulfate under oxygen with given concentrations of [1-^{14}C]lactose (10 mCi/mmol) (*43*).

Binding Affinity Measurements. The K_D for TDG binding was determined in situ by alkylation of given mutants with 0.5 mM [^{14}C]NEM (40 mCi/mmol) in the absence or presence of given concentrations of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) (*15*, *17*, *25*, *44*−*46*).

Flow Dialysis. Binding of [^{3}H]NPG to RSO vesicles containing given LacY mutants was measured by flow dialysis as described (*47*).

RESULTS

Active Transport. As found previously (*37*), *E. coli* T184 (*lacZ*$^-$*Y*$^-$) expressing W151Y or W151F LacY seemingly catalyzes lactose transport at a rate and to a steady-state level of accumulation comparable to cells expressing wild-type LacY (Figure 2). However, when initial rates are measured carefully over the first 15 s, an approximate 2-fold decrease is observed with mutants W151Y and W151F (Figure 2, inset; Table 1).

Kinetic Analyses of Lactose Transport. Initial rates of lactose transport measured over the first 15 s increase in hyperbolic fashion as a function of lactose concentration in wild-type LacY, as well as mutants W151F and W151Y (Figure 3A). Furthermore, as shown in Figure 3B, linear functions are obtained in Eadie−Hofstee plots. The apparent K_m for wild-type LacY derived from the slope is 0.43 mM, and the V_{max} estimated from the *y* intercept is 250 nmol/min/mg protein. The apparent K_m for mutant W151F or W151Y is 2.45 or 1.22 mM, respectively, which is only 6- or 3-fold higher than wild-type LacY (Table 1). The transport activity of mutant W151I is too low for accurate kinetic studies (data not shown).

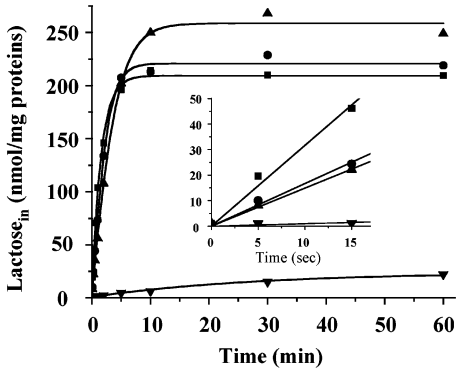

FIGURE 2: Effect of Trp151 replacements on active lactose transport. *E. coli* T184 expressing wild-type permease or given mutants at position 151 were grown at 37 °C, and an aliquot of cell suspension (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ was assayed at 0.4 mM final lactose concentration as described in Experimental Procedures. Inset: initial rates of lactose transport measured over the first 15 s. ■, wild type; ●, W151Y; ▲, W151F; and ▼, pT7-5 with no *lacY* insert.

Table 1: Effect of Trp151 Mutations on Kinetics and Binding Affinity

	initial rate[a] (nmol/s/mg protein)	K_m[a] (mM)	V_{max} (nmol/min/mg protein)	K_D for TDG[b] (mM)
wild type	3.16	0.43	250	0.035[c]
W151F	1.49	2.45	355	1.8
W151Y	1.67	1.22	168	0.68
W151I	0.12	ND	ND	>30

[a] Lactose transport and K_m values were measured in given mutants as described in Figures 2 and 3. [b] K_D values were measured in single-Cys148 background as described in Figure 5. [c] Value for single-Cys148 LacY.

NPG Binding. As shown by flow dialysis measurements with RSO membrane vesicles using [^{3}H]NPG (Figure 4), wild-type LacY exhibits a relatively large increase in the dialysable concentration of [^{3}H]NPG after addition of a saturating concentration of TDG, indicating good binding affinity for NPG. In contrast, with either mutant W151F or W151Y, only a small increase in the dialyzable NPG concentration is observed upon addition of TDG, indicating that NPG binding is largely compromised.

