Binding of Enzyme IIA^{Glc}, a Component of the Phosphoenolpyruvate:Sugar Phosphotransferase System, to the *Escherichia coli* Lactose Permease[†]

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ABSTRACT: Enzyme IIA^{Glc}, encoded by the *crr* gene of the phosphoenolpyruvate:sugar phosphotransferase system, plays an important role in regulating intermediary metabolism in *Escherichia coli* ("catabolite repression"). One function involves inhibition of inducible transport systems ("inducer exclusion"), and with lactose permease, a galactoside is required for unphosphorylated IIA^{Glc} binding to cytoplasmic loops IV/V and VI/VII [Sondej, M., Sun, J. et al. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3525−3530]. With inside-out membrane vesicles containing the permease, [^{125}I]IIA^{Glc} binding promoted by melibiose exhibits an affinity (K_D^{IIA}) of ≈ 1 μM and a stoichiometry of one mole of IIA^{Glc} per six moles of lactose permease. Both the quantity of [^{125}I]IIA^{Glc} bound and the sugar concentration required for half-maximal IIA^{Glc} binding ($K_{0.5}^{IIA}{}_{sug}$) was measured for eight permease substrates. Differences in maximal IIA^{Glc} binding are observed, and the $K_{0.5}^{IIA}{}_{sug}$ does not correlate with the affinity of LacY for sugar. Furthermore, $K_{0.5}^{IIA}{}_{sug}$ does not correlate with sugar affinities for various permease mutants. IIA^{Glc} does not bind to a mutant (Cys154 → Gly), which is locked in an outwardly facing conformation, binds with increased stoichiometry to mutant Lys131 → Cys, and binds only weakly to two other mutants which appear to be predominantly in either an outwardly or an inwardly facing conformation. When the latter two mutations are combined, sugar-dependent IIA^{Glc} binding returns to near wild-type levels. The findings suggest that binding of various substrates to lactose permease results in a collection of unique conformations, each of which presents a specific surface toward the inner face of the membrane that can interact to varying degrees with IIA^{Glc}.

The phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS)[1] is a multifunctional, multicomponent system, the primary function of which is to catalyze the concomitant phosphorylation and translocation of certain carbohydrates across the cytoplasmic membrane of certain bacteria (*1*). The PTS utilizes a phospho-relay system in which phosphoenolpyruvate (PEP) autophosphorylates the cytoplasmic protein Enzyme I, and this phosphoryl group is sequentially transferred to HPr. In the case of glucose transport in *Escherichia coli*, the phosphate is passed to IIA^{Glc} and then to the membrane protein IICB^{Glc}, which catalyzes vectorial phosphorylation (reviewed in ref *2*).

In *E. coli*, there is preferential transport and utilization of glucose via the PTS relative to sugars that are transported by other systems that are generally inducible. This phenomenon involves the phosphorylation state of the glucose-specific PTS protein IIA^{Glc}, encoded by the *crr* gene. When a PTS sugar is transported, IIA^{Glc} becomes predominately unphosphorylated and allosterically inhibits several non-PTS permeases, including those for lactose (LacY), maltose (MalK), melibiose (MelB), and raffinose (RafB). This phenomenon is called "inducer exclusion" (*3*), since the preferred substrate or one of its metabolic derivatives inhibits entry of molecules that induce other metabolic systems. Dephospho-IIA^{Glc} also inhibits phosphorylation of glycerol by glycerol kinase (GK), which catalyzes the first step in glycerol metabolism. Upon depletion of the PTS sugar, IIA^{Glc} accumulates in the phospho-form, which relieves inducer exclusion and activates adenylyl cyclase, thereby increasing synthesis of cAMP. cAMP in turn binds to the catabolite receptor protein (CRP), forming a complex that acts as a transcriptional activator for the *lac* operon, as well as regulating more than 100 other operons in *E. coli* (*4*). Recent genetic evidence (*5*) also suggests that IIA^{Glc} negatively controls translation and transcription of the σ^s subunit of RNA polymerase (*rpoS*), a transcriptional regulator for a set of genes expressed during stress (e.g., nutrient starvation).

X-ray crystallography (*6*, *7*) and NMR spectroscopy (*8*, *9*) have elucidated the structures of both the phospho- and dephospho-forms of IIA^{Glc}, showing that IIA^{Glc} undergoes only minor structural changes upon phosphorylation. In

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[1] Abbreviations: GK, *E. coli* glycerol kinase; IIA^{Glc}, glucose-specific PTS protein; ISO, inside-out; LacY, lactose permease; LacY-6His, wild-type LacY with a 6 His-tag at the C terminus; methyl α-D-gal, methyl α-D-galactopyranoside; methyl β-D-gal, methyl β-D-galactopyranoside; NaPi, sodium phosphate; NEM, *N*-ethylmaleimide; NPG, *p*-nitrophenyl α-D-galactopyranoside; PTS, phosphoenolpyruvate: sugar phosphotransferase system; RSO, right-side-out; and TDG, D-galactopyranosyl β-D-thiogalactopyranoside.

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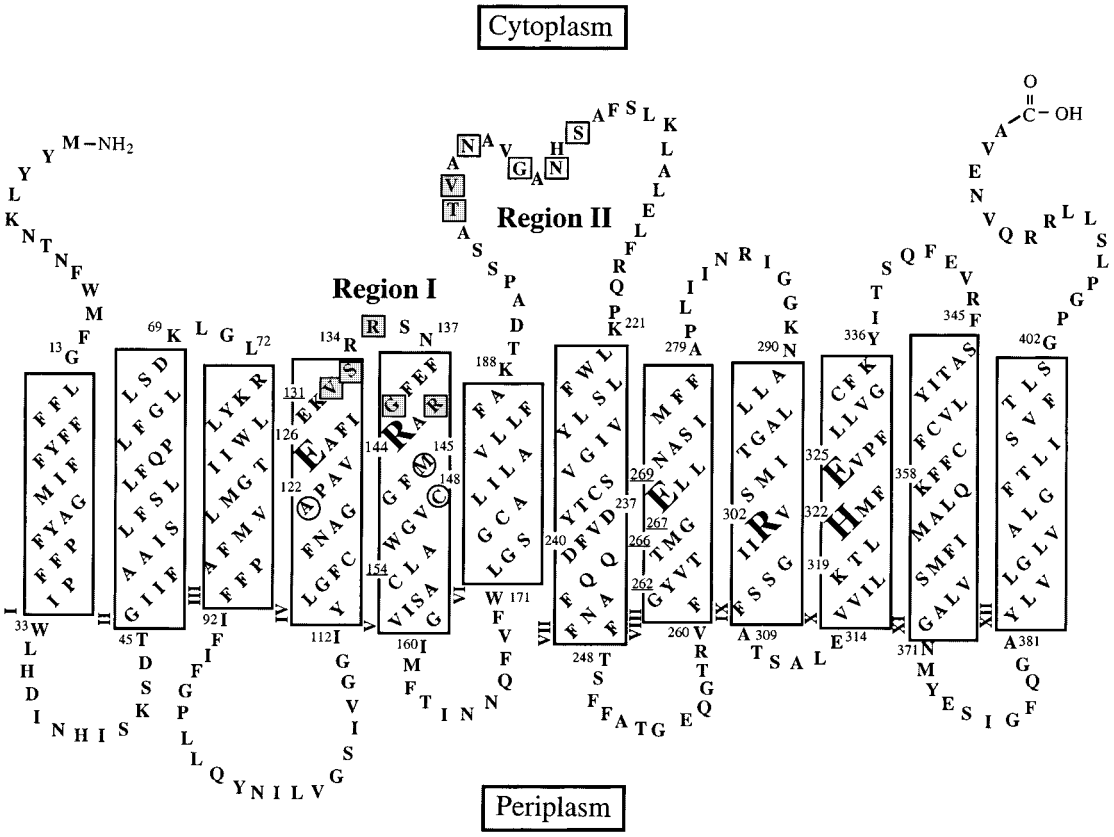

