The C-4 Hydroxyl Group of Galactopyranosides Is the Major Determinant for Ligand Recognition by the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Binding specificity in lactose permease 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 nonspecific hydrophobic interactions with the non-galactosyl moiety [Sahin-Tóth, M., Akhoon, K. M., Runner, J., and Kaback, H. R. (2000) *Biochemistry 39*, 5097−5103]. To characterize the contribution of individual hydroxyls, binding of structural analogues of *p*-nitrophenyl α-D-galactopyranoside (NPG) was examined by site-directed *N*-[^{14}C]ethylmaleimide (NEM) labeling of the substrate-protectable Cys148 in the binding site. NPG blocks NEM alkylation of Cys148 with an apparent affinity of approximately 14 μM. A deoxy derivative at position C-2 binds with 25-fold lower affinity (K_D 0.35 mM), and the deoxy analogue at C-3 exhibits ca. 70-fold decreased binding (K_D 1 mM), while binding of 6-deoxy-NPG is at least 130-fold diminished (K_D 1.9 mM). Remarkably, the C-4 deoxy derivative of NPG binds with almost 1500-fold reduced affinity ($K_D \approx 20$ mM). No significant substrate protection is afforded by NPG analogues with methoxy (CH_3-O-) substitutions at positions C-3, C-4, and C-6. In contrast, the C-2 methoxy analogue binds almost normally (K_D 26 μM). The results confirm and extend the observations that the C-2, C-3, C-4, and C-6 OH groups of galactopyranosides participate in important H-bonding interactions. Moreover, the C-4 hydroxyl is identified as the major determinant of ligand binding, suggesting that sugar recognition in lactose permease may have evolved to discriminate primarily between gluco- and galactopyranosides.

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 permease consists of 12 hydrophobic, membrane-spanning helices connected by hydrophilic loops with both the N and C termini on the cytoplasmic face of the membrane. 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 (*6*−*9*). In addition, experimental observations from structural and extensive mutational analysis have led to a proposed mechanism for lactose/H^+ symport (*10*, *11*).

Specificity of substrate binding to lac permease was initially studied by examining the effects of various galactosides and monosaccharides on lactose transport (*12*, *13*). These studies indicate that ligand binding requires an α- or β-galactopyranosyl ring of the D series, and D-galactose is the smallest specific transport substrate. Furthermore, high-affinity binding is conferred by hydrophobic substituents, aromatic groups in particular, at C-1 of α-galactopyranosides. Subsequently, Wu et al. (*14*) demonstrated that labeling of purified single-Cys148 permease with the fluorescent 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 have no such effect. These studies provided the first indication that substrate specificity of lac permease is directed primarily toward the C-4 OH group of galactose.

More recently, binding of 31 structural analogues of galactose to single-Cys148 permease was examined by site-directed *N*-[^{14}C]ethylmaleimide (NEM) labeling of Cys148 which is protected by substrate (*15*). D-Galactose blocks alkylation of Cys148 with a relatively low affinity of approximately 30 mM. Epimers of D-galactose at C-3 (D-gulose) and C-4 (D-glucose) exhibit no binding whatsoever, while the C-2 epimer α-D-talose binds almost as well as D-galactose. No substrate protection is observed with deoxy derivatives at C-2, C-3, C-4, and C-6 or with L-arabinose, 6-deoxy-6-fluoro-D-galactose, 6-*O*-methyl-D-galactose, or

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[1] Abbreviations: lac permease, lactose permease; NPG, *p*-nitrophenyl α-D-galactopyranoside; NEM, *N*-ethylmaleimide; DDM, dodecyl β-D-maltopyranoside; KP_i, potassium phosphate; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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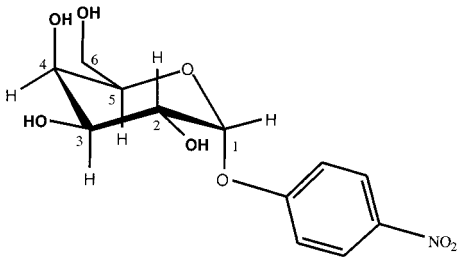