Substrate Protection Against Alkylation of Cys148 by NEM. To determine disassociation constants (K_D), the Typ151 replacement mutations were placed in the single-Cys148 background, and TDG-dependent protection of Cys148 against alkylation by [^{14}C]NEM was quantified. The pseudo-wild type (single-Cys148 LacY with Trp151) exhibits a K_D of 35 μM (Figure 5A; Table 1). Remarkably, mutant W151F (Figure 5B; Table 1) or W151Y (Figure 5C; Table 1) exhibits K_D values of about 1.8 or 0.7 mM, respectively, which corresponds to decreases in affinity by factors of about 50 or 20 relative to the pseudo-wild type (Table 1). Although data are not presented, the W151I mutant exhibits a K_D well in excess of 30 mM.

DISCUSSION

As shown in the earlier studies (*37*), the overall time course of lactose transport by mutants W151F and W151Y at a

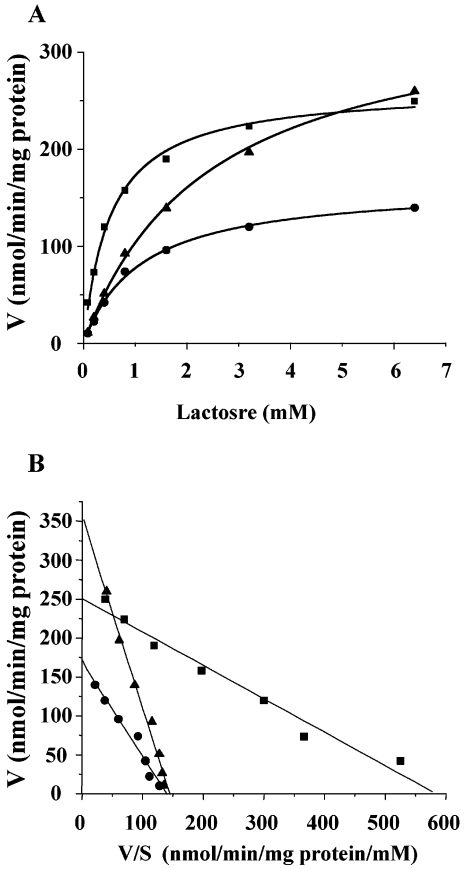

FIGURE 3: Effect of Trp151 replacements on kinetics of lactose transport. RSO membrane vesicles prepared from *E. coli* T184 containing given LacY mutants were assayed for lactose transport as described in Experimental Procedures. Initial rates (V) were measured at 0, 5, 10, and 15 s and plotted as function of V/S, where S represents lactose concentration. Inset: initial rates plotted directly as a function of lactose concentrations. ■, wild type; ▲, W151F; and ●, mutant W151Y.

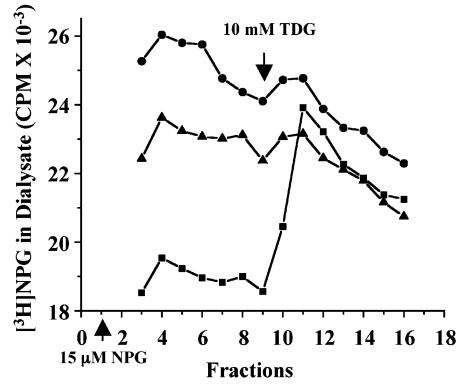

FIGURE 4: Effect of Trp151 mutations on NPG binding. Binding of [^{3}H]NPG to nonenergized RSO vesicles with wild-type permease (■), W151F (▲), or mutant W151Y (●) at a protein concentration of 32 mg/mL was assayed by flow dialysis. [^{3}H]NPG (840 mCi/mmol) at 15 μM final concentration was added at fraction 1. As indicated by the arrow, TDG at 10 mM final concentration was added at fraction 9 to displace bound NPG.

