Characterization of a Lactose Permease Mutant that Binds IIA^{Glc} in the Absence of Ligand†

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ABSTRACT: Enzyme IIA^{Glc} of the *Escherichia coli* phosphoenolpyruvate:glucose phosphotransferase system plays a direct role in regulating inducible transport systems. Dephosphorylated IIA^{Glc} binds directly to lactose permease in a reaction that requires binding of a galactosidic substrate. A double-Cys mutation (Ile129 → Cys/Lys131 → Cys) was introduced into helix IV of the permease near the IIA^{Glc} binding site in cytoplasmic loop IV/V and in the vicinity of the galactoside binding site at the interface of helices IV, V, and VIII. The mutant no longer requires galactoside for IIA^{Glc} binding as demonstrated by both a [^{125}I]IIA^{Glc} binding assay and a newly developed fluorescence anisotropy assay. Further characterization of the mutant shows that it binds substrate with high affinity, but is almost completely defective in all modes of translocation across the cytoplasmic membrane. The data are consistent with the interpretation that the double mutant is locked in an inward-facing conformation.

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

In *E. coli*, IIA^{Glc} also modulates the activity of a number of proteins, resulting in the preferential transport and utilization of glucose via the PTS relative to sugars that are transported by other inducible systems. When a PTS sugar is transported, IIA^{Glc} becomes predominately dephosphorylated 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. Dephosphorylated IIA^{Glc} also inhibits glycerol kinase which catalyzes the first step in glycerol metabolism. Upon depletion of the PTS sugar, IIA^{Glc} accumulates in the phospho form which no longer binds to endogenous inducible transport proteins, thereby allowing inducers to enter the cell, and also activates adenylyl cyclase with release of catabolic repression.

The X-ray structure of the IIA^{Glc}−glycerol kinase complex (*4*) and the NMR structure of the IIA^{Glc}−HPr complex (*5*) have been determined. In addition, mutagenesis experiments have led to the identification of several residues in IIA^{Glc} that are important for binding to LacY (*6*). The majority of these residues are on the same surface of IIA^{Glc} that interacts with both glycerol kinase and HPr and encompasses the IIA^{Glc} phosphorylation site (*5*).

LacY, a cytoplasmic membrane protein with 12 hydrophobic transmembrane helical domains, catalyzes the coupled, stoichiometric translocation of α- or β-D-galactopyranosides with H^+. The H^+ electrochemical gradient, the driving force for sugar accumulation, is not required for IIA^{Glc} binding (*7*); however, specific binding of IIA^{Glc} is observed only in the presence of LacY substrates (*8*−*11*). The major determinants for substrate binding to LacY are located at the interface of helices IV (Glu126 and Ala122), V (Arg144, Cys148, and Trp151), and VIII (Glu269) (see refs *12*−*16*).

Cys-scanning mutagenesis of a functional LacY mutant devoid of native Cys residues (Cys-less LacY) shows that residues located in the cytoplasmic loop between helices IV and V (loop IV−V) in addition to residues in the flanking helical domains and residues in the large central cytoplasmic loop (loop VI−VII) are important for IIA^{Glc} binding (*17*). Though unimportant for IIA^{Glc} binding, two residues, Ile129 and Lys131, located in transmembrane helix IV on the

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[1] Abbreviations: LacY, lactose permease; PTS, phosphoenolpyruvate:sugar phosphotransferase system; IIA^{Glc}, glucose specific PTS protein; 6His-IIA^{Glc}, IIA^{Glc} with an N-terminal six-His tag; ISO, inside-out; RSO, right-side-out; KP_i, potassium phosphate; NaP_i, sodium phosphate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NPG, *p*-nitrophenyl α-D-galactopyranoside; TDG, D-galactopyranosyl β-D-thiogalactopyranoside; TMR, tetramethylrhodamine 5-maleimide.

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cytoplasmic side of the substrate-binding site, exhibit enhanced levels of IIA^{Glc} binding when replaced with Cys in both the Cys-less (*17*) and wild-type background (this work). The double-Cys mutant (I129C/K131C) in a wild-type background also exhibits enhanced levels of IIA^{Glc} binding, and furthermore, IIA^{Glc} binding by the double mutant is independent of substrate binding as judged either by binding of $[^{125}I]IIA^{Glc}$ or by fluorescence anisotropy measurements. Further characterization also reveals that the I129C/K131C mutant exhibits high-affinity substrate binding, but almost no ability to translocate substrate across the membrane.