Abbreviation	Chemical name
NPG	p-nitrophenyl α-D-galactopyranoside
2-deoxy NPG	p-nitrophenyl 2-deoxy-α-D-lyxo-hexopyranoside
3-deoxy NPG	p-nitrophenyl 3-deoxy-α-D-xylo-hexopyranoside
4-deoxy NPG	p-nitrophenyl 4-deoxy-α-D-xylo-hexopyranoside
6-deoxy NPG	p-nitrophenyl α-D-fucopyranoside
2-methoxy NPG	p-nitrophenyl 2-*O*-methyl-α-D-galactopyranoside
3-methoxy NPG	p-nitrophenyl 3-*O*-methyl-α-D-galactopyranoside
4-methoxy NPG	p-nitrophenyl 4-*O*-methyl-α-D-galactopyranoside
6-methoxy NPG	p-nitrophenyl 6-*O*-methyl-α-D-galactopyranoside

FIGURE 1: Structure of *p*-nitrophenyl α-D-galactopyranoside (NPG) and the analogues used in the present study.

D-galacturonic acid. The results indicate that (i) binding of galactopyranosides is governed by H-bond interactions at C-2, C-3, C-4, and C-6 OH groups; (ii) H-bonding at C-2 is nonstereospecific; and (iii) the C-6 OH is an H-bond donor.

Due to the very low affinity of galactose, the reference compound in the studies, quantitative determination of binding affinities for the deoxy sugars was not feasible, and the individual contribution of the different galactosyl hydroxyls to binding could not be assessed. The present study uses a series of deoxy and methoxy analogues of the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside (NPG) to address this question (Figure 1). These sugars have been successfully used previously to characterize the substrate specificity of various α-galactosidases (*16*, *17*). The results identify the C-4 OH group as the major determinant for ligand recognition and confirm that H-bonding at C-2, C-3, and C-6 is also important.

EXPERIMENTAL PROCEDURES

Materials. N-[1-^{14}C]Ethylmaleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, MA). Immobilized monomeric avidin (ImmunoPure) was from Pierce (Rockford, IL). NPG was obtained from Sigma (St. Louis, MO). Synthesis of the NPG analogues used in this study was described previously (*16*, *17*). Sugars were dissolved in dimethyl sulfoxide (DMSO).

Growth of Cells and Preparation of Right-Side-Out (RSO) Membrane Vesicles. E. coli T184 (*18*) expressing single-Cys148 permease with a biotin-acceptor domain at the C terminus (*19*) were grown in Luria−Bertani broth, and RSO membrane vesicles were prepared as described previously (*15*, *20*, *21*). Vesicles were suspended in 100 mM potassium phosphate (KP_i, pH 7.5) 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 presence of given sugars (*15*, *22*, *23*). Single-Cys148 permease which was used for the assays contains a biotin-acceptor domain at the C terminus and is partially biotinylated in vivo. RSO membrane vesicles were preequilibrated in a final volume of 50 μL (containing 0.6 mg of protein) with given sugar concentrations for 5 min at room temperature. Labeling was initiated by addition of 12 μL of [^{14}C]NEM to a final concentration of 0.5 mM (40 mCi/mmol), and the vesicles were incubated for 5 min at room temperature (ca. 24 °C). The final DMSO concentration was 2% in each sample, and control samples were adjusted to the same DMSO concentration. At this concentration DMSO has no effect on [^{14}C]NEM labeling or substrate protection of Cys148. Reactions were quenched by addition of 10 mM dithiothreitol (DTT, final concentration). The vesicles were solubilized with 2% dodecyl β-D-maltopyranoside (DDM, final concentration), and the samples were mixed with immobilized monomeric avidin 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 50 μL of equilibration buffer containing 5 mM D-biotin. After addition of 25 μL of electrophoresis sample buffer (concentrated five times), the sample was analyzed electrophoretically on a sodium dodecyl sulfate ($NaDodSO_4$)/12% polyacrylamide gel. The gel was dried and exposed to a PhosphoImager screen for 4−6 days. Incorporation of [^{14}C]NEM was visualized and quantitated by a Storm 860 PhosphoImager (Molecular Dynamics). Apparent affinity constants (K_D) were determined with the MicroCal Origin (Microcal Software, Inc., Northampton, MA) computer program using nonlinear least-squares curve fitting, as described previously (*10*, *15*).