FIGURE 1: Secondary-structure model for lactose permease. The putative transmembrane domains as determined by Wolin and Kaback (*52*) are indicated by boxes. Essential residues for active transport (reviewed in ref *13*) are enlarged. Residues E126 (helix IV) and R144 (helix V) are essential for substrate binding. Residues E269 (helix VIII), R302 (helix IX), H322, and E325 (helix X) are indispensable for H^+ translocation and/or coupling. Circled residues C148 and M145 (helix V) (*14*) and perhaps A122 (helix IV) (*15*) play important, although nonessential, roles in substrate binding. The charged pairs D237 (helix VII)/K358 (helix XI) and D240 (helix VII)/K319 (helix X) are indicated. Cysteine-scanning mutagenesis has demonstrated that several residues (boxed in) from cytoplasmic loop IV/V and the flanking regions in helices IV and V (region I) and cytoplasmic loop VI/VII (region II) are important for IIA^{Glc} binding (*20*). The location of the mutated residues analyzed in this study are indicated by underlines at the residue number.

addition, the X-ray structure of the IIA^{Glc}-GK complex (*10*) and the NMR structure of the IIA^{Glc}-HPr complex (*11*) have been determined. A direct binding assay (*12*) with IIA^{Glc} mutants was used to identify several residues in IIA^{Glc} that are important for binding to LacY. The IIA^{Glc} binding surface that interacts with GK and HPr is similar and encompasses the IIA^{Glc} phosphorylation site (*11*) along with the residues predicted to be important for binding to LacY (*12*).

LacY is a cytoplasmic membrane protein with 12 hydrophobic transmembrane α-helical domains connected by relatively hydrophilic loops with the N and C termini on the cytoplasmic face of the membrane (Figure 1). LacY catalyzes the coupled, stoichiometric translocation of galactosides such as lactose or melibiose with H^+. Site-directed and Cys-scanning mutagenesis (reviewed in ref *13*) demonstrate that only six residues are irreplaceable with respect to active transport: Glu126 (helix IV) and Arg144 (helix V), which are critical for substrate binding; and Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix X), which are indispensable for H^+ translocation and/or coupling. Cys148 and Met145 (helix V) (*14*) and probably Ala122 (helix IV) (*15*) also play important, although nonessential, roles in sugar binding.

IIA^{Glc} binding to LacY requires a substrate-dependent conformation, since specific binding of IIA^{Glc} is observed only in the presence of LacY substrates (*16*−*18*). Furthermore, the H^+ electrochemical gradient ($\Delta\bar{\mu}_H +$), the driving force for sugar accumulation, is not required for IIA^{Glc} binding (*19*). A mechanism proposed for lactose/H^+ symport by LacY, reviewed in Kaback et al. (*13*), indicates that LacY is protonated prior to ligand binding. In the ground state, the symported H^+ is shared between His322 (helix X) and Glu269 (helix VIII), while Glu325 (helix X) is charge-paired with Arg302 (helix IX). Substrate binding at the outer surface between helices IV (Glu126) and V (Arg144, Cys148) induces a conformational change that leads to transfer of the H^+ to Glu325 and reorientation of the binding site to the inner surface. After release of substrate, Glu325 is deprotonated on the inside due to rejuxtapositioning with Arg302 as LacY returns to the ground state.

Insertional (*18*) and Cys-scanning mutagenesis (*20*) of LacY show that residues located in the cytoplasmic loop between helices IV and V (loop IV/V) along with the flanking helical domains which contain the major determinants for sugar binding and also residues in the large central cytoplasmic loop VI/VII are important for IIA^{Glc} binding to LacY (Figure 1).

This study utilizes a modification of the direct binding assay developed by Seok et al. (*18*) to quantitate binding of IIA^{Glc} to wild-type LacY and certain mutants in the presence of various permease substrates. The results indicate that the available binding sites for IIA^{Glc} vary considerably, depending on the nature of the substrate and on the conformation of the cytoplasmic loops that bind IIA^{Glc}.

EXPERIMENTAL PROCEDURES

Plasmids. A plasmid designed for the expression of IIA^{Glc} with a N-terminal His-tag (6 His residues), pHISIIA, was constructed by inserting the *Nde*I−*Sal*I fragment from pR3 (*21*) into pREHIS (*22*). Plasmid pHISIIA was further modified by using two-step PCR mutagenesis (*23*) to introduce a Tyr codon (TAT) between the start codon (ATG) and the first His residue of the His-tag (pTYRHISIIA). Constructs were verified by Dye Terminator Cycle Sequencing (PE Applied Biosystems). LacY mutant C154G (*24*) was transferred to pT7−5/lacY, and a 10 His-tag was introduced at residue 417. Mutant E325D (*25*) was modified by introducing a 6 His-tag at residue 417. Mutant R302K was constructed as described (*26*). All other plasmids used for overexpression of wild-type and LacY mutants were derived from pT7−5/cassette lacY (EMBL-X56095) and have been described previously: wild-type LacY with a 6 His-tag at the C terminus (*27*); mutants E126D and K131C (*28*); mutants E269D and E269Q (*29*); and mutants R302A, R302S, and E325A (*30*−*32*).

Expression and Purification of $TyrHisIIA^{Glc}$. Plasmid pTYRHISIIA was transformed into *E. coli* GI698 Δ*ptsHIcrr* (*33*) and plated on synthetic medium (*34*) supplemented with 100 μg/mL ampicillin. A single colony isolate was used to inoculate 100 mL of minimal labeling medium (MLM) consisting of 42 mM Na_2HPO_4/22 mM KH_2PO_4/8.6 mM NaCl/19 mM NH_4Cl/1 mM $MgSO_4$/6 μg/mL thiamine/1× trace metals (1000× stock solution: 10 mM $FeCl_3$/0.95 mM $ZnCl_2$/1.2 mM $CoCl_2$/0.83 mM Na_2MoO_4/0.68 mM $CaCl_2$/0.75 mM $CuCl_2$/0.8 mM H_3BO_3/12 mM HCl) supplemented with 100 μg/mL ampicillin/0.5% galactose/2.5 mM cAMP. The culture was incubated at 30 °C for ≈ 3 days and then stored at 4 °C until use. An overnight culture started with the stored culture was used to inoculate 1 L of MLM medium. When the culture reached an OD_{600} of 0.5, 100 μg/mL Trp was added to induce expression of $TyrHisIIA^{Glc}$, and growth at 30 °C was continued overnight. Cells were then harvested, washed, resuspended in 50 mM Tris-HCl (pH 8.0)/300 mM NaCl (buffer A) and lysed by three passages through an Emulsiflex (Avestin) at approximately 18 000 psi. Cell debris was removed by centrifugation at 107000*g*, 4 °C, for 1 h.

The supernatant solution was allowed to bind to Ni−NTA resin (Qiagen) in batch for 1 h with agitation at 4 °C and transferred to a column. The column was washed at room temperature with 20 bed volumes of buffer A, followed by 8 bed volumes of buffer A/10 mM imidazole. $TyrHisIIA^{Glc}$ was eluted with 3 bed volumes of buffer A/100 mM imidazole. The eluate was dialyzed overnight against 10 mM Tris-HCl (pH 7.5)/100 mM NaCl and concentrated using a Macrosep centrifugal concentrator (Pall Gelman). The concentrated sample was passed through an FPLC Hi Prep 16/10 Sephacryl 5−300 high-resolution column (Amersham Pharmacia). Fractions containing $TyrHisIIA^{Glc}$ (as determined by Coomassie Blue staining of a SDS−polyacrylamide gel) were pooled, concentrated, and finally stored at − 80 °C. A yield of 44 mg (Pierce Coomassie Plus Protein Assay) of approximately 99% pure $TyrHisIIA^{Glc}$ was obtained as determined by Coomassie Blue staining of an SDS−12% polyacrylamide gel.