EXPERIMENTAL PROCEDURES

Plasmids and Strains. Plasmids pHISIIA (*E. coli* IIA^{Glc} with an N-terminal six-His tag) (*11*), pTYRHISIIA (*E. coli* IIA^{Glc} with an N-terminal Tyr residue followed by a six-His tag) (*11*), pT7-5/wild-type cassette *lacY* (EMBL-X56095) (*18*), and pT7-5/K131C cassette *lacY* (*19*) have been described previously. LacY mutants I129C and I129C/K131C were derived from pT7-5/wild-type cassette *lacY* using two-step PCR mutagenesis (*20*). pCYSHISIIA (*E. coli* IIA^{Glc} with an N-terminal Cys residue followed by a six-His tag) was constructed in a manner similar to that used for pTYRHISIIA (*11*). Constructs were verified by dye terminator cycle sequencing (PE Applied Biosystems). IIA^{Glc} constructs were transformed into *E. coli* GI698 Δ*ptsHIcrr* (*21*), while LacY constructs were transferred into *E. coli* T184 [$lacI^{+}O^{+}Z^{-}Y^{-}(A)$, *rpsL*, met^{-}, thr^{-}, *recA*, *hsdM*, *hsdR*/F′ $lacI^{q}O^{+}Z^{U118}(Y^{+}A^{+})$] (*22*).

$[^{125}I]IIA^{Glc}$ Binding Assay. TyrHisIIA^{Glc} expression, purification through a Ni−NTA resin (Qiagen) column followed by a pass through an FPLC Hi Prep 16/10 Sephacryl 5-300 high-resolution column (Amersham Pharmacia) and iodination, and preparation of urea-washed inside-out (ISO) membrane vesicles containing overexpressed LacY have been described previously (*11*). IIA^{Glc} binding to LacY was assessed using $[^{125}I]IIA^{Glc}$ (*11*), and the order of addition to the assay (100 μL) was as follows: (i) 100 mM sodium phosphate (NaP_i, pH 7.0), (ii) 2 mM DTT, (iii) 2 mM $MgCl_2$, (iv) 150 mM NaCl, (v) saturating melibiose (5 mM), (vi) urea-washed ISO membrane vesicles containing LacY, and (vii) the specified amount of ^{125}I-labeled IIA^{Glc}. The reaction components were incubated at room temperature for 5 min, centrifuged, and the pellet was left to solubilize overnight at room temperature in 1% Triton X-100 (scintillation grade, RPI). Membrane-bound $[^{125}I]IIA^{Glc}$ was quantitated with a Beckman LS 5000TD liquid scintillation counter.

Fluorescent Labeling of IIA^{Glc}. Since IIA^{Glc} is devoid of Cys residues (*23*), a mutant with a single Cys residue at the N-terminus was constructed, purified (*11*), and labeled overnight in the dark at room temperature in 50 mM NaP_i (pH 7.0) and 100 mM NaCl with a 20-fold molar excess of tetramethylrhodamine 5-maleimide (TMR, from Molecular Probes). Reactions were terminated by addition of 5 mM DTT, followed by a 15 min incubation at room temperature, and unreacted fluorescent probe was removed by extensive dialysis against 25 mM NaP_i (pH 7.0) and 100 mM NaCl at 4 °C. The final concentration of the labeled protein (TMR−IIA^{Glc}) was determined by the Coomassie Plus Protein Assay (Pierce). By using the radiolabeled binding assay (*11*), TMR−IIA^{Glc} competes with $[^{125}I]IIA^{Glc}$ as well as unlabeled, N-terminally His-tagged IIA^{Glc} for binding to wild-type LacY (data not shown). Thus, introduction of the N-terminal Cys residue and labeling with TMR do not alter the ability of TMR−IIA^{Glc} to bind to LacY.

Fluorescence Anisotropy. Steady-state fluorescence anisotropy measurements were performed using a Spex Fluorolog-3 model FL3-22 spectrofluorometer (Spex Industries, Metuchen, NJ) equipped with Glan-Thompson polarizers. Excitation and emission wavelengths were set at 541 and 574 nm with band-pass wavelengths of 2 and 5 nm, respectively. A constant temperature of 25 °C was maintained with a circulating water bath. Data were acquired with the polarizer geometry set in the L format and an integration time of 1 s. Anisotropy values (r) were calculated according to the following equation:

$$r = \frac{I_{VV} + GI_{VH}}{I_{VV} + 2GI_{VV}} \quad (1)$$

where I_{VV} and I_{VH} correspond to the emission intensity when the emission polarizer is parallel and perpendicular, respectively, to the vertically aligned, excitation polarizer. The G-factor serves as an instrumental correction due to differences in the transmission efficiency of the monochromators for vertical and horizontal light, and is defined by the following relation:

$$G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

where I_{HV} and I_{HH} correspond to the emission intensity when the excitation polarizer is oriented parallel and perpendicular, respectively, to the horizontally polarized, excited light. The anisotropy value from each membrane addition is the average of three readings.