RESULTS

NPG Protects Cys148 against NEM Alkylation with High Affinity. Ligand-dependent protection of Cys148 against alkylation by [^{14}C]NEM is used routinely to assess substrate binding to lac permease (*10*, *15*, *22*, *23*). 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. NPG (Figure 1) has been identified previously as a high-affinity substrate of lac permease, and direct binding assays yield an apparent affinity of ca. 10−20 μM (*24*, *25*). As expected, NPG affords protection against NEM labeling of Cys148 with a similarly high affinity (Figure 2), and the apparent K_D (14 μM) obtained from this assay is in very good agreement with earlier determinations.

Deoxy Derivatives of NPG Exhibit Significantly Reduced Binding. Subsequently, deoxy analogues at C-2, C-3, C-4, and C-6 of NPG (Figure 1) were examined in a similar fashion (Figure 3, Table 1). All four analogues bind with significantly decreased but measurable affinities. 2-Deoxy-NPG binds 25-fold weaker (K_D 0.35 mM), and 3-deoxy-NPG exhibits ca. 70-fold decreased binding (K_D 1 mM), while binding of 6-deoxy-NPG is at least 130-fold diminished (K_D 1.9 mM). Dramatically, binding of 4-deoxy-NPG is hardly measurable, and only at very high concentrations (15−20 mM) is substrate protection evident. Due to limitations in solubility, full protection cannot be achieved with this analogue, and binding affinity is only estimated at ca. 20 mM, which corresponds to almost 1500-fold reduced affinity relative to NPG.

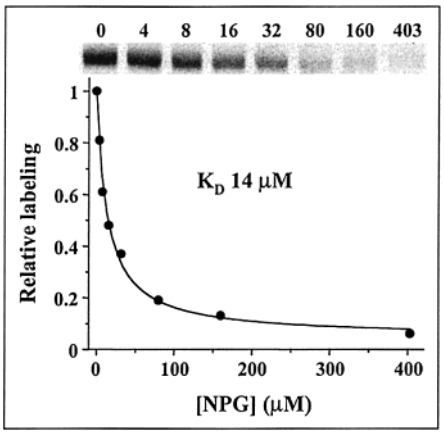

FIGURE 2: Substrate protection against [^{14}C]NEM alkylation of Cys148 by NPG. RSO membrane vesicles containing single-Cys148 permease with a biotin-acceptor domain at the C terminus were incubated in 100 mM KP_i (pH 7.5) with 0.5 mM [^{14}C]NEM for 5 min in the absence or presence of the indicated concentrations of NPG. Reactions were quenched with DTT, and biotinylated permease was solubilized and purified by affinity chromatography on monomeric avidin. Samples were separated on a 12% Na-$DodSO_4$/polyacrylamide gel, and ^{14}C-labeled protein was visualized by autoradiography. Incorporation of [^{14}C]NEM was quantitated by a Storm 860 PhosphoImager, and labeling in the presence of given concentrations of NPG is expressed as percent labeling observed in the absence of the sugar.

Binding of Methoxy Derivatives of NPG. The C-2 methoxy analogue of NPG (Figure 1) binds almost as well as NPG (K_D 26 μM). In remarkable contrast, no significant substrate protection is detectable with methoxy analogues at C-3, C-4, and C-6 (Figure 1) over the concentration range studied (Figure 4). 3-Methoxy-NPG exhibits limited substrate protection at higher concentrations, and binding affinity is estimated to be somewhat above 10 mM. On the other hand, binding affinities of 4-methoxy- and 6-methoxy-NPG are probably well above 20 and 10 mM, respectively, since no significant binding is detected at these concentrations.