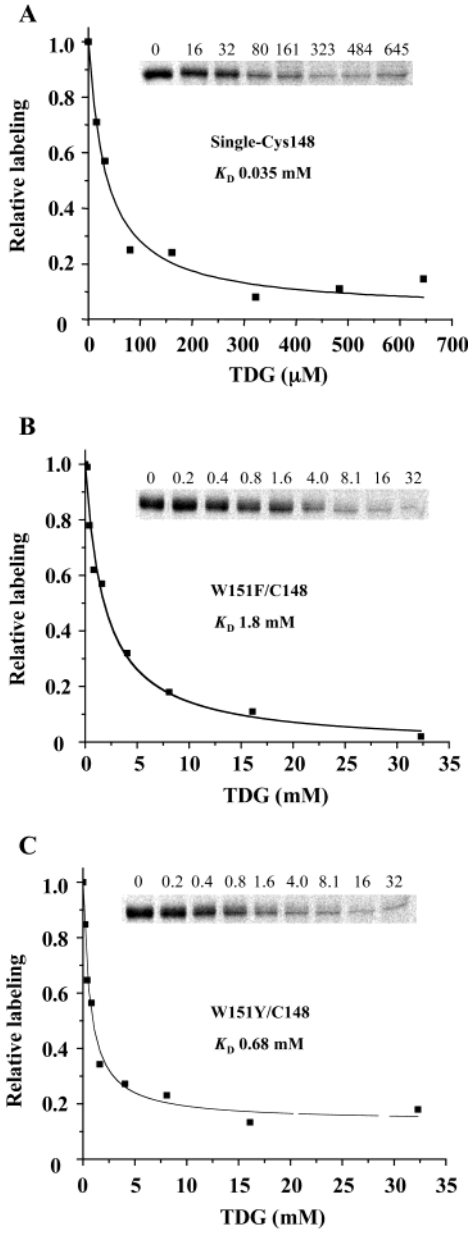

FIGURE 5: Substrate protection against [^{14}C]NEM labeling of single-Cys148 LacY with given replacements for Trp151. RSO membrane vesicles containing given LacY mutants incubated with 0.5 mM [^{14}C]NEM (40 mCi/mmol) for 5 min at pH 7.5 in absence or presence of the given concentrations of TDG. Reactions were quenched with 10 mM DTT, and biotinylated permease was solubilized in dodecyl β,D-maltopyranoside and purified by affinity chromatography on monomeric avidin Sepharose. Samples were subjected to sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis, and [^{14}C]labeled protein was quantitated with a Storm 860 PhosphoImager. Labeling in the presence of a given concentration of sugar is expressed as percent labeling observed in the absence of the sugar. K_D values were determined with the ORIGIN computer program (Microcal Software, Northampton, MA) by using nonlinear least-squares curve fitting to the following user-defined equation: $Y = (1 - P1)/(1 + X/P2) + P1$, where P1 is the residual labeling and P2 is the K_D.

lactose concentration that approximates the K_m is similar to that of the wild type (*40*, *45*). However, careful measurements of initial rates display a modest decrease in the mutants. Consistently, kinetic analyses reveal relatively minor increases in K_m, only about a 6-fold increase for mutant W151F and a 3-fold increase for mutant W151Y (Figure 3; Table 1). Although there is a relatively small difference in the kinetics of transport in the W151F and W151Y mutants, it is apparent that the presence of a Trp at position 151 gives the wild type a distinct advantage in the natural environment.

Strikingly and unexpectedly, however, both mutants exhibit dramatic decreases in binding affinity, at least a 50-