Cell Growth and Transport Assays. For active transport, *E. coli* T184 ($lacZ^{-}Y^{-}$) (*22*) 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 an OD_{600} of ≈0.65 when LacY expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After growth for an additional 3 h, cells were harvested at 6000*g* for 10 min, washed once, and then resuspended in 100 mM KP_i (pH 7.5) and 10 mM $MgSO_4$ to an OD_{600} of 10.0 (0.7 mg of protein/mL). Transport was initiated at room temperature by addition of 0.4 mM [1-^{14}C]lactose (10 mCi/mmol) to 50 μL aliquots. Reactions were quenched at given times with 2.0 mL of 100 mM KP_i (pH 5.5), 100 mM LiCl, and 10 mM $MgSO_4$ and immediate filtration (*24*). Radioactivity retained on the filters was assayed by liquid scintillation spectrometry. LacY expression was assessed by quantitative immunoblot analysis (*25*) with an anti-C-terminal antibody (*11*, *26*).

Flow Dialysis. Binding of [6-^{3}H]-*p*-nitrophenyl α-D-galactopyranoside (NPG, kindly provided by G. LeBlanc, Laboratoire J. Maetz/Commissariat à l'Energie Atomique, Ville Franche-súr-Mer, France) was assessed by flow dialysis (*27*, *28*). The upper chamber contained 250 μL of urea-washed ISO vesicles (10 mg of protein/mL) in 50 mM NaP_i (pH 7.0), and to ensure complete de-energization, 20 μM valinomycin and 0.4 μM nigericin (final concentrations) were

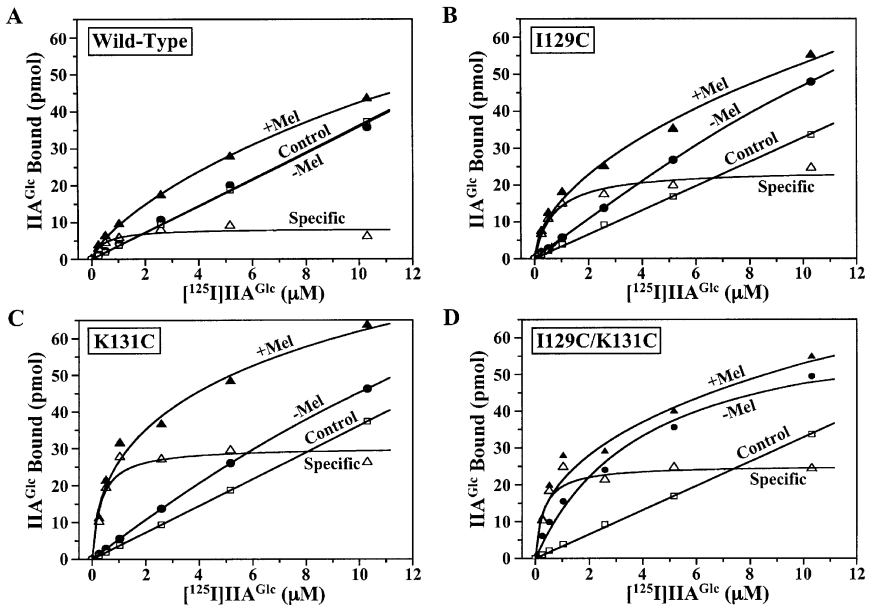

FIGURE 1: Binding of [^{125}I]IIA^{Glc}. The level of LacY expression was determined relative to that of the wild type for each mutant ISO vesicle preparation by quantitative immunoblot analysis (*11*), and an equivalent amount of vesicles was added so that the concentration of LacY was the same in each case. In the case of wild-type LacY, the vesicle concentration that was added equaled 100 μg of total protein, while 84, 109, and 67 μg were added for LacY mutants I129C, K131C, and I129C/K131C, respectively. Given concentrations of [^{125}I]-IIA^{Glc} (914 mCi/mmol) were added to the reaction mixtures to determine the amount of IIA^{Glc} bound in the absence (−Mel) or presence (+Mel) of 5.0 mM melibiose. Also, the amount of IIA^{Glc} bound was measured in ISO vesicles prepared from cells transformed with pT7-5 with no *lacY* (Control) using the same total protein concentration as the vesicles used in that panel. The level of specific binding of [^{125}I]-IIA^{Glc} in the presence of melibiose (Specific) was calculated by subtracting the number of picomoles of IIA^{Glc} bound obtained for control vesicles from that obtained for a particular mutant in the presence of melibiose.

