# Ligand Recognition by the Lactose Permease of *Escherichia coli*: Specificity and Affinity Are Defined by Distinct Structural Elements of Galactopyranosides†

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*Received January 6, 2000; Revised Manuscript Received February 14, 2000*

ABSTRACT: Specificity of substrate recognition in lactose permease is directed toward the galactosyl moiety of lactose. In this study, binding of 31 structural analogues of D-galactose was examined by site-directed *N*-[$^{14}$C]ethylmaleimide-labeling of the substrate-protectable Cys148 in the binding site. Alkylation of Cys148 is blocked by D-galactose with an apparent affinity of approximately 30 mM. Epimers of D-galactose at C-3 (D-gulose) and C-4 (D-glucose) or deoxy derivatives at these positions exhibit no binding whatsoever, indicating that these OH groups participate in essential interactions. Interestingly, the C-2 epimer α-D-talose binds almost as well as D-galactose, while 2-deoxy-D-galactose affords no substrate protection, indicating that nonstereospecific H-bonding at C-2 is required for stable binding. No substrate protection is detected with D-fucose, L-arabinose, 6-deoxy-6-fluoro-D-galactose, 6-*O*-methyl-D-galactose, or D-galacturonic acid, suggesting that the C-6 OH is an essential H-bond donor. Both α- and *β*-methyl D-galactopyranosides bind more strongly than galactose, supporting the notion that the cyclic pyranose conformation is the bound form and that the anomeric configuration at C-1 does not contribute to substrate specificity. However, methyl or allyl α-D-galactopyranosides exhibit 60-fold lower apparent $K_d$'s than D-galactose, demonstrating that binding affinity is significantly influenced by the functional group at C-1 and its orientation. Taken together, the observations confirm and extend the current binding site model [Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802−9807] and indicate that specificity toward galactopyranosides is governed by H-bonding interactions at C-2, C-3, C-4, and C-6 OH groups, while binding affinity can be increased dramatically by hydrophobic interactions with the nongalactosyl moiety.

The lactose permease (lac permease)[1] of *Escherichia coli* is representative of secondary active transport proteins that transduce free energy stored in electrochemical ion gradients into work in the form of a concentration gradient (*1*−*5*). This polytopic membrane protein catalyzes the coupled stoichiometric translocation of lactose or other galactosides and $H^+$. The *lac Y* gene that encodes the permease has been cloned and sequenced (*6*), and the product of the *lac Y* gene has been solubilized, purified, reconstituted into proteoliposomes (*7*), and shown to be solely responsible for galactoside transport as a monomer (*8*). All available evidence (*9*) indicate that the permease consists of 12 hydrophobic, membrane-spanning helices connected by hydrophilic loops with both the N and the C termini on the cytoplasmic face of the membrane.

Although the permease forms two-dimensional crystals (*10*), the protein has been highly resistant to crystallization in a form that diffracts to high resolution. Therefore, alternative approaches have been developed to obtain structural and dynamic information required to understand the transport mechanism. Application of a variety of biochemical, spectroscopic, and immunological techniques to an extensive library of site-directed mutants has allowed the formulation of a helix packing model that includes tilts (*11*−*14*). In addition, experimental observations from structural and extensive mutational analysis have led to a proposed mechanism for energy coupling between sugar and $H^+$ transport (*13, 14*).

Substrate specificity in lac permease was first studied systematically by Sandermann (*15*), who tested a large variety of mono- and disaccharides as inhibitors of facilitated or active transport. The results indicate that inhibition requires an α- or *β*-galactopyranosyl ring of the D-series and a free C-6 OH group, and high-affinity binding is conferred by aromatic groups at C-1 of α-galactopyranosides. In addition, the study demonstrated that the type or size of the aglycon is nonspecific, even though apparent $K_m$ and/or $K_i$ is significantly altered by the different nongalactosyl moieties. Subsequently, Olsen and Brooker studied sugar specificity by competitive inhibition of lactose transport (*16*). D-Galactose was identified as a substrate, and an attempt was made to assign relative functional significance to the different OH groups of the sugar based on the ability of structural analogues to inhibit lactose transport (C-3 > C-4 > C-6 > C-2 > C-1). More recently, Wu et al. (*17*) demonstrated that labeling of purified single-Cys148 permease with the fluo-

† This work was supported in part by NIH Grant DK51131 to H.R.K.

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[1] Abbreviations: lac permease, lactose permease; NEM, *N*-ethylmaleimide; DDM, dodecyl *β*-D-maltopyranoside; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; $KP_i$, potassium phosphate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

10.1021/bi0000263 CCC: $19.00 
Published on Web 04/06/2000

rescent thiol reagent 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) is blocked by D-galactose and other substrates of the permease, while glucose or sucrose has no such effect. These studies not only provided the first indication that substrate specificity of lac permease is directed against C4 of galactose but also demonstrated that substrate-protection experiments are extremely useful for quantitative characterization of ligand binding.