Iodination of IIA^{Glc}. Na[^{125}I] (1.0 mCi; Amersham Pharmacia IMS-30) was activated using an IODO-GEN tube (Pierce) according to the manufacturer's instructions. Activated Na[^{125}I] was added to 1.5 mg of $TyrHisIIA^{Glc}$ in 10 mM Tris-HCl (pH 7.5)/300 mM NaCl (100 μL). The reaction mixture was incubated at room temperature for 9 min, swirling the tube every 30 s. The reaction was terminated by adding 50 μL of Tyr (10 mg/mL) in 25 mM Tris-HCl (pH 7.5)/400 mM NaCl followed by a 5-min incubation at room temperature, swirling the tube at 1 and 4 min. Subsequently, 1 mL of 100 mM Tris-HCl (pH 8.0)/300 mM NaCl (buffer B) was added, and the solution was transferred to 1 mL of Ni−NTA resin. To remove free ^{125}I, [^{125}I]IIA^{Glc} was purified using metal affinity chromatography as described, except that the Ni−NTA column was washed with 30 mL of buffer B and eluted with 5 mL of buffer B/100 mM imidazole. The eluate was dialyzed against buffer B (pH 7.5) for 36 h at 4 °C with three buffer changes. Protein concentration was determined as described above; the average specific activity from three preparations was 1.5×10^6 cpm/μg. Examination of an SDS−12% polyacrylamide gel with a Storm 860 phosphoimager showed little sign of [^{125}I]IIA^{Glc} degradation after storage for 2 months at 4 °C.

Preparation of Inside-Out (ISO) Membrane Vesicles. *E. coli* T184 (*lacI* $^{+}$*O* $^{+}$*Z* $^{-}$*Y* $^{-}$(*A*), *rpsL*, *met* $^{-}$, *thr* $^{-}$, *recA*, *hsdM*, *hsdR*/F′ *lacI* ^{q}*O* $^{+}$*Z* ^{U118}(*Y* $^{+}$*A* $^{+}$)) (*35*) harboring given mutations was grown overnight at 37 °C in Luria-Bertani medium containing 100 μg/mL ampicillin. The overnight culture was diluted (OD_{600} = 0.15−0.2) and grown to OD_{600} ≈ 0.65; then LacY expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After growth for an additional 4 h, cells were harvested at 6000*g* for 15 min, washed in 100 mM sodium phosphate (NaPi; pH 7.0), resuspended in cold 100 mM NaPi (pH 7.0)/5 mM DTT (buffer C), and then subjected to one pass through an Emulsiflex at ≈12 000−13 000 psi. Cell debris was removed by centrifugation at 10000*g*, 4 °C for 10 min. The supernatant was centrifuged overnight at 70000*g*, 4 °C, the pellet was resuspended by homogenization in buffer C, and 10 M urea was added slowly to a final concentration of 5 M. The mixture was stirred on ice for 30 min and centrifuged at 144000*g*, 4 °C, for 2.5 h. To remove residual urea from the membranes, the pellet was resuspended by homogenization in buffer C, and centrifugation was repeated. The final pellet was resuspended by homogenization in 1/1000th the volume of the induced culture in 100 mM NaPi (pH 7.0)/1 mM DTT, immediately frozen in liquid N_2 and stored at −80 °C. The total protein concentration (usually 10−20 mg/mL) was measured using the BCA protein assay (Pierce). The ISO orientation of a urea-extracted wild-type vesicle preparation containing LacY was verified by flow dialysis (*36*) after treatment with 70 μM dicyclohexylcarbodiimide for 30 min (*37*). The preparations accumulate [^{14}C]thiocyanate in the presence of ascorbate and phenazine methosulfate under oxygen, but do not accumulate [^{3}H]tetraphenylphosphonium.

To use equivalent amounts of wild-type and the various mutant forms of LacY, expression was determined relative to wild-type LacY by quantitative immunoblot analysis. A sample (20 μg) from each membrane preparation was separated by electrophoresis on an SDS−12% polyacrylamide gel, electroblotted onto a PVDF membrane (Immobilon-P, Millipore), and probed with either rabbit polyclonal

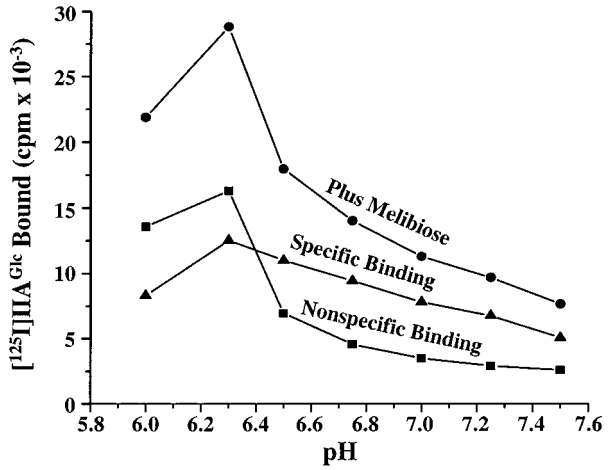

FIGURE 2: pH-dependent binding of IIA^{Glc}. The binding of [^{125}I]-IIA^{Glc} (0.5 μM) to ISO membrane vesicles containing wild-type LacY was measured in the absence (indicated as nonspecific binding) and in the presence of saturating melibiose (4 mM) as a function of pH. The pH of the NaPi buffer was varied from 6 to 7.5. The amount of [^{125}I]IIA^{Glc} specifically bound to LacY was determined by subtracting the counts (cpm) observed in the absence of melibiose (nonspecific) from the counts observed in the presence of sugar.

antibody to the C terminus of LacY (*38*) followed by treatment with Protein A-conjugated horseradish peroxidase or with Penta·His Antibody (Qiagen) followed by an anti-mouse IgG peroxidase-linked antibody. The immunoblots were developed with Supersignal West Pico chemiluminescent substrate (Pierce), and the LacY bands on the exposed film were quantitated by spot densitometry using a Chemi-Imager 4000. The same immunblot assay was also used for stoichiometry determinations where the amount of LacY in the membrane preparations was quantitated by comparing known amounts (0.05−0.5 μg) of purified wild-type LacY with a 6 His-tag at the C terminus (LacY-6His) to the amount of LacY-6 His in a particular membrane preparation.