added. While being stirred constantly, 50 mM NaP_i (pH 7.0) was pumped through the lower chamber at a flow rate of 0.5 mL/min, and 1.0 mL fractions were collected. Scintillation fluid (5 mL of Fisher ScintiSafe Econo2) was added to a 0.9 mL aliquot of each fraction, and the samples were assayed for radioactivity by liquid scintillation spectrometry.

RESULTS

[^{125}I]IIA^{Glc} Binding. By using the assay described previously (*11*), binding of [^{125}I]IIA^{Glc} to ISO membrane vesicles containing wild-type LacY and Cys replacement mutant I129C, K131C, or I129C/K131C was examined in the absence or presence of saturating concentrations of melibiose (*11*) and compared to binding observed with an equivalence (total protein) of control vesicles (from cells transformed with the pT7-5 vector without a *lacY* insert) (Figure 1). Control vesicles exhibit nonspecific binding of IIA^{Glc}, since the extent of binding increases linearly with increasing amounts of [^{125}I]-IIA^{Glc} and shows no tendency to saturate. Levels of IIA^{Glc} binding observed for wild-type LacY vesicles in the absence of melibiose are identical to those observed for control vesicles (Figure 1A). However, in the presence of melibiose, a significant increase in the level of IIA^{Glc} binding is observed which is consistent with previous studies (*8*, *11*, *29*) demonstrating that substrate (e.g., melibiose) is required for specific binding of IIA^{Glc} to wild-type LacY. With the LacY mutants (Figure 1B−D), significant binding is observed in the absence of sugar which varies with each mutant (I129C/K131C > I129C > K131C). Although the level of binding of IIA^{Glc} to mutants I129C and K131C increases when melibiose is present, surprisingly, mutant I129C/K131C shows only a small increase in the level of binding in the presence of melibiose (Figure 1D).

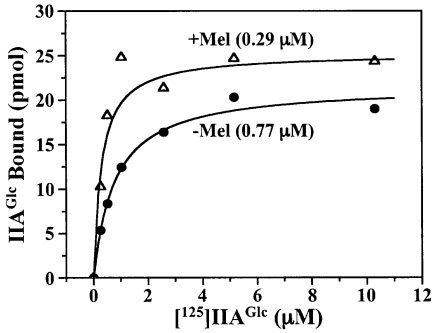

FIGURE 2: Specific binding of IIA^{Glc} to mutant I129C/K131C. The level of specific binding of [^{125}I]IIA^{Glc} to mutant I129C/K131C in the absence (−Mel) or presence (+Mel) of melibiose was calculated from the data presented in Figure 1D. Conditions are the same as those described in the legend of Figure 1. The data were fit to a standard hyperbolic curve (*11*) to determine the apparent affinity for IIA^{Glc} (K_D^{IIA}, in parentheses).

Since the LacY mutants exhibit IIA^{Glc} binding in the absence of sugar, the level of "specific binding" was determined by subtracting the value obtained for control membranes from the value obtained for a given mutant in the presence of melibiose. The three LacY mutants manifest approximately the same maximum level of specific binding which is approximately 3−4-fold higher than that of wild-type LacY. With mutant I129C/K131C, both melibiose-dependent and -independent IIA^{Glc} binding approach saturation. Therefore, specific binding was compared in the absence or presence of melibiose (Figure 2). As shown, specific binding clearly saturates both in the absence and in the presence of the sugar, and the apparent affinity for IIA^{Glc} is ca. 2.5-fold greater in the presence of melibiose (i.e., the K_D^{IIA} is ca. 2.5-fold lower).