On the basis of these sugar specificity studies as well as detailed characterization of mutants at Cys148 (*17, 18*), Glu126, and Arg144 (*19*−*22*), the following model for galactoside binding was postulated (*14, 20*): (i) One of the guanidino $NH_2$ groups of Arg144 forms a H-bond with the OH group at the C-4 and/or C-3 position(s) of the galactosyl moiety of the substrate, an interaction that plays a key role in substrate specificity. (ii) The other guanidino $NH_2$ of Arg144 forms a salt bridge with Glu126, and the interaction holds Arg144 and Cys148 in an orientation that allows specific interaction with the galactosyl moiety. One of the oxygen atoms of the carboxylate at position 126 could also act as an H-bond acceptor from the C-6 OH of the galactosyl moiety. (iii) Cys148, which is protected by substrate against alkylation by *N*-ethylmaleimide (NEM), interacts hydrophobically with the galactosyl end of lactose and other galactosides. Although interactions with the nongalactosyl moiety are not clearly understood, Met145, which is on the same face of helix V as Cys148, is thought to be important in this respect.

The binding site model makes explicit predictions with regard to H-bonding interactions between Glu126−Arg144 and certain OH groups of galactose. To test these assumptions, binding of 31 structural analogues of galactose was examined. The results confirm and extend the predictions of the binding-site model and provide more evidence that specificity and affinity are determined by distinct structural elements of galactopyranosides.

## EXPERIMENTAL PROCEDURES

*Materials. N*-([$^{14}$C]Ethyl)maleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, MA). [$^{125}$I]Streptavidin and horseradish peroxidase-conjugated avidin were from Amersham (Arlington Heights, IL). Immobilized monomeric avidin was from Pierce (Rockford, IL). Sugars were obtained from Sigma or Aldrich (St. Louis, MO).

*Growth of Cells and Preparation of Right-Side-Out (RSO) Membrane Vesicles. E. coli* T184 (*23*) expressing single-Cys148 permease with a biotin-acceptor domain at the C terminus (*24*) were grown in Luria−Bertani broth, and RSO membrane vesicles were prepared as described previously (*21, 25, 26*) with the following modification: To prevent Cys oxidation during vesicle preparation, 5 mM dithiothreitol (DTT) was included in all buffers. At the end of the preparation, vesicles were washed with 100 mM potassium phosphate ($KP_i$; pH 7.5) to remove the DTT, resuspended in the same buffer at a protein concentration of 15 mg/mL, frozen in liquid $N_2$, and stored at −80 °C until use.

*[$^{14}$C]NEM-Labeling.* Reactivity of Cys148 with [$^{14}$C]NEM in situ was determined in the absence and in the presence of given sugars (*21, 27*). Single-Cys148 permease that was used for the assays contains a biotin-acceptor domain at the C terminus and is biotinylated in vivo. RSO membrane vesicles were preequilibrated in a final volume of 50 $\mu$L (containing 0.6 mg of protein) with given sugar concentrations for 10 min at room temperature. Labeling was initiated by addition of 12 $\mu$L of [$^{14}$C]NEM to a final concentration of 0.5 mM (40 mCi/mmol), and the vesicles were incubated for 5 min at 25 °C. Reactions were quenched by addition of 10 mM DTT (final concentration). The vesicles were solubilized with 2% dodecyl $\beta$-D-maltopyranoside (DDM, final concentration), and the samples were mixed with immobilized monomeric avidin (avidin-Sepharose) equilibrated with 50 mM $NaP_i$ (pH 7.5)/0.1 M NaCl/0.02% DDM (w/v). After a 15-min incubation at 4 °C, the resin was washed with 5 mL of equilibration buffer, and biotinylated permease was then eluted with 2 × 50 $\mu$L of equilibration buffer containing 5 mM D-biotin. After addition of 25 $\mu$L of electrophoresis sample buffer (concentrated 5×), an 100 $\mu$L aliquot was analyzed electrophoretically on a sodium dodecyl sulfate (SDS)−12% polyacrylamide gel. The gel was dried and exposed to a PhosphoImager screen for 5−8 days. Incorporation of [$^{14}$C]NEM was visualized and quantitated by a Storm 860 PhosphoImager (Molecular Dynamics). Apparent affinity constants ($K_{d(app)}$) were determined with the MicroCal Origin (Microcal Software, Inc., Northampton, MA) computer program using nonlinear least-squares curve fitting to the following user-defined equation:

$$N = \frac{1 - M}{1 + \frac{[\text{ligand}]}{K_{d(app)}}} + M$$

where $N$ is NEM-labeling in relative units, and $M$ is residual NEM-labeling at infinite ligand concentrations in relative units.