IIA^{Glc} Binding to LacY. The assay used to measure the binding of [^{125}I]IIA^{Glc} to LacY was modified from the original assay using [^{3}H]IIA^{Glc} (*18*). The order of addition to the assay (100 μL) was (i) 100 mM NaPi (pH 7.0); (ii) 2 mM DTT; (iii) 2 mM $MgCl_2$; (iv) 150 mM NaCl; (v) urea washed inside-out membrane vesicles containing LacY (equivalent LacY expression of 250 μg of total protein for wild-type or wild-type LacY-6His membrane preps); (vi) 0.5 μM [^{125}I]-labeled IIA^{Glc}; (vii) a given LacY substrate (at a concentration equivalent to the $K_{0.5}^{IIA}{}_{sug}$; see Figure 3) unless otherwise noted. The reaction components were added to a Beckman polycarbonate tube (8 × 34 mm), vortexed, incubated at room temperature for 5 min, and centrifuged in a Beckman Optima ultracentrifuge at 290000*g*, 4 °C, for 20 min. The supernatant solution was discarded. The tube was gently rinsed with 150 μL of H_2O before 100 μL of 1% Triton X-100 (scintillation grade, RPI) was added, and the pellet was left at 4 °C to solubilize overnight. The sample was then vortexed and transferred to a scintillation vial. The tube was rinsed with 150 μL of H_2O, which was transferred to the same vial. Scintillation cocktail (5 mL of Fisher ScintiSafe Econo2) was added, and membrane-bound [^{125}I]-IIA^{Glc} was quantitated (cpm) by using a Beckman LS 5000TD liquid scintillation counter. Specific binding of [^{125}I]-IIA^{Glc} to LacY was determined by subtracting cpm obtained

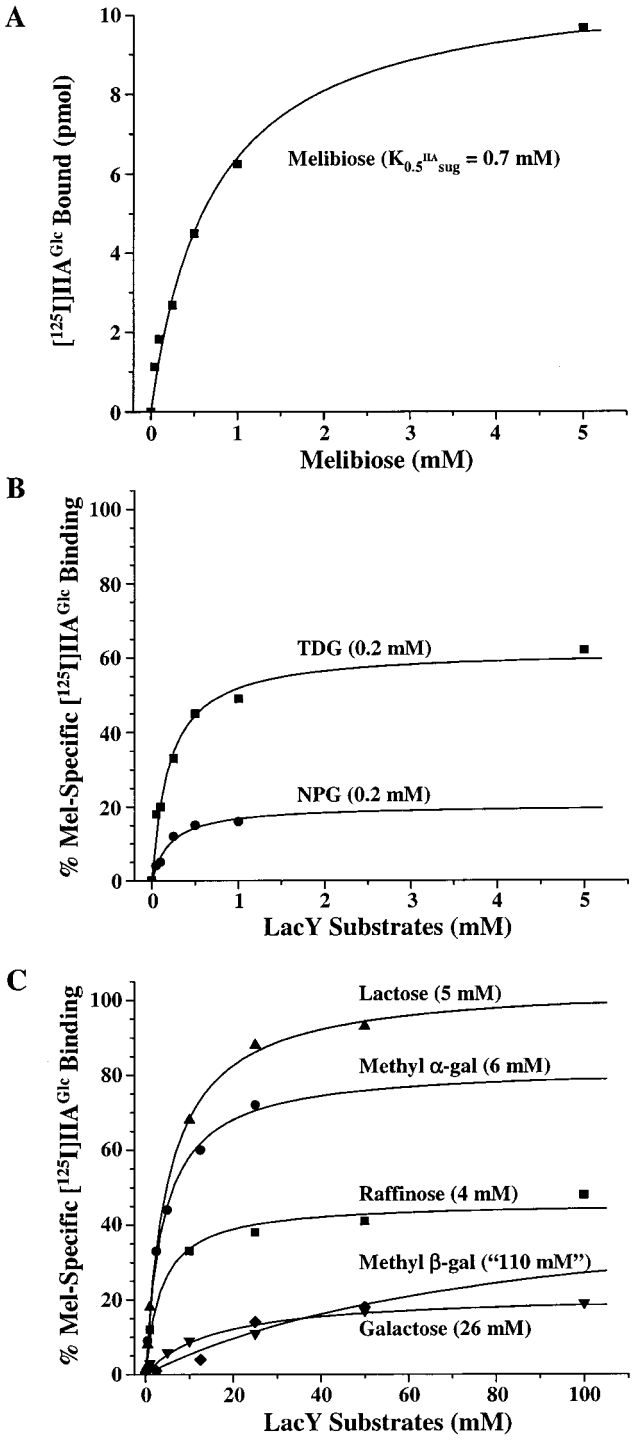

FIGURE 3: Substrate dependence of IIA^{Glc} binding to LacY. Binding of subsaturating levels of IIA^{Glc} (0.5−1 μM) to LacY is examined in the presence of varying concentrations for the given LacY substrates. Specific binding of [^{125}I]IIA^{Glc} to wild-type LacY (expressed as pmoles of IIA^{Glc} bound) as a function of melibiose concentration is shown in panel A. The data in panels B and C are plotted as the percentage of maximum melibiose-specific [^{125}I]IIA^{Glc} binding (10.9 ± 0.4 pmol). The data for each LacY substrate was fitted to a standard hyperbolic curve (see Experimental Procedures) to determine both maximum [^{125}I]IIA^{Glc} binding and the concentration of substrate (indicated in parentheses) at which half-maximal [^{125}I]IIA^{Glc} binding ($K_{0.5}^{IIA}{}_{sug}$) is observed. Panel B shows data for LacY substrates that exhibit relatively high affinities with respect to promoting IIA^{Glc} binding, while panel C shows data for substrates with lower affinities. Since the steady-state level of IIA^{Glc} binding promoted by methyl β-D-galactopyranoside was extrapolated from this curve-fitting analysis, that $K_{0.5}^{IIA}{}_{sug}$ value is an estimate.

in the absence of sugar from cpm obtained in the presence of a given sugar. Membranes prepared from cells transformed with pT7−5 with no *lacY* insert exhibited the same amount of nonspecific IIA^{Glc} binding as membranes incubated in the absence of sugar. The $[^{125}I]IIA^{Glc}$ binding data was fit with MicroCal Origin software (MicroCal Software, Inc, Northampton, MA) and the user-defined equation for a standard hyperbolic curve:

$$Y = (X^*P1)/(X + P2)$$

where P1 is the value for maximum $[^{125}I]IIA^{Glc}$ binding and P2 is the concentration of substrate at which half-maximal $[^{125}I]IIA^{Glc}$ binding ($K_{0.5}^{IIA_{sug}}$) is observed. To study the effect of phosphorylated IIA^{Glc}, 2 mM phosphoenolpyruvate, 0.2 μg of HPr (*21*), and 10 μg of Enzyme I (*21*) were also added to the reaction mixture, which was incubated at room temperature for 10 min before the addition of LacY substrate.

In some of the binding experiments, the possibility was considered that the sugar concentration at the binding site might not be at steady-state after 5 min, resulting in decreased IIA^{Glc} binding. To rule out this possibility, ISO vesicles containing wild-type LacY or LacY mutants C154G, K131C, and T266G/M267G/E269D were incubated overnight in the presence of 15 mM lactose before adding $[^{125}I]IIA^{Glc}$. The amount of lactose-specific IIA^{Glc} binding was identical for vesicles equilibrated overnight in the presence of lactose relative to the typical 5-min incubation. Therefore, it is probable that the sugars completely equilibrate with the intravesicular space within 5 min.

Affinity of Melibiose or Raffinose for LacY. Growth of *E. coli* T184 transformed with single-Cys148 LacY, preparation of right-side-out membrane vesicles and substrate protection against alkylation with [1-^{14}C]*N*-ethylmaleimide (NEM) was carried out as previously described (*14*, *39*, *40*).