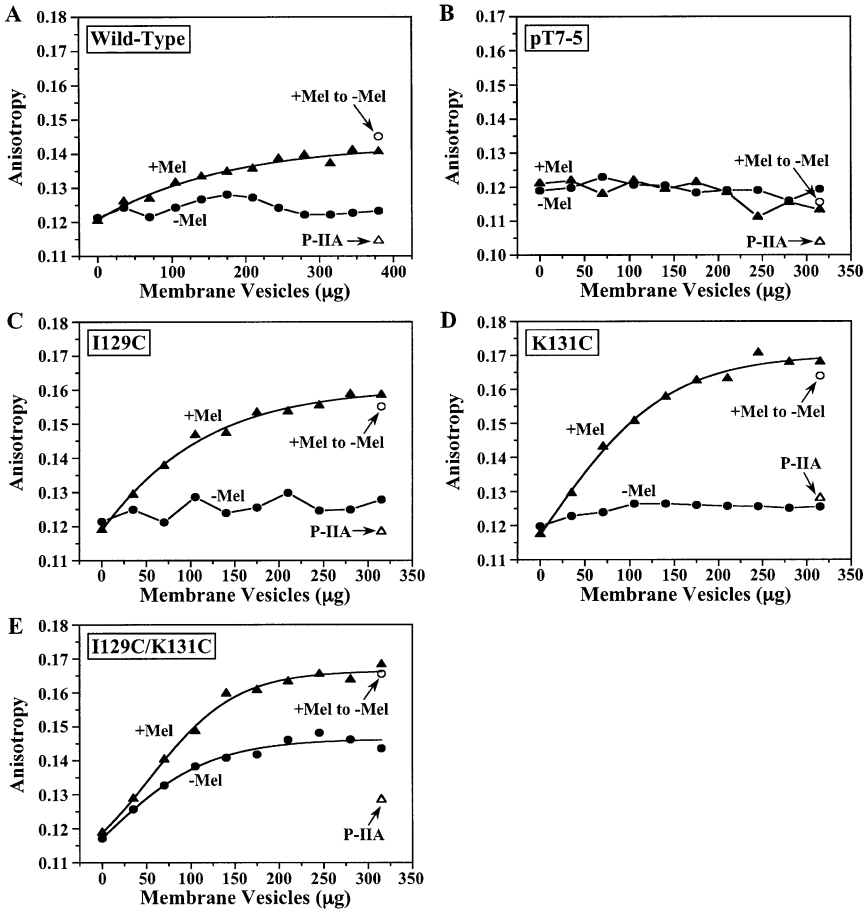

FIGURE 3: Level of IIA^{Glc} binding measured by fluorescence anisotropy. TMR−IIA^{Glc} (final concentration of 0.5 μM) was titrated in the absence and presence of a saturating melibiose concentration (2 mM), with given amounts of ISO vesicles containing (A) wild-type LacY, (B) control (pT7-5 with no *lacY* insert), (C) mutant I129C, (D) mutant K131C, or (E) mutant I129C/K131C in 50 mM NaP_i (pH 7.0). Melibiose (2 mM) was added to the final data point for the study carried out in the absence of melibiose (+Mel to −Mel). TMR−IIA^{Glc} in the presence of melibiose was phosphorylated by the addition of 2 mM phosphoenolpyruvate, 0.19 μM HPr (*37*), and 1.4 μM Enzyme I (*37*) which was then incubated at room temperature for 10 min before the anisotropy value was measured (P-IIA). Data are the averages of two independent runs.

Fluorescence Anisotropy. To further characterize IIA^{Glc} binding to the mutated permeases, an independent assay was developed to examine changes in the steady-state fluorescence anisotropy of TMR−IIA^{Glc} upon binding to LacY. When a fluorescently labeled protein is excited with polarized light, the emitted light is depolarized with respect to the incident light as a result of the mobility of both the labeled protein and the fluorophore (*30*). The anisotropy value (*r*; see Experimental Procedures) is calculated from the ratio of vertically polarized to horizontally polarized emission of the fluorophore after excitation with vertically polarized light. Lower anisotropy values represent a high mobility of the fluorophore, while high values represent a low mobility. Therefore, binding of TMR−IIA^{Glc} to LacY in ISO vesicles should decrease the mobility of TMR−IIA^{Glc} and results in higher anisotropy values.