*Western Blot Analysis.* An aliquot (10−20 $\mu$L) of purified [$^{14}$C]NEM-labeled permease was electrophoresed on an SDS−12% polyacrylamide gel, electroblotted to poly(vinylidene diflouride) membranes (Immobilon-PVDF; Millipore), and treated with horseradish peroxidase-conjugated avidin. The PVDF membrane was subsequently developed with fluorescent substrate (Renaissance, DuPont NEN) and exposed to film. Alternatively, protein on the PVDF membrane was treated with [$^{125}$I]streptavidin, autoradiographed, and visualized and quantitated with a Storm 860 PhosphoImager (Molecular Dynamics).

*Synthesis of 3-Deoxy-D-galactose and Allyl 4-Deoxy-α-D-galactopyranoside.* Reactions involving moisture-sensitive reagents were performed in oven-dried or flame-dried glassware under a positive pressure of argon. All reagents were obtained from commercial sources and used as received. Thin-layer chromatography was performed on Whatman (F254) 0.25-mm coated silica gel plates and visualized by using 254-nm ultraviolet light or by heating samples stained with a vanillin−$H_2SO_4$ solution. Column chromatography was carried out using 230−245 mesh silica gel (Bodman Scientific, Aston, PA). The chemical identity of each sugar synthesized was verified by $^1$H and $^{13}$C NMR spectroscopy. 3-Deoxy-D-galactose was prepared from 1,2,5,6-di-*O*-isopropylidene-α-D-glucofuranose as described (*28*). Allyl 4-deoxy-α-D-galactopyranoside was synthesized by using a modified procedure of Lee and Lee (*29*) and Mulard et al. (*30*) as follows.

*Synthesis of Allyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside.* This sugar was synthesized by limited benzoylation of allyl α-D-galactopyranoside as described (*29, 31*). To a stirred solution of allyl α-D-galactopyranoside (0.5 g, 2.6 mmol) in anhydrous pyridine (5.2 mL), benzoyl chloride (1.0 mL, 8.9 mmol) was added at 0 °C under argon. The resulting mixture was stirrred at room temperature for 48 h. The reaction was quenched with a saturated solution of sodium bicarbonate and extracted 3 times with methylene chloride. The combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel using hexane−ethyl acetate (6:1) as the eluent and crystallized from methanol/hexane to yield 56.4% of protected sugar.

*Synthesis of Allyl 2,3,6-Tri-O-benzoyl-4-O-(imidazol-1-ylthiocarbonyl)-α-D-galactopyranoside.* Allyl 2,3,6-tri-*O*-benzoyl-α-*D*-galactopyranoside (0.4 g, 0.9 mmol) in anhydrous 1,2-dichloroethane (40 mL) was refluxed overnight with *N*,*N*′-thiocarbonyldiimidazole. The reaction mixture was allowed to cool to room temperature, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using hexane−ethyl acetate (5:1) as eluent to give 0.5 g (90.2%) of imidazolide.

*Synthesis of Allyl 2,3,6-Tri-O-benzoyl-4-deoxy-α-D-galactopyranoside*. Allyl 2,3,6-tri-*O*-benzoyl-4-*O*-(imidazol-1-ylthiocarbonyl)-α-*D*-galactopyranoside (0.3 g, 0.47 mmol) in anhydrous toluene (11.6 mL) was added dropwise to a refluxing solution of tributyltin hydride (0.32 g, 1.1 mmol) in anhydrous toluene (17.5 mL). The resulting mixture was heated at reflux for 4 h. The reaction mixture was allowed to cool to room temperature, and the solvent was evaporated. The residue was purified by column chromatography on silica gel using hexane−ethyl acetate (5:1) as the eluent to give a deoxygenated, benzoylated product (0.16 g, 65.6%).

*Synthesis of Allyl 4-Deoxy-α-D-galactopyranoside.* Sodium methoxide (2.0 mL, 1.0 mmol) was added dropwise to a cooled (0 °C) solution of allyl 2,3,6-tri-*O*-benzoyl-4-deoxy-α-D-galactopyranoside (0.16 g, 0.32 mmol) in anhydrous methanol (1.36 mL). The reaction was stirred overnight at room temperature, and the resultant mixture was neutralized with methanol-washed IRC 50 ($H^+$) resin. The resin was filtered off, and the filtrate was concentrated in vacuo. The residue was purified by column chromatography on silica gel using chloroform−methanol (10:1) as the eluent to yield 61.2 g (94.5%) of unprotected sugar.