RESULTS

Binding of IIA^{Glc} to LacY. An assay to measure specific binding of IIA^{Glc} to LacY by using [^{3}H]labeled-IIA^{Glc} was developed by Seok et al. (*18*). Because the specific activity achieved by that method was relatively low, making the assay relatively insensitive, the assay has been modified by utilizing $[^{125}I]IIA^{Glc}$. IIA^{Glc} lacks Tyr (*41*); therefore, a Tyr residue for iodination was introduced between the N-terminal Met and the 6 His-tag used for affinity purification. Since N-terminal His-tagged IIA^{Glc} competes with $[^3H]IIA^{Glc}$ as well as wild-type IIA^{Glc} for binding to LacY using the original $[^3H]IIA^{Glc}$ binding assay described by Seok et al. (*18*), the N-terminal His-tag on IIA^{Glc} has no effect on affinity. The 6 N-terminal residues of TyrHisIIA^{Glc} were confirmed by protein sequencing (Midwest Analytical), and thus, the His-tag allows for purification of full-length IIA^{Glc}, also called the "slow form" due to its electrophoretic mobility (*42*). The "fast form" of IIA^{Glc}, a proteolytically cleaved product missing the N-terminal seven amino acid residues, has 3−5 times less inhibitory effect on LacY than full-length IIA^{Glc} (*43*). Recent NMR studies demonstrate that the N terminus of IIA^{Glc} forms an amphipathic helix, which functions as a membrane anchor (*44*). It is required for efficient phosphoryl transfer from IIA^{Glc} to $IICB^{Glc}$ (glucose PTS) (*42*) and probably has a similar function for the interaction between IIA^{Glc} and LacY.

Other modifications introduced to reduce nonspecific binding include addition of 150 mM NaCl and the use of urea-washed ISO membrane vesicles (*37*). The optimum pH for binding in the presence of melibiose is 6.3 (Figure 2), in agreement with previous observations (*16*, *45*) (Y.-J. Seok and A. Peterkofsky, unpublished observations); these studies were carried out at pH 7.0 to minimize nonspecific binding. The modified assay exhibits the same substrate-dependent binding of $[^{125}I]IIA^{Glc}$ to LacY as observed in the original assay. To determine if phospho-IIA^{Glc} binds to LacY, PTS phospho-transfer components (PEP, Enzyme I, and HPr) were included in the reaction mixture. Phosphorylation of $[^{125}I]IIA^{Glc}$ completely abolishes melibiose-specific binding to LacY, while omitting either PEP or Enzyme I restores normal IIA^{Glc} binding (data not shown). Thus, as shown previously (*16*, *45*), it is the unphosphorylated form of IIA^{Glc} that binds to LacY.

IIA^{Glc} Binding as a Function of LacY Substrate Concentration. Seok et al. (*18*) observed varying levels of IIA^{Glc} binding to LacY for several different LacY substrates (melibiose > D-galactopyranosyl β-D-thiogalactopyranoside (TDG) > lactose > raffinose at concentrations of 2 mM each). This phenomenon was explored in more detail (Figure 3). $[^{125}I]$-IIA^{Glc} binding was determined as a function of sugar concentration at subsaturating levels of IIA^{Glc}. Shown in panel A is the specific binding of $[^{125}I]IIA^{Glc}$ to wild-type LacY as a function of melibiose concentration. The other substrates tested fall into three categories relative to melibiose-specific binding (panels B and C): (i) substrates that induce the highest IIA^{Glc} binding, which include lactose (104%) and methyl α-D-galactopyranoside (methyl α-D-gal; 82%); (ii) substrates that induce intermediate levels of IIA^{Glc} binding, which include TDG (62%), methyl β-D-galactopyranoside (methyl β-D-gal; extrapolated to 50%), and raffinose (46%); and (iii) substrates that induce low IIA^{Glc} binding, which include galactose (21%) and *p*-nitrophenyl α,D-galactopyranoside (NPG; 20%). These results differ from those observed by Seok et al. (*18*), since the concentration of LacY substrate used in that study was at saturating levels only for melibiose- and TDG-induced IIA^{Glc} binding.

The substrate concentrations at which half-maximal $[^{125}I]$-IIA^{Glc} binding ($K_{0.5}^{IIA_{sug}}$) is observed are indicated in parentheses in Figure 3. Substrates that exhibit relatively high affinity (panels A and B) with respect to promoting $[^{125}I]$-IIA^{Glc} binding are NPG (0.2 mM), TDG (0.2 mM), and melibiose (0.7 mM), while raffinose (4 mM), lactose (5 mM), and methyl α-D-gal (6 mM) are intermediate, and galactose (26 mM) and methyl β-D-gal (110 mM) exhibit low affinity (panel C) with respect to $[^{125}I]IIA^{Glc}$ binding.

IIA^{Glc} Binding Affinity. Since only one concentration of $[^{125}I]IIA^{Glc}$ is examined in the study shown in Figure 3, a possible explanation for the differences in IIA^{Glc} binding in the presence of various LacY substrates is that IIA^{Glc} affinity for LacY may vary with each sugar. Therefore, binding of full-length IIA^{Glc} to wild-type LacY as a function of IIA^{Glc} concentration is determined in the presence of saturating melibiose or TDG (Figure 4). In the presence of melibiose, the affinity of IIA^{Glc} is 1.0 ± 0.2 μM, while in the presence of TDG, IIA^{Glc} affinity is 2.6 ± 0.3 μM. A similar apparent K_D^{IIA} was measured in the presence of melibiose by competition assays in which bound $[^{125}I]IIA^{Glc}$ (0.25 μM, final concentration) is displaced by concentrations of unlabeled

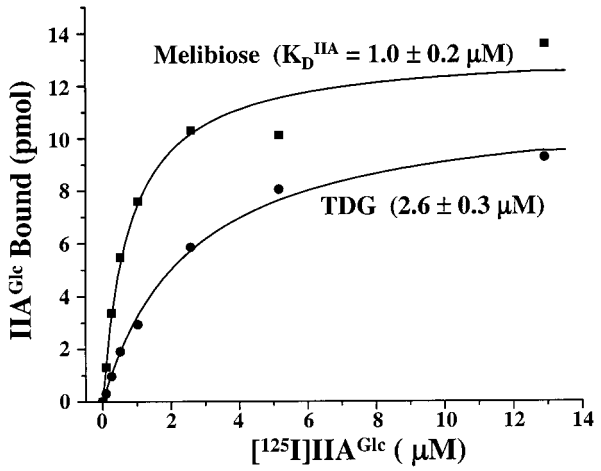

FIGURE 4: Affinity for IIA^{Glc} binding to LacY. The affinity (apparent K_D^{IIA}) of IIA^{Glc} binding to LacY at pH 7.0 was determined in the presence of saturating melibiose (2.1 mM) and TDG (600 μM). The [^{125}I]IIA^{Glc} was diluted 1:10 with unlabeled IIA^{Glc}, and varying amounts (0.1−13 μM) of this diluted [^{125}I]IIA^{Glc} was added to the assay mixture. The data are expressed as the amount of [^{125}I]-IIA^{Glc} specifically bound to wild-type LacY ISO vesicles.

IIA^{Glc} ranging from 0.1 to 13 μM (data not shown). Other results using indirect methods to measure TDG-induced binding of *Salmonella typhimurium* IIA^{Glc} to *E. coli* LacY yield an apparent K_D^{IIA} of 5 to 16 μM by using rocket immunoelectrophoresis to quantify IIA^{Glc} binding (*16*). The apparent K_D^{IIA} obtained in that study is expected to be higher than the value obtained here, as the purified IIA^{Glc} used by Nelson et al. was a heterogeneous mixture of full-length and proteolytically cleaved IIA^{Glc}. Values of 20−45 μM were also reported (*45*) from measurements of the PTS activity of IIA^{Glc} released from membranes containing LacY by treatment with 1 M NaCl. The data presented here indicate that, at sufficiently high concentrations of IIA^{Glc}, the differences in maximal binding become minor.

It would have been desirable to measure the affinity of IIA^{Glc} in the presence of substrates that showed the lowest maximum IIA^{Glc} binding (i.e., NPG or galactose). However, the required dilution of [^{125}I]IIA^{Glc} specific activity for this experiment, reduces the sensitivity of the assay to a level that makes IIA^{Glc} binding in the presence of these sugars barely detectable. Thus, a more sensitive methodology for exploring this problem is necessary.