Anisotropy (*r*) values observed at 0.5 μM TMR−IIA^{Glc} are plotted versus increasing concentrations of ISO vesicles (total protein) containing wild-type LacY or a given mutant in the absence or presence of melibiose (Figure 3). IIA^{Glc} binding by control membranes in the absence or presence of melibiose is essentially identical (Figure 3B, vector without a *lacY* insert). Similarly, data generated in the absence of melibiose for ISO vesicles containing wild-type LacY (Figure 3A) or mutants I129C (Figure 3C) and K131C (Figure 3D) are similar to the data obtained for the control vesicles (Figure 3B). Only the I129C/K131C mutant (Figure 3E) exhibits a significant increase in anisotropy in the absence of melibiose which plateaus as a function of vesicle concentration. In the presence of melibiose, the wild-type and all three mutants exhibit a significant increase in anisotropy which saturates as a function of vesicle concentration. In addition, when melibiose is added to the last data point obtained in the absence of melibiose (Figure 3, +Mel to −Mel), in all cases, the anisotropy value increases to the level observed in the presence of melibiose. Finally, since only dephosphorylated IIA^{Glc} binds to LacY (*8*, *11*, *29*), it is not surprising that phosphorylation of TMR−IIA^{Glc} almost completely abolishes the melibiose-induced increase in anisotropy (Figure 3), as well as the melibiose-independent increase observed with mutant I129C/K131C (data not shown).

Transport Activity. To obtain a qualitative estimate of downhill transport activity, *E. coli* HB101 ($lacY^{-}Z^{+}$) cells harboring plasmid pT7-5 encoding wild-type LacY or a given mutant were grown on MacConkey indicator plates containing 1% lactose, and colonies were observed after 20 h at 37 °C. Red colonies indicate that LacY can catalyze downhill translocation of lactose into the cell which is hydrolyzed by cytosolic β-galactosidase with acidification of the medium

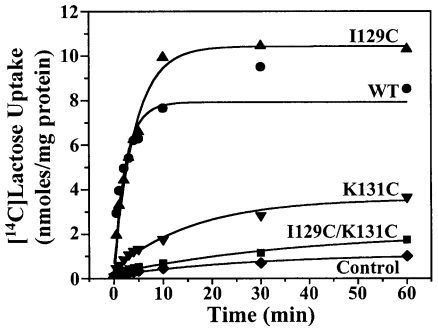

FIGURE 4: Transport activity. Time courses of active lactose transport by *E. coli* T184 expressing wild-type permease (WT), Control (pT7-5 with no *lacY* insert), mutant I129C, mutant K131C, or mutant I129C/K131C. Cells were grown at 37 °C, and aliquots of cells (50 μL containing ~35 μg of protein) suspended in 100 mM KP_i (pH 7.5) and 10 mM $MgSO_4$ were assayed at a final external concentration of lactose of 0.4 mM as described in Experimental Procedures.

due to further metabolism of the monosaccharides. Transformants expressing wild-type LacY or mutant I129C exhibit a dark red phenotype, while cells transformed with a vector devoid of a *lacY* insert exhibit a white phenotype. Colonies expressing mutant K131C grow as colonies with a red center and a white periphery (haloed), and colonies that express mutant I129C/K131C are largely white with a few haloed colonies.

Quantitative assays of active lactose transport are carried out with *E. coli* T184 (*lacY*$^-$*Z*$^-$) expressing a given LacY construct (Figure 4). Mutant I129C accumulates lactose about as well as wild-type LacY; mutant K131C accumulates lactose poorly, and the double mutant I129C/K131C accumulates only slightly more lactose than control cells transformed with pT7-5 with no *lacY* insert. The results are not due to differences in the level of LacY expression. Thus, cells with wild-type permease and mutants I129C and K131C exhibit nearly identical levels of LacY expression as determined by immunoblot analysis, while cells with mutant I129C/K131C exhibit approximately half the level of expression.

Although data are not presented, the mutants were also tested for the ability to catalyze lactose efflux down a concentration gradient in symport with H^+ and equilibrium exchange where the LacY recycles in the protonated state (*31*−*33*). Mutant I129C catalyzes efflux and exchange about as well as wild-type LacY. However, both modes of translocation in mutants K131C and I129C/K131C are extremely slow relative to those in wild-type LacY.