## RESULTS

*Galactose Protects Cys148 against NEM Alkylation.* Ligand-dependent protection of Cys148 against alkylation by [$^{14}$C]NEM is used routinely to assess substrate binding to lac permease (*20, 21, 27*). At 25 °C, labeling with 0.5 mM NEM is linear for up to 10 min at pH 7.5, and when ligand protection is tested within this time frame, quantitative determinations of apparent affinity can be obtained (*21, 27*). In the assay, protection against [$^{14}$C]NEM-labeling at different sugar concentrations is visualized by autoradiography and quantitated by using a PhosphoImager. The ligand concentration required for 50% protection is then used to characterize the apparent affinity of a given sugar (apparent $K_d$). D-Galactose at sufficiently high concentrations (ca. 200 mM) affords almost complete protection against NEM-labeling, demonstrating that this monosaccharide has all the essential properties of any substrate of the permease, and the apparent $K_d$ is ca. 30 mM (Figure 1). The result confirms previous observations that all the specificity of the permease is directed against the galactosyl moiety (*15*−*17*).

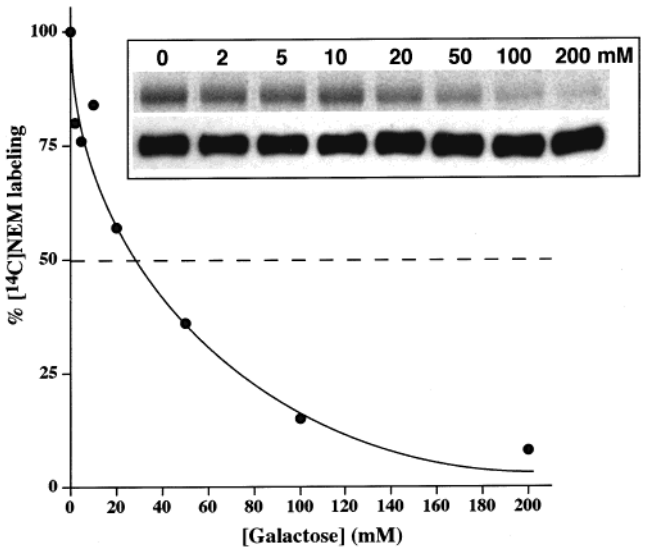


FIGURE 1: Substrate protection against [$^{14}$C]NEM alkylation of Cys148 by D-galactose. RSO membrane vesicles containing single-Cys148 permease with a biotin-acceptor domain at the C terminus were incubated in 100 mM $KP_i$ (pH 7.5) with 0.5 mM [$^{14}$C]NEM at 25 °C for 5 min in the absence or the presence of the indicated concentrations of galactose. Reactions were quenched with DTT, and biotinylated permease was solubilized and purified by affinity chromatography on monomeric avidin. Aliquots of protein were separated on a 12% SDS/polyacrylamide gel, and $^{14}$C-labeled protein was visualized by autoradiography (insert, upper panel). A fraction of the protein was analyzed by Western blotting to determine the amount of permease in each sample (insert, lower panel). Incorporation of [$^{14}$C]NEM was quantitated by a Storm 860 PhosphoImager, and labeling in the presence of given concentrations of galactose is expressed as percent labeling observed in the absence of the sugar.

Subsequently, 31 monosaccharide analogues of galactose were examined in similar fashion. The data presented in Figure 2 are representative experiments in which the ability of six different monosaccharides to protect Cys148 against alkylation is compared to that of galactose. In this example, 100 mM D-galactose and α-D-talose provide significant protection (81% and 71%, respectively), while only 8−20% protection is observed with the other sugars tested. Typically, sugar binding was analyzed at 100 and 200 mM, and the great majority of sugars tested exhibit only 5−20% protection at these concentrations. The low levels of apparent substrate protection reflect experimental variations in labeling and/or nonspecific effects and are not due to weak sugar binding, since no significant difference is observed between 100 or 200 mM sugar concentrations (not shown). Monosaccharides with no detectable affinity for the permease are presented in Table 1.