Apparent Affinity of LacY for Melibiose and Raffinose. With the exception of melibiose and raffinose, the apparent K_{Dsug} for substrate binding to right-side-out (RSO) membranes vesicles containing LacY has previously been determined (*14*, *40*) for the LacY substrates used in this study (Table 1). The affinity of LacY for a given substrate is reflected by the ability of that substrate to protect against alkylation of single-C148 LacY by *N*-[^{14}C]ethylmalemide (NEM), since Cys148 interacts hydrophobically with the β face of the galactose moiety of LacY substrates (*14*, *39*, *40*). Using the same method, the apparent K_{Dsug} (the substrate concentration at which 50% protection of single-C148 LacY against NEM labeling is observed) is determined to be 85 μM for melibiose and 800 μM for raffinose (Figure 5). The affinity measured here for melibiose binding is higher than the previously published value of 510 μM (*46*); however, the affinity measured for raffinose is similar to the reported value of 980 μM. For the majority of the sugars, the affinity of the permease for a particular sugar does not correlate with concentration at which half-maximal [^{125}I]IIA^{Glc} binding ($K_{0.5}^{IIA}{}_{sug}$) is observed (Table 1) for that same sugar.

LacY Mutants and IIA^{Glc} Binding. In an attempt to gain further insight into the substrate-dependent conformation of LacY recognized by IIA^{Glc}, two groups of LacY mutants were examined for IIA^{Glc} binding. The first group of mutants, K131C and C154G, were examined for IIA^{Glc} binding in the presence of lactose, melibiose, NPG, and TDG (Figure 6). Mutant K131C exhibits from 5- to 17-fold increases in IIA^{Glc} binding over wild-type for these sugars, while mutant C154G exhibits no IIA^{Glc} binding. To further characterize mutant K131C, this mutant is examined for [^{125}I]IIA^{Glc} binding as a function of sugar concentration. Unlike wild-type (Figure 3), mutant K131C has approximately the same maximum IIA^{Glc} binding for all the sugars tested (data not shown). In addition, the substrate concentration at which half-maximal [^{125}I]IIA^{Glc} binding ($K_{0.5}^{IIA}{}_{sug}$) was determined (Table 1). Mutant K131C has 9−27-fold lower $K_{0.5}^{IIA}{}_{sug}$ values compared to wild-type LacY for all the substrates with the exception of methyl β-D-gal which is 129-fold lower.

Transport of lactose by mutant C154G is almost completely abolished, although this mutant binds substrate with high-affinity (*24*, *47*). Mutant C154G is thought to be locked in the outwardly facing conformation (i.e., the binding site is exposed on the inside of ISO vesicles). The inability to bind IIA^{Glc} to this mutant form of LacY is probably related to its inability to undergo the substrate-dependent conformational change necessary for IIA^{Glc} binding.

The second group of LacY mutants are a series of E269D rescue mutants (*48*) which allow examination of lactose-dependent IIA^{Glc} binding (Figure 7) to several different, stable conformations of LacY. In the presence of lactose, mutant E269D, which is deficient in lactose transport due to a defect in protonation, exhibits no IIA^{Glc} binding. Mutants G262A/E269D and T266G/M267G/E269D which exhibit partial rescue of lactose transport activity manifest a low level of IIA^{Glc} binding, while for mutant G262A/T266G/M267G/E269D, which exhibits near wild-type transport activity, IIA^{Glc} binding is completely restored to wild-type levels. An interpretation of these results in the context of conformational changes is treated in the discussion.

Stoichiometry of IIA^{Glc} Binding to LacY. The stoichiometry of IIA^{Glc} binding to LacY was determined from quantitative immunoblot analyses of LacY in conjunction with direct [^{125}I]-IIA^{Glc} binding measurements (Figure 8). The C-terminal His-tag in the LacY construct used in these determinations, does not affect the affinity for IIA^{Glc} (Figures 4 and 8). Also, it has been shown that IIA^{Glc} binding to ISO vesicles with LacY is linear up to at least 0.6 mg of membrane protein (*18*). The binding stoichiometry is one mole of IIA^{Glc} bound per six moles of wild-type LacY, indicating that most of the LacY is not capable of binding IIA^{Glc}. For mutant K131C, which binds much higher levels of IIA^{Glc} (Figure 6), the stoichiometry is increased to 1:2. Moreover, the apparent K_D^{IIA} is approximately the same for both preparations. The same stoichiometries are obtained when the vesicles are incubated with melibiose for prolonged periods of time (>1 h) to ensure equilibration of the sugar with the intravesicular space or when the melibiose concentration is increased to 20 mM (data not shown).

Table 1: Comparison of the Substrate Concentration Required for Half Maximal IIA^{Glc} Binding to Wild-Type (Wt) and Mutant K131C LacY with Sugar Affinity for Wt LacY[a]

LacY substrates	$K_{0.5}^{IIA}{}_{sug}$ for Wt LacY ISO vesicles	$K_{0.5}^{IIA}{}_{sug}$ for K131C LacY ISO vesicles	K_{Dsug} for Wt LacY RSO vesicles
methyl β-d-Gal	"110 mM"	856 μM	10 mM
galactose	26 mM	3 mM	30 mM
methyl α-d-Gal	6 mM	326 μM	0.5 mM
lactose	5 mM	273 μM	3−5 mM
raffinose	4 mM	315 μM	800 μM
melibiose	700 μM	26 μM	85 μM
NPG	200 μM	9 μM	≈ 20 μM
TDG	200 μM	10 μM	≈ 20 μM

[a] Shown in the first two columns, respectively, is the apparent $K_{0.5}^{IIA}{}_{sug}$ (the substrate concentration at which half-maximal [^{125}I]IIA^{Glc} binding is observed) for the Wt (Figure 3) and mutant K131C LacY ISO vesicle preparations. The third column shows the apparent affinity (K_{Dsug}) for substrate binding to wild-type LacY RSO vesicles determined from the substrate concentration at which 50% protection of single-Cys148 against alkylation by [^{14}C]NEM is observed. With the exception of melibiose and raffinose (Figure 5), these values were previously determined (*14*, *40*).

DISCUSSION

There are three major findings presented here that require interpretation: (i) The amount of IIA^{Glc} bound varies with the substrate used. (ii) Certain amino acid replacements in LacY influence the amount of IIA^{Glc} binding. (iii) The stoichiometry for IIA^{Glc} binding to wild-type LacY falls far below unity.

The level of IIA^{Glc} binding to wild-type LacY varies with different LacY substrates. The possibility that these differences are related to the affinity of the substrates for LacY has been considered and eliminated. As discussed previously, the same levels of binding are observed even when the standard assay is modified by incubating the reaction mixtures overnight or by using higher substrate concentrations. Consequently, the observed differences in binding are unlikely to be related to unfavorable equilibria between different conformations of LacY.

All of the specificity for substrate recognition by LacY is directed toward the galactosyl moiety of substrate (*14*, *50*). Thus, galactose is the most specific substrate for LacY, but has very low affinity. However, affinity can be increased up to 3 orders of magnitude by hydrophobic (particularly aromatic) substituents at the one position of the galactosyl moiety. The interactions with the nongalactosyl moiety must vary from one substrate to another, and it is likely that different galactoside analogues introduce a slightly different conformation of LacY around the binding site. These conformational differences could result in different degrees of exposure of the IIA^{Glc} binding site.