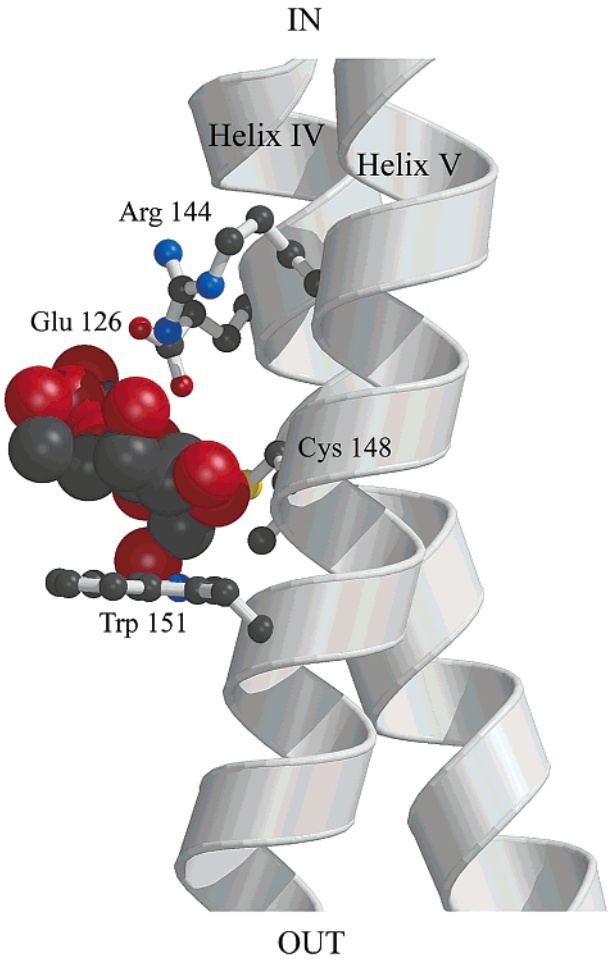

FIGURE 6: Side view of putative binding site in LacY showing helices IV and V with bound lactose. The sugar is shown in space filling configuration and relevant side chains as balls and sticks. The hydrophobic face of the galactopyranosyl ring stacks with Trp151 which forms a platform, and Cys148 is at the bottom of a notch in close proximity to the 1 position of the galactopyranosyl ring. Also shown are Glu126 (helix IV) and Arg144 (helix V).

fold increase in K_D for TDG in mutant W151F and a 20-fold increase in W151Y (Table 1). It is noteworthy that the order of efficacy of the side chain at position 151 (Trp $\gg$ Tyr $>$ Phe) is consistent with the aromaticity of the three residues. Thus, the distance and orientation of Trp151 is optimal for van der Waals interactions followed by Tyr and Phe. The results clearly support the notion that Trp151 stacks hydrophobically with the galactopyranosyl ring (Figure 1). By this means, Trp151 orients the galactosyl ring in such a position that important H-bond interactions between the OH groups on the ring and the side chains on LacY can be formed. In this regard, recent studies (Weinglass, A. B., Whitelegge, J. P., Hu, Y., Faull, K. F., and Kaback, H. R., submitted for pubication) demonstrating ligand protection of Glu269 (helix VIII) against modification by hydrophobic carbodiimides by mass spectrometry also suggest that this residue may form an H-bond with the C-3 OH of the galactopyranosyl ring. Moreover, since the C-3 OH is close to Glu269 but at an angle (Figure 1), it is reasonable to suggest that a water molecule may mediate the interaction.

For additional clarity, a side view of the putative binding site depicting only helices IV and V is presented in Figure 6 with the sugar shown in space-filling form and relevant side chains as balls and sticks. In addition to illustrating clearly how the hydrophobic face of the galactopyranosyl ring might stack hydrophobically with Trp151, there appears to be a hydrophobic notch with the aromatic side chain forming a shelf and the side of the galactopyranosyl ring interacting with Cys148 in the vicinity of the 1 position of the ring.

Since Trp residues tend to localize at the membrane/aqueous interface in known 3-D structures of membrane proteins (*48*), it is of interest that out of the six Trp residues in LacY, five are located at or near the ends of helices, while Trp151 is present within transmembrane helix V (*49*, *50*). Furthermore, multiple sequence alignment of the 10 members of the Oligosaccharide/H^+ Symport subfamily of the MFS (Clustal W) reveals that the amino acid side chains involved in substrate specificity are conserved to a high degree in all orthologues of LacY that recognize galactopyranosides. Thus, a Glu residue is present at position Glu126, and an Arg residue is conserved at position 144 in all instances. A Cys residue is largely conserved at position 148, an aromatic group— Trp or in two instances Tyr—is present at position 151, and Glu269 is also very largely conserved. In contrast, Ala122, which interacts with the nongalactopyranosyl moiety of substrate and plays little or no role in specificity, is found in only three homologues, with Ser in four and Ala or Cys in two others. It is also noteworthy that the remaining irreplaceable residues—Arg302, His322, and Glu325—which are involved in H^+ translocation but do not make direct contact with the galactopyranosyl moiety of substrate are also highly conserved. Therefore, as in the case of soluble sugar binding proteins, it seems likely that aromatic stacking may play an important role in sugar binding by membrane transport proteins as well.

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