*C-3 and C-4 OH Groups Are Essential Specificity Determinants for Galactoside Recognition.* Epimers of D-galactose at C-3 (D-gulose) and C-4 (D-glucose) exhibit no binding whatsoever (Table 1), indicating that these OH groups are essential for specificity. There are at least two reasons why glucose and/or gulose may not bind to the permease: (i) the

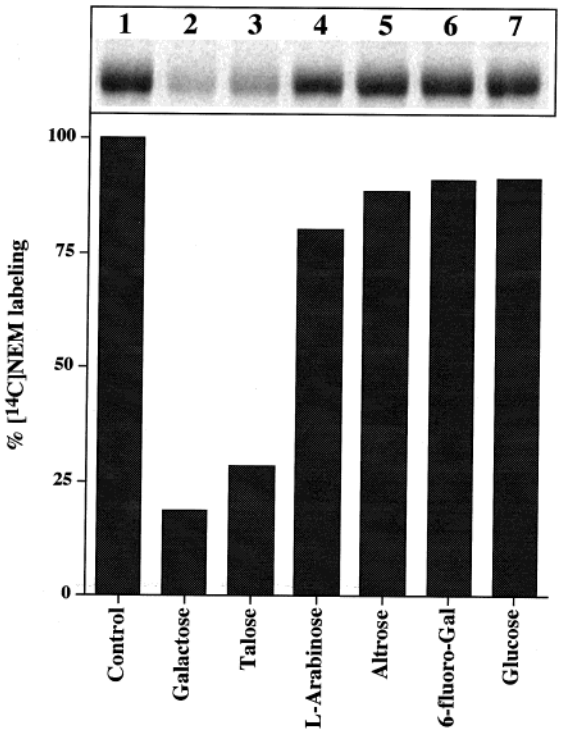


FIGURE 2: Effect of various monosaccharides on NEM-labeling of Cys148. RSO membrane vesicles were incubated in 100 mM $KP_i$ (pH 7.5) with 0.5 mM [$^{14}$C]NEM at 25 °C for 5 min in the absence or the presence of 100 mM concentration of the indicated sugars. Reactions were quenched with DTT, samples were processed as described in Figure 1, and $^{14}$C-labeled permease was visualized by autoradiography. Although not shown, a fraction of the protein was analyzed by Western blotting to determine the amount of permease in each sample; no significant differences were observed. Incorporation of [$^{14}$C]NEM was quantitated by a Storm 860 PhosphoImager, and labeling in the presence of given sugars is expressed as percent labeling observed in the control sample. Lane 1, control; lane 2, D-galactose; lane 3, α-D-talose; lane 4, L-arabinose; lane 5, D-altrose; lane 6, 6-deoxy-6-fluoro-D-galactose; lane 7, D-glucose.

OH group(s) at C-3 and/or C-4 participate in an important H-bond(s), and this bond(s) cannot form with the sterically displaced OH in the epimers; or (ii) the OH groups are not essential in galactose; however, steric clash between the epimeric OH and one or more side chains in the binding site prohibits binding. To distinguish between the two possibilities, 3-deoxy-D-galactose and allyl 4-deoxy-α-D-galactopyranoside were synthesized (see Experimental Procedures), and their ability to block NEM-labeling relative to D-galactose and allyl α-D-galactopyranoside, respectively, was examined (Figure 3). Binding of allyl 4-deoxy-α-D-galactopyranoside is severely diminished relative to allyl α-D-galactopyranoside, even though the allyl group at C-1 significantly increases binding affinity (Figure 4, see below). Similarly, 3-deoxy-D-galactose exhibits no measurable binding (Table 1). Taken together, the results indicate that both C-3 and C-4 OH groups participate in essential H-bonding interactions. This conclusion is further substantiated by the observations that six other glucose analogues (2-deoxy-D-glucose, 3-deoxy-D-glucose, 6-deoxy-D-glucose, 4-deoxy-4-fluoro-D-glucose, 3-deoxy-3-fluoro-D-glucose, and 6-deoxy-6-fluoro-D-glucose) exhibit no substrate protection whatsoever (Table 1). Similarly, β-D-allose (the combined 3,4-epimer of D-galactose) and 2-epimers of D-glucose (D-idose), D-gulose (D-mannose), or D-allose (D-altrose) are also completely inactive.

Table 1: Monosaccharides That Do Not Bind to Lac Permease[a]

| common name | structural features (relative to D-galactose) |
|---|---|
| 3-deoxy-D-galactose | 3-deoxy |
| D-gulose | 3-epimer |
| D-glucose | 4-epimer |
| D-idose | 2,3-epimer |
| D-mannose | 2,4-epimer |
| β-D-allose | 3,4-epimer |
| D-altrose | 2,3,4-epimer |
| 2-deoxy-D-glucose | 2-deoxy,4-epimer |
| 3-deoxy-D-glucose | 3-deoxy,4-epimer |
| 6-deoxy-D-glucose | 6-deoxy,4-epimer |
| 4-deoxy-4-fluoro-D-glucose | 4-deoxy,4-fluoro,4-epimer |
| 3-deoxy-3-fluoro-D-glucose | 3-deoxy,3-fluoro,4-epimer |
| 6-deoxy-6-fluoro-D-glucose | 6-deoxy,6-fluoro,4-epimer |
| 2-deoxy-D-galactose | 2-deoxy |
| D-fucose | 6-deoxy |
| L-arabinose | D-galactose without C-6 |
| D-xylose | 4-epimer of L-arabinose (D-glucose without C-6) |
| 6-deoxy-6-fluoro-D-galactose | 6-deoxy,6-fluoro |
| 6-*O*-methyl-D-galactose | 6-*O*-methyl |
| D-galacturonic acid | 6-COOH |
| D-arabinose | L-galactose without C-6 |
| L-fucose | L-galactose 6-deoxy |
| L-galactose | enantiomer |
| L-mannose | L-galactose 2,4-epimer |
| L-talose | L-galactose 2-epimer |