Table 1: Apparent Binding Affinities of NPG and Its Deoxy and Methoxy Derivatives to Lac Permease

compound	K_D
NPG	14 μM
2-deoxy-NPG	0.35 mM
3-deoxy-NPG	1 mM
4-deoxy-NPG	≈20 mM
6-deoxy-NPG	1.9 mM
2-methoxy-NPG	26 μM
3-methoxy-NPG	>10 mM
4-methoxy-NPG	≫20 mM
6-methoxy-NPG	≫10 mM

DISCUSSION

This study analyzes the binding of a series of NPG analogues (Figure 1) to characterize quantitatively the contribution of individual OH groups to affinity. In these NPG derivatives one of the OH groups at position C-2, C-3, C-4, or C-6 is replaced either by H (deoxy analogues) or by CH_3-O (methoxy analogues). The results confirm previous observations that OH groups at these positions are essential for high-affinity binding (*15*) and indicate that the C-4 OH

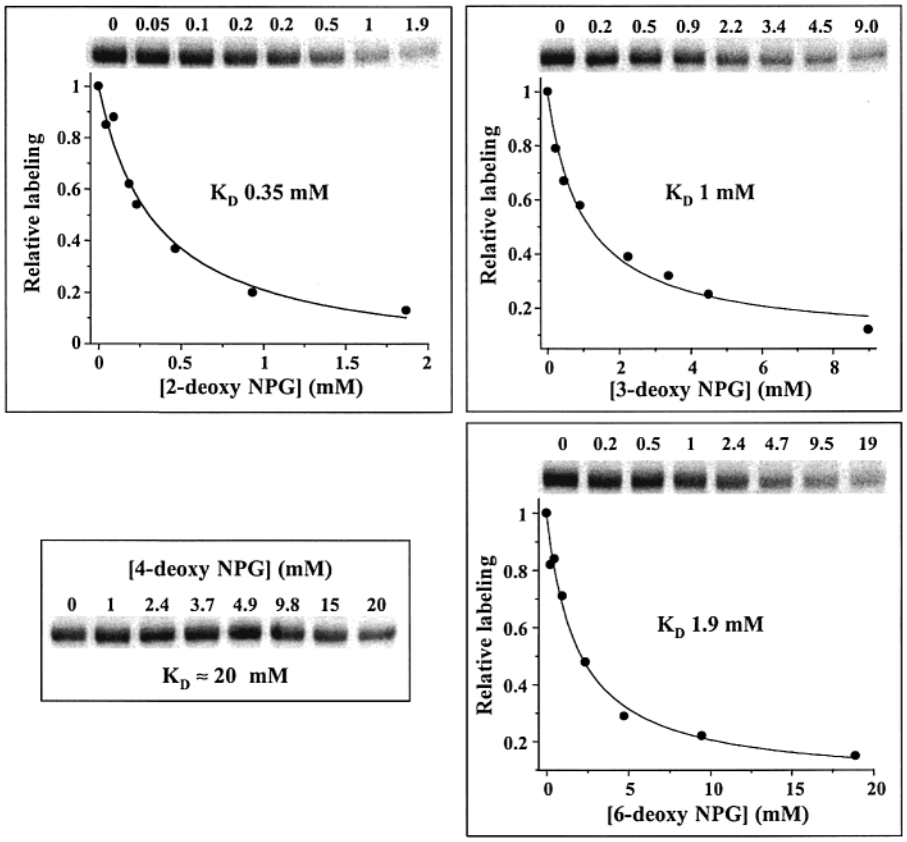

FIGURE 3: Effect of deoxy derivatives of NPG on NEM labeling of Cys148. RSO membrane vesicles were incubated in 100 mM KP_i (pH 7.5) with 0.5 mM [^{14}C]NEM for 5 min in the absence or presence of the indicated sugar at the given concentrations. Reactions were quenched with DTT, and samples were processed as described in Figure 2. Incomplete protection observed with 4-deoxy-NPG does not allow the generation of plots suitable for curve fitting. Therefore, the K_D value shown is only an estimate.

is the major determinant for ligand recognition. Thus, all four deoxy and three of the four methoxy analogues exhibit significantly decreased binding (see Table 1), and the determined K_D values indicate characteristic differences in the contribution of the individual positions to binding affinity (C-2 < C-3 < C-6 ≪ C-4).