Certain amino acid replacement in LacY influence the amount of IIA^{Glc} binding. An interesting LacY mutant that has been characterized previously with respect to LacY function is C154G (*24*, *47*). This mutant has a 4-fold higher affinity for ligand than wild-type LacY, but does not translocate substrate (*47*) nor binds IIA^{Glc} in the absence or presence of substrate (Figure 6). Similarly, another LacY mutant (lacY^{59}) (*16*) that binds, but does not transport galactosides, also does not bind IIA^{Glc} in the presence of NPG. Both mutants are probably locked in an outward-facing conformation that binds substrate, but is not recognized by IIA^{Glc}.

Another interesting LacY mutant is K131C, a position that is in close proximity to the substrate-binding site (region I in Figure 1). This mutant manifests a low but significant initial rate and steady-state level of lactose accumulation (*28*). Remarkably, this mutant binds approximately 5−17 times more IIA^{Glc} than wild-type LacY in the presence of lactose, melibiose, NPG, or TDG (Figure 6) and exhibits at least a 9-fold decrease in $K_{0.5}^{IIA}{}_{sug}$ relative to wild-type LacY (Table 1). Unlike wild-type LacY, maximum IIA^{Glc} binding is approximately the same with each substrate, suggesting that all substrate-dependent conformations of mutant K131C are comparable.

Recently, a series of LacY rescue mutants for the E269D mutation were described (*48*) that allow three general substrate-bound conformations to be studied. Mutant E269D, whose primary defect appears to be a decrease in protonation, exhibits poor lactose accumulation, efflux, and exchange and does not bind [^{125}I]IIA^{Glc} in the presence of lactose (Figure 6). The triple mutant T266G/M267G/E269D restores the ability to accumulate lactose from the outside, but efflux and exchange are hardly altered, suggesting that the conformation equilibrium is shifted toward the outwardly facing conformation. For this mutant, only a low level of lactose-specific [^{125}I]IIA^{Glc} binding (∼30% of wild-type) is restored. This is consistent with the observation that the C154G LacY mutant, which is locked completely in the outwardly facing conformation, also binds no IIA^{Glc}. The reciprocal phenotype is mutant G262A/E269D where efflux and exchange are largely restored with only a small increase in the rate of lactose accumulation. This mutant likely favors the inwardly facing conformation and like mutant T266G/M267G/E269D, exhibits partial restoration of [^{125}I]IIA^{Glc} binding (10% of wild-type). Finally, all lactose transport functions (i.e., initial rate and steady-state level of lactose accumulation, efflux, and exchange) are restored when both sets of mutations are combined in mutant G262A/T266G/M267G/E269D (*48*), and likewise, IIA^{Glc} binding is restored to wild-type levels. The data suggest that neither the outwardly facing nor the inwardly facing conformation is recognized by IIA^{Glc}.

One straightforward possibility for why wild-type LacY never achieves a 1:1 binding stoichiometry with IIA^{Glc} is that the ISO vesicle preparations may be contaminated with RSO vesicles. However, as reported previously (*36*), the vesicles accumulate thiocyanate in the presence of ascorbate and phenazine methosulfate under oxygen, indicating that the polarity of $\Delta\bar{\mu}_H+$ is interior positive, and no tetraphenylphosphonium uptake is demonstrable. Thus, the preparations are not contaminated to a significant extent with RSO vesicles. In addition, when ISO vesicles with wild-type LacY

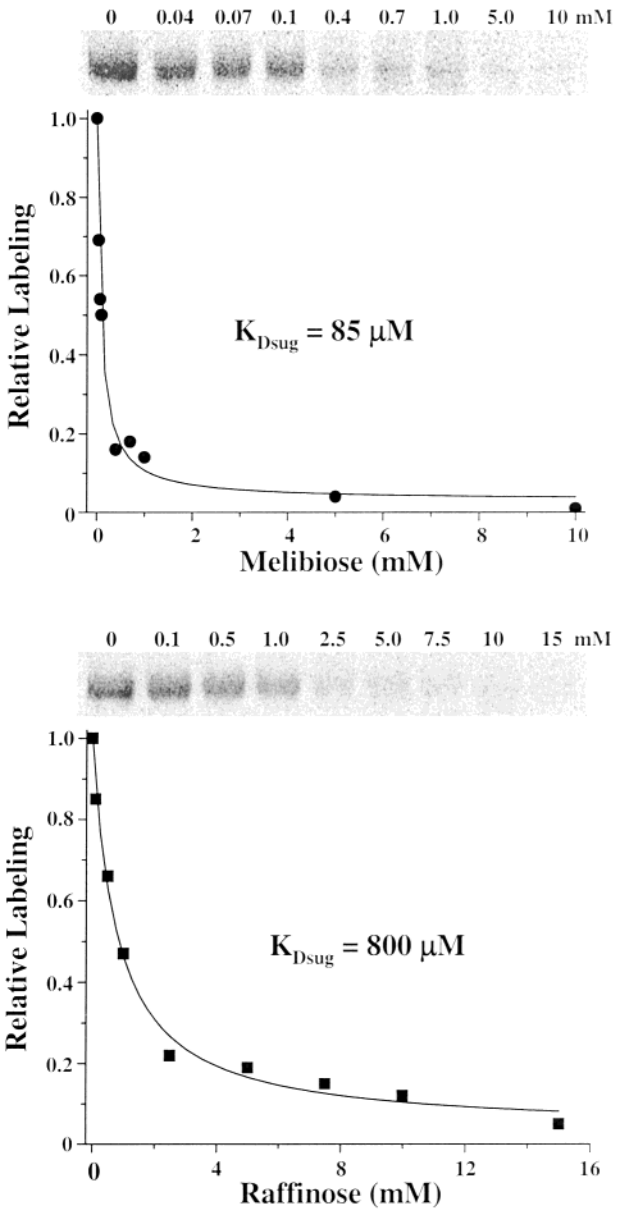

FIGURE 5: Apparent affinity of LacY for melibiose and raffinose. Substrate protection provided by melibiose and raffinose against [^{14}C]NEM alkylation of residue C148 of LacY is shown. This method has been described in detail (*14*) and involves incubating RSO membrane vesicles containing single-C148 permease with the biotin acceptor domain at the C terminus, with [^{14}C]NEM in the presence of varying amounts of LacY substrate. After quenching, the biotinylated permease is affinity purified, separated on a SDS−12% polyacrylamide gel, and the ^{14}C-labeled permease is visualized by phosphoimaging (top panel). The percent of labeling, as compared to the labeling seen in the absence of substrate (lane 1), is plotted for the various concentrations of substrate examined. Also indicated is the concentration at which 50% protection against NEM labeling is observed and is equal to the affinity for substrate binding (apparent K_{Dsug}).

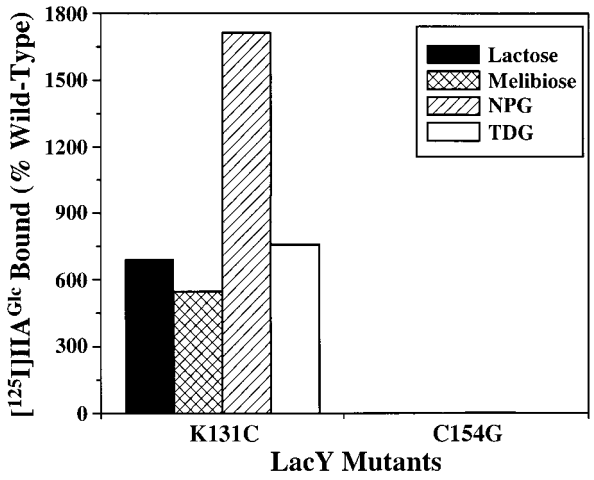

FIGURE 6: Binding of IIA^{Glc} to LacY mutants. Two mutants and wild-type LacY (Wt) were examined for their ability to bind [^{125}I]-IIA^{Glc} (0.5 μM) in the presence of LacY substrates: lactose, melibiose, NPG, and TDG. LacY substrates were used at the $K_{0.5}^{IIA}{}_{sug}$ concentration determined for wild-type LacY in Figure 3. Values shown are the percent of [^{125}I]IIA^{Glc} binding to wild-type LacY vesicles for that particular substrate.