Substrate Binding. One potential explanation for the transport defect observed with mutants K131C and I129C/K131C is that substrate binding is compromised. To examine binding, ISO membrane vesicles containing either wild-type LacY or given mutants were assayed by flow dialysis for binding of the high-affinity ligand [^{3}H]NPG under nonenergized conditions (Figure 5) (*14*, *27*). At the inception of each experiment, [^{3}H]NPG was added to the upper chamber of a flow dialysis apparatus containing membrane vesicles without or with saturating concentrations of IIA^{Glc}, as indicated. Radioactivity in the lower chamber dialysates increases linearly, reaches a maximum (fractions 4 and 5), and then decreases at a slow rate. When a saturating concentration of TDG is added to the upper chamber (fraction 8), bound [^{3}H]NPG is displaced, and the concentration of dialyzable radioactivity increases. In the absence of IIA^{Glc}, addition of excess TDG results in displacement of ~2500 cpm for wild-type LacY (Figure 5A), while mutants I129C (Figure 5B), K131C (Figure 5C), and I129C/K131C (Figure 5D) exhibit displacements of ~2000, ~1000, and ~7500 cpm, respectively. In the presence of IIA^{Glc}, there is relatively little change in TDG displacement of [^{3}H]NPG with the wild-type (ca. 2000 cpm) or mutant I129C/K131C (ca. 8700 cpm); however, mutants I129C and K131C exhibit significant increases in dialyzable radioactivity upon addition of TDG (ca. 4700 and 3900 cpm, respectively).

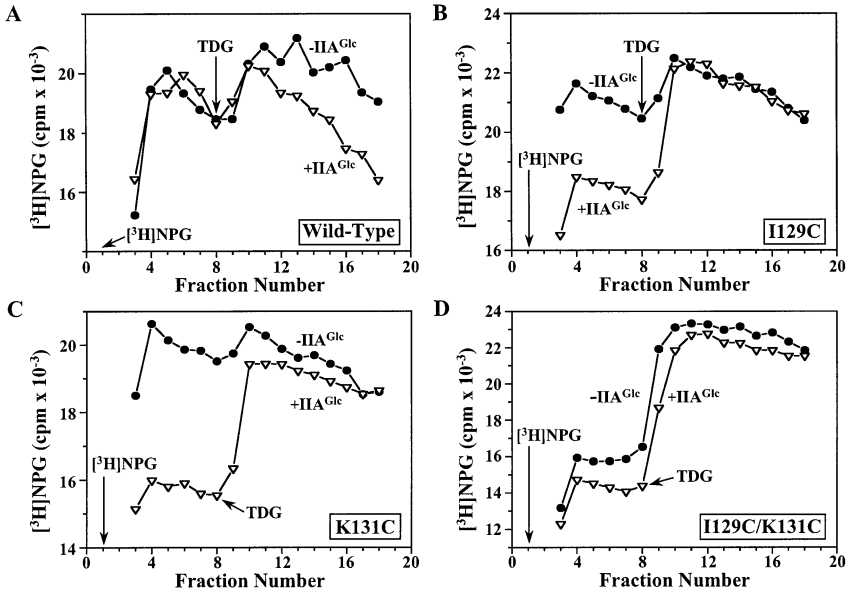

FIGURE 5: Effect of IIA^{Glc} on NPG binding. Binding of [^{3}H]NPG to nonenergized ISO vesicles was assayed in the absence and presence of IIA^{Glc} (10 μM) by flow dialysis as described in Experimental Procedures. [^{3}H]NPG (0.86 Ci/mmol, final concentration of 16 μM) was added to the upper chamber as indicated, and TDG (final concentration of 10 mM) was added at fraction 8 as indicated. LacY expression, as determined from immunoblot analysis, was comparable for all the vesicle preparations. Note that the scales for the *y*-axis differ for each of the panels.

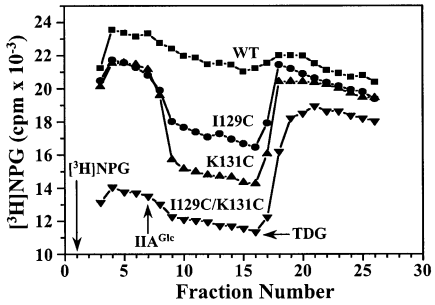

FIGURE 6: Effect of IIA^{Glc} on NPG binding. [^{3}H]NPG binding to nonenergized ISO vesicles was assayed by flow dialysis as described in the legend of Figure 5 with the following modifications. As indicated by the arrows, IIA^{Glc} (10 μM) was added at fraction 7 and TDG (10 mM) was added at fraction 16. The LacY content of ISO vesicles with the wild type, mutant K13C, or mutant I129C/K131C was equivalent, but approximately 2-fold lower in vesicles with mutant I129C as judged by quantitative immunoblots.