Among the four positions studied, the C-2 OH is probably the least important in determining galactopyranoside recognition, even though binding of 2-deoxy-NPG is 25-fold reduced. On the other hand, the 2-methoxy substitution does not affect significantly binding, and the 2-epimer of galactose (talose) also binds well (*15*). Taken together, the findings suggest that the C-2 OH acts as an H-bond acceptor, an interaction required for high-affinity binding but one which also exhibits unique flexibility. Relative to C-2, the C-3 OH plays a more significant role. 3-Deoxy-NPG exhibits 70-fold decreased binding, and 3-methoxy-NPG binds at least 700 times weaker. In addition, 3-deoxy-galactose or the 3-epimer of galactose (gulose) does not bind (*15*). Similar properties are observed with galactopyranosides modified at C-6. 6-Deoxy-NPG binds weakly (ca. 135-fold lower affinity), and 6-methoxy-NPG exhibits no measurable binding up to 10 mM. Various substitutions at C-6 of galactose also abolish binding (*15*). The observations are consistent with the notion that the C-6 OH of galactopyranosides is an essential H-bond donor. Finally, the most important determinant of galactopyranoside binding appears to be the OH group at the C-4 position. Binding of 4-deoxy-NPG is 1500-fold weaker, and 4-methoxy-NPG has no measurable affinity to lac permease. Glucose, the 4-epimer of galactose, or glucopyranosides do not bind either (*12*, *14*, *15*).

The observation that the C-4 OH is the major determinant of substrate binding in lac permease suggests that ligand recognition in this transporter evolved primarily to distinguish between glucose (glucosides) and galactosides. While the evolutionary rationale for this strict distinction may not be readily apparent, it seems relevent that glucose plays a central role not only as a carbon source but also as a regulator of sugar transport and metabolism in *E. coli*. In the presence of glucose, lactose uptake is inhibited by dephosphorylated enzyme IIA^{Glc}, a component of the phosphoenolpyruvate glucose phosphotransferase system (PTS), and expression of the entire *lac* operon is repressed (inducer exclusion and catabolite repression). Under these conditions, glucose is preferentially utilized, and lactose uptake and metabolism are turned on only after depletion of glucose in the medium (diauxie). In addition, the phosphorylation state of enzyme IIA^{Glc} regulates a number of other metabolic processes (for a review see ref *26*). It is conceivable that such a tight regulatory mechanism could not be maintained if glucose was also transported by other non-PTS transport systems, e.g., lac permease.

How do galactopyranosides interact with lac permease? Mutagenesis combined with NEM labeling and spectroscopic studies has led to the notion that Glu126 (helix IV) and Arg144 (helix V) form a salt bridge that is mandatory for ligand binding (Figure 5) (*23*, *27*−*31*). Since Lys substitution for Arg144 maintains the salt bridge but leads to complete loss of binding (*23*, *27*, *28*), the guanidino group of Arg144 is likely to be the principal site of interaction with the OH groups of galactopyranosides. Since the C-4 OH is the most important galactosyl hydroxyl, it seems reasonable to assume