[a] Substrate protection of Cys148 by 100 and 200 mM sugar concentrations was assayed as described in Experimental Procedures. Sugars affording less than 20% protection are listed.

*C-2 and C-6 OH Groups Are Required for Galactose Binding.* Binding properties of galactose analogues at C-2 show an interesting pattern. The C-2 epimer α-D-talose binds almost as well as D-galactose ($K_d \cong 60$ mM, data not shown), D-galactosamine (2-deoxy-2-amino-D-galactose) binds weakly ($K_d \cong 150$ mM), while 2-deoxy-D-galactose exhibits no binding. The observations indicate that H-bonding ability at C-2 is required for binding, but the permease exhibits no epimeric specificity with respect to C-2 of D-galactose. As for C-6, no substrate protection is detected with D-fucose (6-deoxy-D-galactose), L-arabinose (D-galactose without C-6), 6-deoxy-6-fluoro-D-galactose, 6-*O*-methyl-D-galactose, or D-galacturonic acid (C-6 OOH). The data indicate that a C-6 OH is mandatory for galactose binding, in all likelihood as an H-bond donor.

*Anomeric Functional Groups Increase Affinity for Galactoside Binding.* In contrast to galactose, methyl galactopyranosides have no open forms and cannot undergo mutarotation. To examine the significance of the anomeric OH in galactose binding, substrate protection by methyl α- and β-D-galactopyranosides was compared. Both analogues bind better than galactose, indicating that (i) the pyranose ring structure, not the open form or the furanose ring structure, is the bound species; (ii) a H-bonding ability at C-1 is unlikely to be important for binding; and (iii) anomeric orientation does not contribute to specificity. Interestingly, however, methyl α-D-galactopyranoside and allyl α-D-galactopyranoside ($K_d \cong 0.5$ mM) exhibit 20-fold better apparent affinity (i.e., 20-fold lower $K_d$) than methyl β-D-galactopyranoside ($K_d \cong 10$ mM), demonstrating that binding affinity is significantly influenced by the functional group at C-1 and its orientation (Figures 3 and 4).

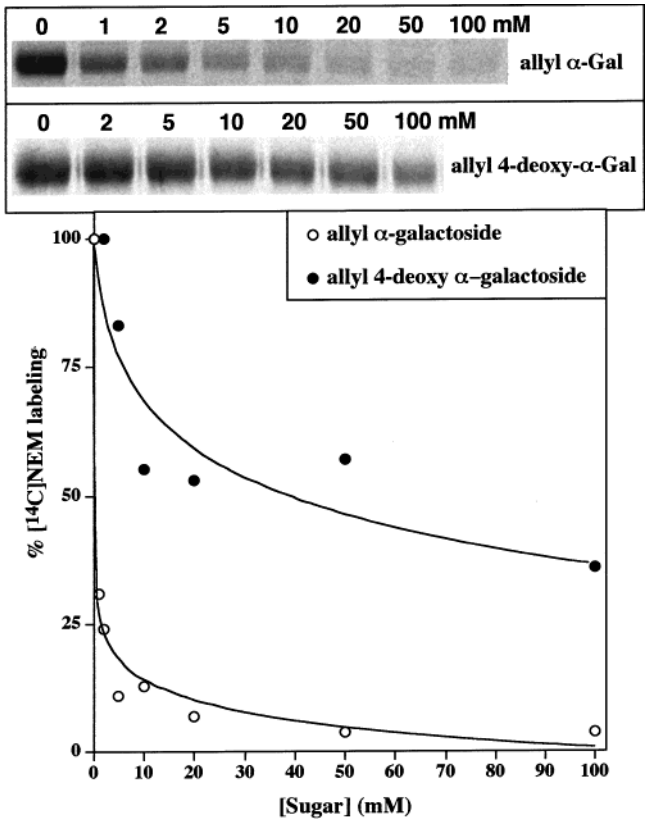


FIGURE 3: Effect of allyl α-D-galactopyranoside and allyl 4-deoxy-α-D-galactopyranoside on NEM-labeling of Cys148. RSO membrane vesicles were incubated in 100 mM $KP_i$ (pH 7.5) with 0.5 mM [$^{14}$C]NEM at 25 °C for 5 min in the absence or the presence of the indicated sugar at a given concentration. Reactions were quenched with DTT, samples were processed as described in Figure 1, and $^{14}$C-labeled permease was visualized by autoradiography. Although not shown, a fraction of the protein was analyzed by Western blotting to determine the amount of permease in each sample; no significant differences were observed. Incorporation of [$^{14}$C]NEM was quantitated by a Storm 860 PhosphoImager, and labeling in the presence of sugars is expressed as percent labeling observed in the control sample.