Another means of examining the effect of IIA^{Glc} on substrate binding is to add the protein in the middle of the flow dialysis run (Figure 6). At fraction 8, saturating concentrations of IIA^{Glc} were added to the upper chamber; no significant change is observed with wild-type LacY and a slight decrease in dialyzable NPG with mutant I129C/K131C, and significant decreases are observed with mutants I129C and K131C. Finally, when a saturating concentration of TDG is added at fraction 16, displacements of approximately 1000, 5000, 6200, and 7600 cpm are observed in the presence of IIA^{Glc} with wild-type, I129C, K131C, and I129C/K131C LacY, respectively.

DISCUSSION

As demonstrated here, by using either a direct binding assay with [^{125}I]IIA^{Glc} or fluorescence anisotropy measurements with TMR−IIA^{Glc}, the double-Cys mutant I129C/K131C exhibits a high level of specific IIA^{Glc} binding in the absence of a sugar substrate (Figures 1−3). In contrast, specific binding of IIA^{Glc} to wild-type LacY and to mutants I129C and K131C is observed only in the presence of sugar substrates. In addition, mutants I129C, K131C, and I129C/K131C exhibit 3−4 times more IIA^{Glc} binding than wild-type LacY in the presence of melibiose. Previous work (*11*) demonstrated that this difference in the maximal level of IIA^{Glc} binding was due to differences in the stoichiometry. Wild-type LacY has a stoichiometry of 1 mol of IIA^{Glc} per 6 mol of LacY, while the stoichiometry of the K131C mutant is 1:2. Therefore, the results we obtained with our newly developed fluorescence assay are consistent with the results obtained with the [^{125}I]IIA^{Glc} binding assay, but are inconsistent with the stoichiometry of 1:1 determined by Nelson et al. (*8*). For a complete discussion of this inconsistency, please refer to ref *11*.

Previous work (*11*) showed that the maximal level of IIA^{Glc} binding varies with different LacY substrates for wild-type LacY but not mutant K131C. Therefore, we tested IIA^{Glc} binding to mutants I129C and I129C/K131C in the presence of other LacY substrates (lactose, TDG, and NPG) besides melibiose. The maximum level of IIA^{Glc} binding is the same regardless of the substrates that were tested (data not shown); thus, these mutants behaved in a manner similar to that of mutant K131C.

Although maximum melibiose-dependent IIA^{Glc} binding is very similar with the three mutants (Figures 1 and 3), transport activities differ markedly (Figure 4). Mutant I129C transports lactose as well or slightly better than wild-type LacY; mutant K131C accumulates a low but significant amount of lactose, and mutant I129C/K131C is essentially unable to translocate lactose, as well as melibiose (data not shown), across the membrane. Strikingly, however, the double mutant exhibits an approximately 7-fold increase in the level of NPG binding compared to that of wild-type LacY in the absence of IIA^{Glc}, while NPG binding in the absence of IIA^{Glc} by either the I129C or K131C mutant approximates that of wild-type LacY (Figure 5).

Nelson et al. (*8*) have reported that IIA^{Glc} binding increases the affinity of NPG for wild-type LacY by ~3.5-fold. However, no significant difference is observed here for wild-type LacY or mutant I129C/K131C (Figures 5 and 6). However, IIA^{Glc} markedly increases the level of NPG binding by mutant I129C or K131C. It was shown previously (*11*) that the substrate concentration for half-maximal IIA^{Glc} binding ($K_{0.5}^{IIA}{}_{sug}$) is decreased by a factor of at least 9 with mutant K131C compared to that of wild-type LacY, and it was proposed that IIA^{Glc} binding increases the affinity of the mutant for the substrate.

High-affinity binding of the substrate with almost complete loss of all modes of translocation across the membrane exhibited by mutant I129C/K131C closely resembles that of mutant C154G (*34*−*36*). However, in striking contrast to mutant I129C/K131C, IIA^{Glc} does not bind to C154G LacY which is locked in an outward-facing conformation and binds sugar at least as well as wild-type LacY, but exhibits none of the long-range conformational changes observed upon sugar binding (*36*). The loss of sugar-dependent IIA^{Glc} binding by mutant C154G likely reflects an inability to undergo the substrate-dependent conformational change(s) necessary for both transport and IIA^{Glc} binding. Since mutant I129C/K131C binds NPG well, but IIA^{Glc} binding is largely independent of substrate, it seems reasonable to suggest that the double mutant is locked in a predominantly inward-facing conformation (i.e., both the IIA^{Glc} binding site and the substrate binding site are exposed on the outer surface of ISO vesicles).

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