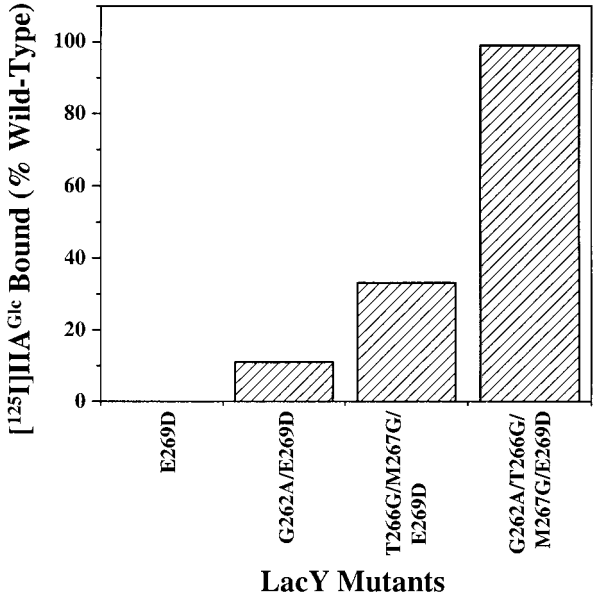

FIGURE 7: Binding of IIA^{Glc} to LacY E269D rescue mutants. IIA^{Glc} binding was examined for these mutants in the presence of lactose under the conditions described in Figure 6. Values are listed as percent of [^{125}I]IIA^{Glc} binding to wild-type LacY vesicles in the presence of lactose. As with mutant C154G, no change in IIA^{Glc} binding is observed for mutant T266G/M267G/E269D when the vesicles are incubated overnight with lactose.

are sonicated, no increase in IIA^{Glc} binding is observed. Another possibility is that urea extraction damages the cytoplasmic loops involved in binding IIA^{Glc}. However, the amount of melibiose promoted IIA^{Glc} binding is the same in ISO vesicles that are not treated with urea. Therefore, urea extraction reduces nonspecific IIA^{Glc} binding with no effect on sugar-dependent binding. Finally, it is noteworthy that there is a stoichiometric relationship between the amount of LacY in the membrane, as determined immunologically, and binding of ligand (NPG), as determined by flow dialysis (*38*, *49*). Furthermore, NEM labeling of single-Cys148 LacY is abolished completely at high sugar concentrations. Therefore, the low stoichiometry observed for IIA^{Glc} binding to wild-type LacY cannot be due to a population of damaged LacY molecules that are incapable of binding substrate.

Nelson et al. (*16*) determined a stoichiometry of 1:1 by using an assay involving rocket immunoelectrophoresis to determine *S. typhimurium* IIA^{Glc} bound and [^{3}H]NPG binding to quantitate the amount of LacY. More specifically, they used the relative peak heights of the immunoprecipitin profiles for their calculations. However, it is necessary to measure the areas subtended by immunoprecipitin profiles

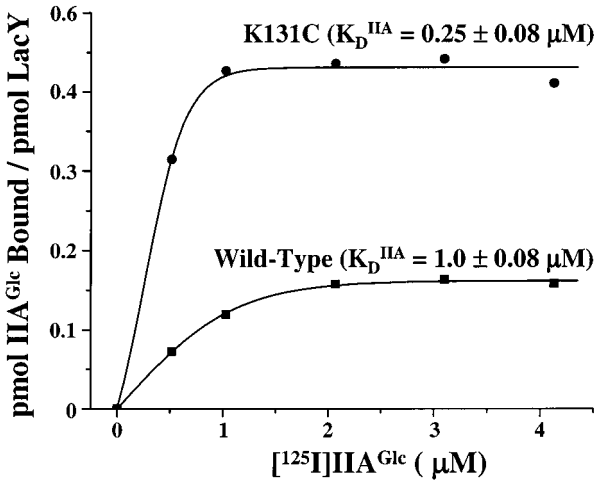

FIGURE 8: Stoichiometry of IIA^{Glc} binding to LacY. In the presence of saturating melibiose (2.1 mM), the maximal level of $[^{125}I]IIA^{Glc}$ binding to 150 μg of total membrane protein for ISO vesicles containing wild-type LacY was 25 pmol and for ISO containing mutant K131C LacY was 35 pmol for these particular vesicle preparations. Both LacY constructs have a 6-His tag at the C termini that was used to measure, by quantative immunoblot analysis, as described in Experimental Procedures, the amount of LacY. By this method, the concentrations determined were wild-type, 150 pmol of LacY/150 μg of protein and K131C, 85 pmol of LacY/150 μg of protein. The data are plotted as pmol of $[^{125}I]IIA^{Glc}$ specifically bound per pmol of LacY. The affinity for IIA^{Glc} binding (K_D^{IIA}) is indicated in parentheses.

to obtain quantitative data (*51*). If a stoichiometry of 1:1 for wild-type LacY is correct, it is difficult to explain how mutant K131C can bind significantly more IIA^{Glc} than wild-type LacY. Strikingly, mutant K131C binds about 3-times more IIA^{Glc} than wild-type LacY and exhibits a stoichiometry of 1:2 (Figure 8). Therefore, as suggested by Sondej et al. (*20*), the arrangement of the residues in helix IV, appears to place a limitation on IIA^{Glc} binding to wild-type LacY. A Cys replacement at residue 131 may change the tilt of helix IV and/or influence the orientation of loop IV/V with respect to loop VI/VII. Thus, upon sugar binding, a larger fraction of the IIA^{Glc} binding sites for mutant K131C is available for interaction compared to wild-type LacY.

The degree of inhibition of LacY activity by IIA^{Glc} (inducer exclusion) is related to the ratio of the two interacting components (*2*). It has been observed that cells are more sensitive to inducer exclusion when either the amount of IIA^{Glc} is increased above chromosomal level or the amount of a target protein (i.e., LacY) is decreased. This phenomenon has been rationalized with regard to the formation of a stoichiometric complex of IIA^{Glc}:LacY and its dissociation constant. Using the values of 25−50 μM for the cellular concentration of IIA^{Glc}, 5−16 μM for K_D^{IIA} and 0.2 nmol for the number of LacY molecules in lactose-induced cells per mg of membrane protein, it has been proposed that essentially all the LacY would be complexed to IIA^{Glc}, resulting in essentially complete inhibition of lactose transport.

The results presented here lead to a somewhat different perspective on the simplified model presented above. The observations that the stoichiometry of IIA^{Glc} binding to LacY varies from 1:6 to 1:2 are consistent with the idea that overexpression of LacY is associated with an escape from inducer exclusion (*19*). The explanation for this may lie in the unique substrate-induced structure that allows only a fraction of the LacY molecules to interact with and be inhibited by IIA^{Glc}. Consequently, this property of LacY may serve as a modulatory influence on the process of inducer exclusion.

In summary, the findings reported here are consistent with the idea that LacY forms unique liganded structures with each of its sugar substrates. These structures can be distinguished by the different levels of IIA^{Glc} binding to the various sugar-permease complexes. Furthermore, certain amino acid replacements in LacY lead to modifications in the nature of these structures with respect to IIA^{Glc} binding. Particularly noteworthy are mutant C154G, which binds, but does little or no translocation of substrate, along with not binding IIA^{Glc}, and mutant K131C, which exhibits a higher level of IIA^{Glc} binding, but does not form unique substrate-dependent conformations.

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