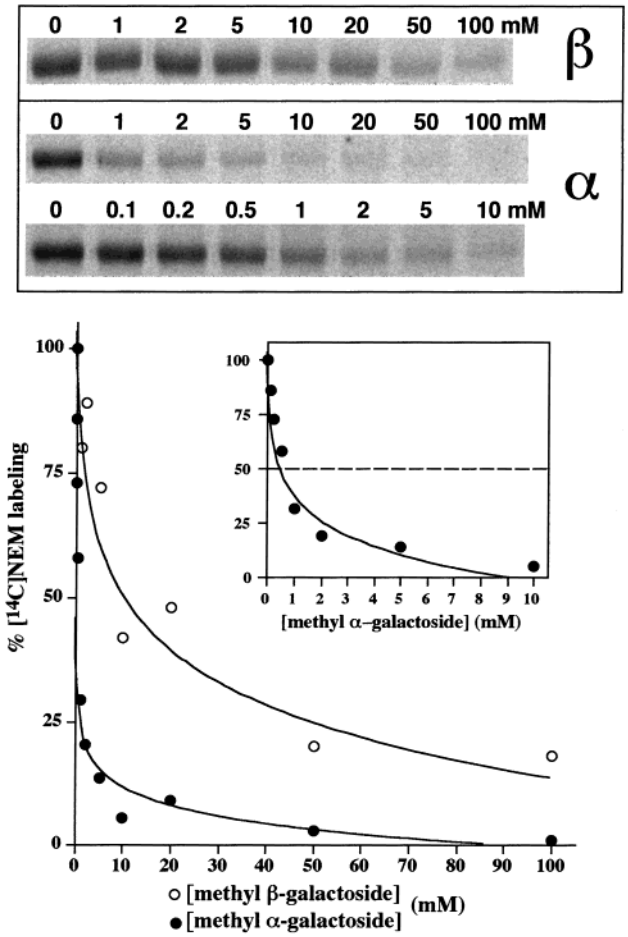


FIGURE 4: Effect of methyl α-D-galactopyranoside and methyl β-D-galactopyranoside on NEM-labeling of Cys148. See Figures 1 and 3 for experimental conditions. For the higher affinity methyl α-D-galactopyranoside, two concentration ranges (0.1−10 and 1−100 mM) were tested. The inset demonstrates the results in the 0.1−10 mM range.

## DISCUSSION

This paper is the first attempt to use binding assays in the systematic study of the structural characteristics of galactosides that play a role in substrate specificity and affinity by lac permease. Previous studies (*15*, *16*) approached this problem by examining the effects of various galactosides and galactose analogues on lactose transport only, and no binding experiments were performed. Consequently, quantitative interpretation of results from these studies with respect to binding specificity per se is difficult. In addition, the studies were carried out on whole cells, where addition of certain monosaccharides can elicit profound metabolic changes that, in turn, significantly influence lactose transport. One noteworthy example is that glucose was demonstrated to inhibit lactose transport, and this property was attributed to glucose binding and competitive inhibition (*16*). Metabolic effects of glucose were not considered, particularly the phenomenon of inducer exclusion, where lac permease is inhibited by unphosphorylated Enzyme $IIA^{Glc}$, a component of the phospho*enol*pyruvate glucose phosphotransferase system. Subsequently, Wu et al. (*17*) demonstrated that the native Cys148 in the binding site is fully protected against MIANS-labeling by substrates of the permease; however, glucose affords no protection whatsoever, indicating that glucose does not bind, and this conclusion is supported by the studies presented here.