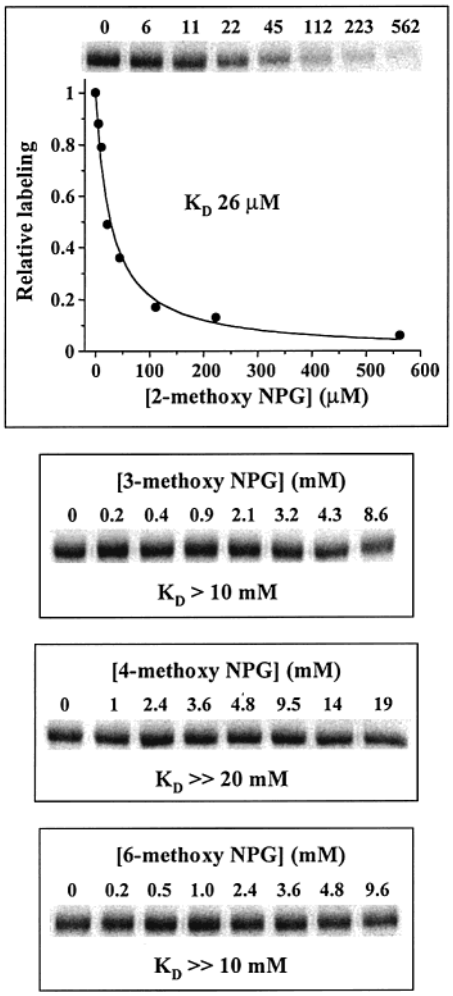

FIGURE 4: Effect of methoxy analogues of NPG on NEM labeling of Cys148. See Figures 2 and 3 for experimental conditions. Minimal or incomplete protection observed with 3-, 4-, and 6-methoxy-NPG does not allow the generation of plots suitable for curve fitting. Therefore, the K_D values shown are only estimates.

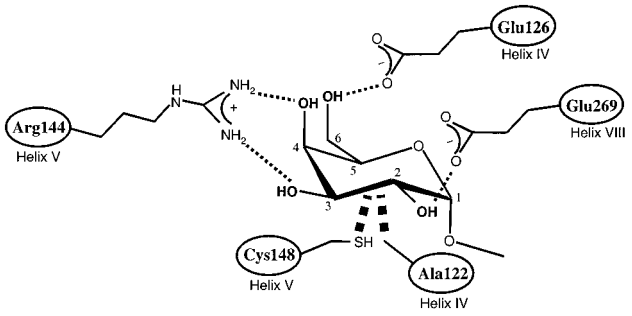

FIGURE 5: Putative binding interactions between a D-galactopyranoside and amino acid side chains in lac permease. As an example, methyl α-D-galactopyranoside is shown. As indicated, the guanidino group of Arg144 H-bonds with the OH groups at the C-3 and C-4 positions. One of the carboxylate O atoms of Glu126 acts as an H-bond acceptor from the C-6 OH. The carboxylate of Glu269 H-bonds to the C-2 OH. Ala122 and Cys148 interact with the hydrophobic (*β*) face of the pyranose ring. See text for details.

that the guanidino group of Arg144 interacts primarily with this position. Structural modeling suggests that the guanidino group may also interact with the C-3 OH, and one of the carboxyl O atoms of Glu126 may H-bond to the OH group at C-6. Properties of mutants in Glu269 (helix VIII) suggest that this carboxylate may also H-bond with the galactosyl

end, and this interaction is tentatively assigned to the C-2 OH (see discussion in ref *15*). In addition to H-bonds, at least two hydrophobic interactions have been identified. Reaction of Cys148 (helix V) with NEM or other thiol reagents abolishes transport and ligand binding, and substrates of the permease protect against sulfhydryl inactivation or labeling (*14*, *15*, *22*, *32*). Furthermore, replacement of Cys148 with small hydrophobic amino acyl side chains (Ala, Val) results in preserved function (*33*). On the basis of these observations it was suggested that Cys148 interacts with the hydrophobic β face of the pyranose ring. More recently, Ala122, which is located in helix IV at approximately the same level in the membrane as Cys148, was shown to possess similar properties (*34*). Thus, NEM abolishes transport activity of the A122C single-Cys mutant, and ligand affords complete protection against NEM inactivation or [^{14}C]NEM labeling. Therefore, it is likely that Ala122 plays a similar role in ligand binding as Cys148 and participates in a hydrophobic interaction with the pyranose ring. Since single-Cys mutant M145C is partially protected against MIANS or [^{14}C]NEM labeling by TDG, it was suggested that Met145 might interact with the non-galactosyl end of galactosides (*14*). However, TDG binding appears to be unaltered in mutants M145A and M145S, indicating that the hydrophobic Met side chain is not required for high-affinity binding (M. Sahin-Tóth and H. R. Kaback, unpublished observations).

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