The results of the present study are in remarkably good agreement with the predictions of a recently postulated model for the substrate binding site (*14*, *20*) (Figure 5). Thus, specificity toward D-galactose and D-galactopyranosides is determined by H-bonding interactions between C-3 and C-4 OH groups and Arg144. Removal of either OH (deoxy derivatives) or epimers at these positions (D-gulose, D-glucose) markedly diminishes binding. The C-6 OH donates an essential H-bond to Glu126, and any modification of galactose at this position abolishes binding. In addition, sugar binding is stabilized by H-bonding with the C-2 OH; however, the permease does not discriminate between epimers (galactose vs talose) at this position. The proposed interactions in the binding site model are generally similar to those observed in sugar-binding proteins. Guanidino (Arg)- and/or carboxylate (Asp, Glu)-containing side chains are always present in sugar binding sites of lectins (*32*−*35*), periplasmic sugar binding proteins (*36*−*38*), glycoporins (*39*−*42*), and repressor molecules (*43*, *44*), usually as H-bond donors and acceptors, respectively. One notable example is the lac repressor protein, in which Arg197 interacts with the

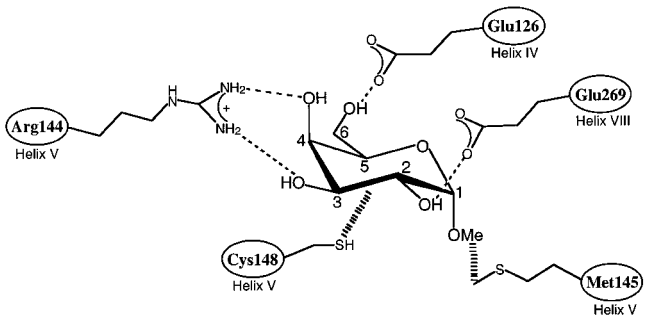


FIGURE 5: Putative binding interactions between methyl α-D-galactopyranoside and amino acid side chains in lac permease. As indicated, the guanidino group of Arg144 H-bonds with the OH groups at the 3 and 4 positions of the pyranose ring and forms an electrostatic interaction (salt bridge) with Glu126. One of the carboxylate O atoms of Glu126 acts as an H-bond acceptor from the C-6 OH, and the carboxylate of Glu269 H-bonds to the C-2 OH group. Cys148 interacts hydrophobically with the hydrophobic (*β*) face of the galactose ring, and the side chain of Met145 interacts hydrophobically with the methyl group.

C-3 and C-4 OH groups and Asp149 H-bonds to the C-6 OH of bound isopropyl 1-thio-*β*-galactopyranoside (*43*). Hydrophobic interactions are also observed in lac permease (Cys148, Met145; see Figure 5) as well as in binding proteins, in which commonly aromatic side chains play this role (aromatic stacking).

Which amino acid side chain interacts with the C-2 OH? Although at this time direct experimental evidence is lacking, it is intriguing to speculate that Glu269 in helix VIII might be responsible for stabilizing galactose binding through H-bonding with the OH at C-2. There are at least two indications supporting this notion: (i) Mutants at Glu269 are severely defective with respect to ligand binding and transport (*45*, *46*). In this regard, Glu269 closely resembles Glu126 or Arg144, suggesting that this residue might participate directly in substrate binding. (ii) The galactose-binding (chemoreceptor) protein from *E. coli* binds both glucose and galactose (*36*). Recognition of both epimers is achieved by utilizing H-bonding to different O atoms of Asp14; glucose interacts with Oδ1, and galactose interacts with Oδ2. This scenario can be easily adapted to lac permease, in which recognition of the C-2 epimers galactose and talose might occur in a similar manner involving the carboxylate group of Glu269.

One of the most interesting results of this study is the dramatic difference between the affinities of methyl α- and *β*-galactopyranosides. While both anomers bind better than galactose, methyl α-galactopyranoside exhibits a 20-fold higher affinity than *β*. Taken together with previous observations demonstrating that a variety of aromatic aglycons in the α position confer high-affinity binding (*15*, *47*−*49*), the results warrant the conclusion that affinity and specificity are distinct properties determined by different interactions. Thus, in lac permease, affinity is primarily determined by the anomeric substituent(s), while these functional groups play no role whatsoever in specificity. A strikingly similar observation with respect to the substrate specificity of *β*-galactosidase has been described recently (*50*). Finally, computer modeling of the relatively small methyl group into the binding site might allow identification of amino acids responsible for the high-affinity hydrophobic interaction(s) observed with methyl α-galactopyranoside. As shown in Figure 5, one such candidate is the hydrophobic side chain of Met145. Met145 is on the same face of helix V as Cys148, and the disaccharide *β*-D-galactopyranosyl 1-thio-*β*-D-galactopyranoside (TDG) partially protects M145C against labeling with MIANS (*17*) or NEM (K.-C. Zen and H.R.K, unpublished observations). Current experiments are underway to evaluate how replacement of Met145 with short hydrophobic side chains (Ala, Val) or bulky aromatic residues (Trp, Phe) influences binding of methyl α- and *β*-galactopyranosides.

## ACKNOWLEDGMENT

We are indebted to Tatsushi Toyokuni for critically reviewing this manuscript and to Arthur Karlin for suggesting the equation used for curve fitting.

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BI0000263